Doctoral Thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

The Role of Mammalian Target of Rapamycin in the Regulation of Amino Acid Transporters in the Human Placenta

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ABSTRACT

Abnormal fetal growth, which is associated with both perinatal morbidity as well as metabolic diseases in adulthood, is an important clinical problem affecting as many as 15% of all pregnancies. However, to this date, there is no specific treatment of this condition. Fetal growth is intimately linked to the nutrient transport functions of the placenta and placental amino acid transporter activity is known to be altered in cases of abnormal fetal growth. Therefore, detailed information on the mechanisms regulating placental amino acid transporters will increase our understanding of how abnormal fetal growth develops and may provide new targets for therapeutic intervention.

The focus of this study was to identify factors, such as hormones and growth factors, regulating three key amino acid transporters in the human placenta; system L, system A, and system β. The central hypothesis was that mammalian target of rapamycin (mTOR) signaling regulates placental amino acid transporters in the human placenta in response to nutrient availability and growth factors such as insulin and IGF-I. To test this hypothesis, we have used cultured primary trophoblast cells, primary villous fragments, and homogenates, all from the human placenta, to study the regulation of amino acid transport.

We show that the mTOR signaling pathway constitutes an important positive regulator of the placental amino acid transporters system A, system L, and the taurine transporter (system β). Furthermore, we demonstrate that these amino acid transporters are regulated by nutrients, such as glucose, and growth factors, such as insulin and IGF-I, in an mTOR dependent manner. Placental mTOR activity was found to be decreased in intrauterine growth restriction (IUGR), which may explain the down-regulation of placental amino acid transporters in this pregnancy complication.

We propose a model in which placental mTOR functions as a nutrient sensor linking maternal nutrient and growth factor concentrations to amino acid transport in the placenta. Since fetal growth is critically dependent on placental nutrient transport, these data suggest that placental mTOR signaling plays an important role in the regulation of fetal growth.

The regulation of amino acid transport is important not only in the placenta. Our results were obtained in primary human tissue fragments and cells from the placenta, however, we believe that findings in this study are also relevant for other human tissues such as the skeletal muscle and liver. Furthermore, the growth of many tumor cells is dependent on a high expression of amino acid transporters and detailed information on the mechanisms of regulation of these transporters may facilitate the development of new interventions.

Keywords: amino acids, fetal growth restriction, human, mammalian target of rapamycin, membrane transporters, metabolism, placenta, pregnancy, system A, system L, taurine transporter

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, published or in manuscript, which will be referred to by their Roman numerals:

I. **Roos S**, Powell TL & Jansson T. (2004). **Human placental taurine transporter in uncomplicated and IUGR pregnancies: cellular localization, protein expression, and regulation.** *Am J Physiol Regul Integr Comp Physiol* 287, R886-893.

II. **Roos S**, Jansson NL, Palmberg I, Säljö K, Powell TL & Jansson T. (2007). **Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth.** *J Physiol* 582, 449-459.

III. **Roos S**, Kanai Y, Prasad PD, Powell TL & Jansson T (2008). **Regulation of placental amino acid transporter activity by mammalian target of rapamycin.** *Accepted.*

IV. **Roos S**, Lagerlöf O, Wennergren M, Powell TL & Jansson T (2008). **Regulation of amino acid transporters by glucose and growth factors in cultured primary human trophoblast cells is mediated by mTOR signaling.** *Submitted.*

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LIST OF ABBREVIATIONS

4E-BP1	eukaryotic initiation factor 4E-Binding Protein 1
4F2hc	4F2 heavy chain
ABC	Avidin:Biotinylated enzyme Complex
AGA	Appropriate for Gestational Age
ANOVA	ANalysis Of VAriance
BM	Basal plasma Membrane
C	Cesarean section
cDNA	complimentary DeoxyriboNucleic Acid
CHT	CHeleryThrine
cpm	counts per minute
DAB	DiAminoBenzidine
DMEM	Dulbecco's Modified Eagle's Medium
DTT	DiThioThreitol
EGF	Epidermal Growth Factor
eIF4E	eukaryotic initiation factor 4E
FBS	Fetal Bovine Serum
GDM	Gestational Diabetes Mellitus
GH	Growth Hormone
h	hour
HBSS	Hank's Balanced Salt Solution
hCG	human Chorionic Gonadotropin
IDDM	Insulin Dependent Diabetes Mellitus
IGF	Insulin-like Growth Factor
IL	InterLeukin
IUGR	IntraUterine Growth Restriction
kDa	kilo Dalton
LAT	Large neutral Amino acid Transporter
LDH	Lactate DeHydrogenase
LGA	Large for Gestational Age
MeAIB	MethylAminoIsoButyric acid
mRNA	messenger RiboNucleic Acid
mTOR	mammalian Target Of Rapamycin
MVM	MicroVillous Membrane
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	PhosphoInositide 3-Kinase
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
RT	Reverse Transcriptase
S6K1	ribosomal protein S6 Kinase 1
SEM	Standard Error of the Mean
SD	Standard Deviation
SDHA	Succinate DeHydrogenase complex, subunit A
SDS	Sodium Dodecyl Sulphate
SIN-1	3-morpho-linoSydnonImiNe
SNAT	Sodium-coupled Neutral Amino acid Transporter
TAUT	TAUrine Transporter
TBP	TATA Box binding Protein
TBS	Tris Buffered Saline
TNF-α	Tumor Necrosis Factor-α

INTRODUCTION

The beginning of a new life is one of life's great miracles. Optimal growth in utero is of utmost importance for the developing fetus. However, as many as 15% of all pregnancies result in abnormal intrauterine growth, either intrauterine growth restriction (IUGR) or fetal overgrowth (3, 4). IUGR and fetal overgrowth represent two clinically important pregnancy complications, because babies subjected to abnormal intrauterine growth are at risk for short- as well as long-term complications. IUGR fetuses can be defined as having failed to reach their genetic growth potential (145) and are associated with an increased risk of perinatal morbidity (18) as well as the risk of developing metabolic abnormalities in adult life, such as type 2 diabetes and cardiovascular disease (7). Fetal overgrowth is a risk factor for traumatic birth injuries (23), and large babies are predisposed to develop the metabolic syndrome in childhood (14) and diabetes and obesity in later life (35, 136). As of today, there is no specific treatment for abnormal fetal growth. Resent research suggests that changes in placental amino acid transporter activity directly contribute to aberrant fetal growth (79, 157). However, the cellular mechanisms underlying altered placental transport, and consequently fetal growth, remain to be established. This information is critical for designing new intervention strategies.

The primary determinant of fetal growth is the availability of nutrients, whereas the fetal genome plays a more limited role. Fetal nutrient availability is in turn directly dependent on the transport functions of the placenta. Studies in plasma membranes isolated from the polarized syncytiotrophoblast, the transporting epithelium of the human placenta, have shown that abnormal fetal growth is associated with alterations in specific placental nutrient transporters (Reviewed in (31, 79-81, 157)). In general, these studies show that IUGR is characterized by a decreased activity of placental amino acid transporters whereas fetal overgrowth is associated with an up-regulation of placental amino acid transporters.

These *in vitro* findings are compatible with observations *in vivo* showing lower fetal plasma concentrations of amino acids in IUGR (24, 25, 41) as well as reduced placental transfer of the essential amino acids leucine and phenylalanine (132). The critical importance of placental system A (a sodium-dependent transporter for small, neutral amino acids) transport for fetal growth has been supported by experimental studies. Cramer and co-workers infused α-(methylamino)isobutyric acid (MeAIB), a non-metabolizable amino acid analogue specific for system A, into the maternal circulation of pregnant rats in order to achieve competitive inhibition of the system A transporter, which resulted in intrauterine growth restriction (34). Placental system A activity has been shown to be reduced and maternal plasma amino acid concentrations were largely maintained in pregnant rats fed a low protein diet, a model of IUGR (114). These findings suggest that the growth restriction in response to maternal

protein malnutrition may be caused by a restricted placental nutrient transport capacity rather than a reduction in circulating maternal amino acid levels or a decrease in delivery of amino acids due to reduced placental blood flow. We have recently shown that in pregnant rats fed a low protein diet throughout pregnancy, the activity of placental system A was reduced before the development of growth restriction in the fetuses (76), strongly supporting down-regulation of placental system A as a causative factor in this model of IUGR. Therefore, information on factors regulating placental amino acid transporters as well as the underlying cellular mechanisms are important to better understand the pathophysiology of altered fetal growth and its links to adult disease.

Amino acids are not only important in intermediary metabolism, as building blocks for proteins and as energy substrates, they also play a key role in the regulation of cell function (70). For example, amino acids are potent modulators of insulin secretion and they are involved in the activation of the ribosomal protein S6 kinase (S6K1). Amino acid transporter overexpression may be involved in human disease, such as cancer and immune diseases (42). Therefore, the regulation of amino acid transporters needs further attention.

The human placenta

Placental function

The placenta is the main interface between the mother and the fetus. In intrauterine life, the placenta performs functions that in postnatal life are taken over by the lungs, the gastrointestinal tract, the kidneys, and the endocrine glands of the neonate. Its three primary functions are to provide an immunological barrier between the mother and fetus, produce and secrete hormones and cytokines, and mediate the transfer of nutrients, oxygen, and waste products.

Placental morphology

Placental blood flow is established at the end of the first trimester, when the trophoblast plugging of the maternal spiral arteries is released. The invasion of extravillous trophoblasts in these blood vessels helps to create a low-resistance conduit, which maximizes the blood flow to the intervillous space. Fetal blood enters the placenta through two umbilical arteries, which branch and form a capillary network in the villi. These villi are covered by the syncytiotrophoblast and are in direct contact with the maternal blood in the intervillous space. In order for nutrients in maternal blood to reach fetal blood, they are transported across two cell layers, the syncytiotrophoblast and the endothelium of the fetal capillaries. This type of endothelium allows for relatively unrestricted passage of nutrients, such as amino acids, through pores within the interendothelial cleft (106). Therefore, it is the syncytiotrophoblast with its two polarized plasma membranes, the microvillous and the basal plasma membranes, that constitute

the placental barrier, which limits the transplacental transport of for example amino acids.

Placental transport

The syncytiotrophoblast is a true syncytium, generated by fusion of underlying cytotrophoblast cells, and constitutes a relatively tight barrier, since there are no intercellular spaces available for transport. There is some evidence suggesting transfer across the syncytiotrophoblast via water-filled channels (the 'paracellular' pathway), however transcellular transport through the syncytiotrophoblast is the primary route for nutrient transport (Fig. 1).

Models of transport functions

The concentration of amino acids is higher in the fetus than in the mother, suggesting that amino acid transport in the human placenta is an active process (138). The energy-requiring step of placental amino acid transport is in the microvillous plasma membrane (MVM), since amino acid concentrations in the placenta are markedly higher than in both the mother and the fetus. This makes the uptake across the MVM an important factor in limiting the transplacental transfer of amino acids. This in turn allows modeling of maternal-to-fetal transport of amino acids in both healthy and complicated pregnancies from transporter expression and activity studies in isolated membrane vesicles, cultured primary trophoblast cells, and primary villous fragments.

General principles

Transplacental transport can be either flow-limited, i.e. limited by the blood flow transporting the molecule to and from the barrier, or diffusion-limited, i.e. limited by the rate of transfer across the membrane. Small molecules like O_2, CO_2, and urea can readily diffuse through plasma membranes, and are therefore limited by delivery to the plasma membrane, i.e. by blood flow. Transport of nutrients, like amino acids, is however limited by the transport functions of the plasma membrane. Specialized membrane transport proteins are responsible for transferring nutrients across plasma membranes. There are two major classes of plasma membrane transport proteins, carrier proteins (transporting most nutrients and some ions) and channel proteins (transporting some ions and water).

Placental transfer of glucose and lactate are examples of carrier-mediated passive transport (facilitated diffusion). The driving force for this type of transport is the concentration gradient of substrates and is not dependent on hydrolysis of ATP.

As in all cells, the electric potential of the interior of the trophoblast cell is negative with respect to the outside. Ions and other charged molecules, such as anionic and cationic amino acids, transferred across the placenta are therefore influenced both by the concentration gradient and the electrical gradient.

Active transport, defined as an energy-requiring transport of a solute against its concentration gradient, can be divided into primary and secondary active transport. Primary active transport directly utilizes energy from the hydrolysis of ATP. The Na^+/K^+-ATPase and the Ca^{2+}-ATPase are examples of primary active transporters in the placenta. Transport of amino acids constitutes secondary active transport, is mediated by carriers, and indirectly dependent on the energy stored in ATP. These transporters transfers the solutes across the membrane by undergoing reversible conformational changes, and this transfer is dependent on the transport of a second solute, which constitutes the driving force of the transporter. The second solute can either be transferred in the same direction as the first; these transporters are called symporters, or in the opposite direction, performed by antiporters.

Some molecules are transported across the plasma membrane by endocytosis, such as iron. Fe^{2+}transferrin in the maternal plasma binds to the transferrin receptor present on the MVM and the resulting complex is internalized (160). The iron is then released and transferred to the cytosol. Iron transfer mechanisms across the basal plasma membrane remain to be established.

Placental ion and nutrient transport

Ions

Both transcellular and paracellular routes for placental ion transport have been described (164). Sodium is the main extracellular cation and its steep electrochemical gradient across the plasma membrane is used as a driving force for the transport of a number of substances, such as amino acids. The Na^+/H^+ exchanger, which is involved in maintenance of intracellular pH, is primarily distributed to the MVM, and IUGR is associated with a reduced protein expression and activity of this transporter (88). The Na^+/K^+-ATPase transports sodium out of cells and is present in both plasma membranes of the syncytiotrophoblast, but polarized to the MVM (89). The MVM Na^+/K^+-ATPase activity is decreased in IUGR (90). Calcium is transported into the syncytiotrophoblast by channels across the MVM (28) and the Ca^{2+}-ATPase is the primary transporter that extrudes Ca^{2+} across BM into the fetal compartment (163). The activity of the BM Ca^{2+}-ATPase is increased in IUGR (162).

Glucose

About 1/3 of the glucose taken up by the placenta is metabolized and the rest is delivered to the fetus. A maternal-fetal glucose concentration gradient is present, and glucose is transported by facilitated diffusion. At term, GLUT1 is the major glucose transporter present in the human placenta and it is present in both MVM and BM, although polarized to the MVM (83). The activity and expression of placental glucose transporters are unaltered in IUGR (83, 86) and up-regulated in

insulin-dependent diabetes (IDDM) associated with accelerated fetal growth, but not in gestational diabetes (GDM) (78, 85).

Lipids

Fatty acids are circulating in maternal blood as triacylglycerols in lipoproteins or as free fatty acids bound to albumin. Maternal triacylglycerols are either taken up intact or hydrolyzed by placental lipases. Placental fatty acid transfer involves diffusion as well as membrane and cytosolic fatty acid binding proteins (Reviewed in (58)). It has been shown that the MVM activity of the lipoprotein lipase is reduced in IUGR (110).

Amino acid transport

Delivery of amino acids to the fetus has been shown to be an important determinant of fetal growth. Amino acids are not only critical for fetal protein synthesis, but are also important as insulin secretagogues and energy metabolites. As much as 20-40% of the total energy requirement for fetal growth supplied from the maternal circulation derives from amino acids (8). Some amino acids are essential and cannot be synthesized by the fetus, while others, which in adult-life are regarded as non-essential, can be regarded as conditionally essential because of the high rate of protein synthesis in the fetus. Amino acid transporters have been classified into distinct 'systems', depending on their substrate specificity, transporter mechanisms, and sodium dependence. This study has focused on three key amino acid transporters, a sodium-dependent transporter of neutral amino acids (system A), a sodium-independent transporter of neutral amino acids (system L), and a sodium- and chloride-dependent transporter, mediating the uptake of taurine (system β) (Fig. 1).

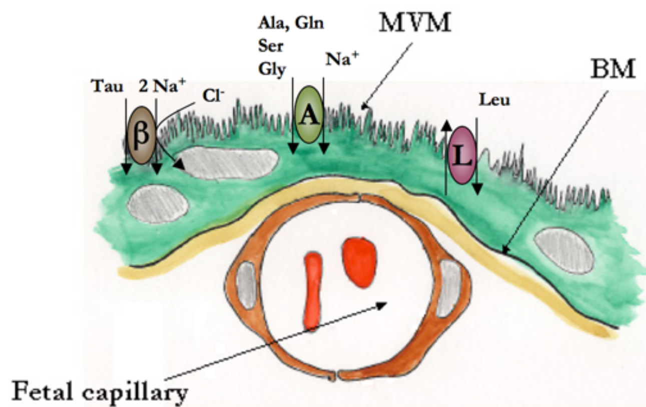

Fig. 1. Placental amino acid transporters. In the human placenta, transfer of nutrients from the maternal to the fetal circulation requires movement across two cell layers, the syncytiotrophoblast and the endothelium of the fetal capillaries. The syncytiotrophoblast has two polarized plasma membranes, the MVM and the BM and it is primarily the properties of these membranes that limit the transplacental transport of amino acids. Placental amino acid transport, mediated by transporter proteins in MVM and BM, is an active process resulting in fetal amino acid concentrations higher or much higher than maternal. The uptake of amino acids from the maternal blood into the syncytiotrophoblast cell constitutes the active step in placental amino acid transfer and the figure depicts three key placental amino acid transporter systems in this plasma membrane. The system β transports β-amino acids such as taurine, and system A mediates the uptake of neutral amino acids like alanine, serine, and glutamine. Both system A and system β are sodium-dependent. System L is sodium independent and transports neutral amino acids with bulky side chains, such as leucine. For simplicity, transporter proteins mediating the facilitated transfer across BM have not been included. Reproduced from Läkartidningen 2003 (84), with permission.

System A

System A mediates the cellular uptake of small, neutral amino acids with short, unbranched side-chains such as alanine, serine, and glycine, by co-transporting sodium (118). Three isoforms of the system A transporter are present in the human placenta, SNAT1, SNAT2, and SNAT4, which are encoded by the genes *Slc38a1*, *Slc38a2*, and *Slc38a4* (38, 63, 171). SNAT1 and 2 operate via similar mechanisms (63, 171), while SNAT4 has a lower affinity for neutral amino acids than SNAT1 and 2 and interacts with cationic amino acids in a sodium-independent manner resembling system y^+L (62). System A has a unique ability to transport MeAIB, a non-metabolizable amino acid analogue, which has been used extensively to study system A transporter activity in a wide range of cells and tissues, including the placenta. All isoforms interact with MeAIB, but SNAT4 more weakly than SNAT1 and 2 (62, 63, 171). System A activity, measured as Na^+-dependent MeAIB uptake, has been shown to increase over gestation in isolated MVM vesicles (112), whereas this gestational increase was not observed in primary villous fragments (46). Placental SNAT1 and 2 show no gestational changes in mRNA expression whereas SNAT4 mRNA expression is significantly higher in first trimester as compared with term tissue. In contrast, SNAT4 protein expression changes in the opposite direction with higher protein levels at term (38). These observations indicate that placental transporter gene expression, protein expression, and activity do not necessarily change in parallel, indicating regulation at the posttranscriptional and posttranslational level. SNAT2 is localized to the MVM (26) and SNAT4 transporters are present in

both MVM and BM (38). SNAT1 expression has not been previously investigated due to lack of antibodies.

System A activity is particularly important, because system A transporter substrates can be transported by the exchanger system L. System L transporters are obligatory exchangers, and can only exchange one amino acid molecule from outside the cell for one from the inside of the cell. As a result of system A activity, a steep outwardly directed concentration gradient of some non-essential amino acids, such as glycine, is created. Glycine molecules can bind to system L transporters on the inside of the cell and be exchanged for extracellular essential amino acids, and thus drive the uptake of essential amino acids against their concentration gradients.

The MVM activity of the system A transporter is reduced in IUGR (39, 54, 86, 113), a finding which has been confirmed in primary villous fragments (153). In fetal overgrowth associated with diabetes, MVM activity of system A is up-regulated (77). However, in one study, system A activity has been shown to be reduced in MVM from pregnancies where the mothers had insulin-dependent diabetes (104). The reason for these discrepancies are unclear, but may be related to the different study populations which is supported by the fact that placental weight was increased in one study (77) and unaffected in the other (104).

System L

System L transporters function as exchangers and transport branched-chain and aromatic amino acids, such as leucine, phenylalanine, isoleucine, and tyrosine, independent of sodium (118). System L is composed of two subunits, one catalytic light chain and the heavy chain, 4F2hc, encoded by the gene *Slc3a2*, which is critical for the trafficking of the light chain to the plasma membrane. These two subunits are covalently linked through a disulfide bridge to form a heterodimer. Two isoforms of the light chain subunit are expressed in the placenta, LAT1 and LAT2, which are encoded by the genes *Slc7a5* and *Slc7a8* (139, 146). The exact localization of the light chain proteins is however not clear. On the basis of functional studies, Kudo & Boyd (100) have suggested that LAT1 is present in MVM and LAT2 in BM, whereas others have suggested the opposite (29, 107). The LAT1 protein has been detected in MVM and its protein expression increases over gestation (130). The heavy chain has been observed in both membranes (130), whereas the protein localization of LAT2 is currently unknown. LAT1/4F2hc transports large-chain neutral amino acids while LAT2/4F2hc has a broader specificity as it transfers large neutral amino acids as well as alanine, serine, glycine, and glutamine (22). System L amino acid transport mediated by LAT1 and LAT2 is inhibited by BCH. Recently LAT3 and LAT4 have been demonstrated at the mRNA level in the human placenta (12, 30) and they have been suggested to be involved in the transport of amino acids over BM.

The activity of system L is decreased in both plasma membranes of the syncytiotrophoblast in IUGR (82) and the activity is increased in MVM vesicles isolated from placentas from mothers with gestational diabetes giving birth to LGA infants (77).

System β

System β, encoded by the gene *Slc6a6*, transports β-amino acids such as taurine and β-alanine together with sodium and chloride in a 2:1:1 Na^+:Cl^-:taurine stoichiometry (142). Taurine can be regarded as an essential amino acid during fetal life, because the fetus lacks the enzyme cysteine-sulfinic acid decarboxylase needed to synthesize taurine. Animal experiments have shown that taurine deficiency during pregnancy is associated with growth failure (165). Functional studies have suggested that the taurine transporter is almost exclusively polarized to MVM (128). The taurine transporter activity is decreased in MVM in IUGR (128).

Alterations in activity of nutrient and ion transporters in MVM and BM of placentas from IUGR pregnancies and from pregnancies associated with fetal overgrowth in diabetes are summarized in Table 1 and Table 2, respectively.

Table 1. *Changes in activity of transporters in the microvillous (MVM) and basal (BM) plasma membrane of placentas from IUGR pregnancies as compared with normal pregnancies*

Transporter	MVM	BM	Ref
System A	↓	-	(39, 54, 86, 112)
System L	↓	↓	(82)
System β	↓	-	(128)
GLUT1	-	-	(83)
Na^+/K^+-ATPase	↓	-	(90)
Ca^{2+}-ATPase	Not measured	↑	(162)
Na^+/H^+ exchanger	↓	Not measured	(54, 88)
Lipoprotein lipase	↓	Not measured	(110)

Increased (↑), no change (-), or reduced (↓) transporter activity.

Table 2. *Changes in activity of transporters in the microvillous (MVM) and basal (BM) plasma membrane of placentas from pregnancies complicated by diabetes and fetal overgrowth as compared with normal pregnancies*

Transporter	MVM	BM	Ref
System A	↑	-	(77)
System L	↑[a]	-	(77)
System β	-	-	(77)
GLUT1	-	↑[b]	(78, 85)
Na^+/K^+-ATPase	-	-	(135)
Ca^{2+}-ATPase	Not measured	↑[b]	(162)
Na^+/H^+ exchanger	-	Not measured	(104)
Lipoprotein lipase	↑[b]	Not measured	(110)

Increased (↑), no change (-), or reduced (↓) transporter activity.
[a] Only GDM
[b] Only IDDM

Regulation of amino acid transport

In order to understand the mechanisms underlying abnormal fetal growth, it is critical to identify the factors regulating placental nutrient transporters. System A is subjected to extensive regulation, whereas information is lacking regarding the transporters for essential amino acids.

System A

Several hormones and growth factors have been shown to stimulate placental system A transporter activity in cultured trophoblasts and placental explants, including insulin, IGF-I, EGF, and leptin (11, 75, 93, 94, 99, 123). System A is also under hormonal control in L6 myotubes (69), hepatocytes (56), and human skeletal muscle (13). It is well established that the activity of system A is up-regulated in response to amino acid deprivation, a phenomenon called adaptive regulation. This has been demonstrated in many cell types, such as hepatocytes (15), L6 myotubes, adipocytes (68), and rat C6 glioma cells (108), as well as trophoblast cells (92). In trophoblast cells, SNAT1 and SNAT2 mRNAs are differentially regulated by amino acid deprivation. SNAT2 mRNA expression is up-regulated by depriving cells of amino acids, whereas SNAT1 mRNA is down-regulated by depriving cells of non-essential amino acids. Both angiotensin II concentrations and oxygen levels have been shown to affect system A activity in vitro (125, 154). Angiotensin II has been reported to decrease system A activity

through AT1-R activation, the authors did however not investigate the effect on the different transporter isoforms (154). Low oxygen levels decrease system A activity through down-regulation of both SNAT1 and SNAT2 mRNAs (125). The effect of glucocorticoids on system A activity seems to be dependent on length of exposure. In the BeWo cell model, 24-h cortisol treatment increases system A activity and SNAT2 mRNA expression (91), while 1-h cortisol exposure had no effect on system A uptake in term primary villous fragments (75). In isolated rat hepatocytes, glucocorticoids have been shown to enhance system A activity (118). Information on system A regulation by cytokines is lacking, although there is one study demonstrating that incubating BeWo cells with IL-1β results in a decrease in system A activity as well as a decrease in SNAT1 and SNAT2 mRNA expression (166). Incubating primary villous fragments with SIN-1 which releases NO and O_2^- has been shown to inhibit system A uptake, which was proposed to be a direct effect of free radicals on the transporter (95). In a rat model, brief hyperglycemia in early pregnancy has been shown to reduce placental system A activity in late gestation (47). In diabetes and a model of obesity, system A activity has been shown to be up-regulated in liver and skeletal muscle (118).

System L

Regulation of system L has not been extensively studied. It has been reported that lowering the extracellular pH, treating cells with the PKC activator PMA, and calmodulin antagonists all stimulate system L transport in the human placental choriocarcinoma cell line JAR (16, 140). However, system L activity in placental membrane vesicles does not seem sensitive to changes in extracellular pH (100). Combined treatment of BeWo cells with PMA and a calcium ionophore stimulated system L activity by increasing mRNA and protein expression of both 4F2hc and LAT1 (130). To our knowledge, hormonal regulation of placental system L has not been studied. Prolonged treatment of Caco-2 cells with EGF and a phorbol ester stimulated system L activity (131) and system L activity in CHO cells was stimulated by lowering the extracellular pH (156).

System β

The regulation of system β in the human placenta is not well established. Incubation of MVM vesicles with Ca^{2+} has been shown to inhibit taurine uptake by decreasing the affinity of the transporter for taurine as well as reducing the rate of the transporter (103). Treatment of JAR cells with PMA and cyclosporine A inhibit taurine uptake (102, 141) as well as treating primary villous fragments with SIN-1 (95). The taurine transporter has also shown adaptive responses. Exposure of JAR cells to taurine decreased taurine transporter activity and mRNA expression (87). In cell lines, TAUT activity has been shown to be regulated by, for example, cytokines, glucose, and nitric oxide (17, 27, 161).

Mammalian target of rapamycin (mTOR)

An increasing body of evidence suggest that mTOR is a central controller of cell growth (151) by regulating translation, actin organization, nutrient transporter trafficking, and transcription in response to nutrients. Recent reports have indicated a role for mTOR in determining organ and organism size. Studies in *Drosophila melanogaster* have shown that a decrease in the TOR signaling pathway in the fat body (an organ comparable to mammalian fat and liver tissue) causes a decrease in overall body size (32). In the rat, activation of mTORC1 in the hypothalamus controls feeding behavior (33). Dysregulation of the mTOR pathway has been found in many human tumors (36). In addition, mTOR signaling has recently been shown to link nutrient overload to insulin resistance, an effect mediated by inhibitory phosphorylation of IRS-1 (insulin receptor substrate-1) (Reviewed in (61, 168)).

Downstream targets of mTOR

The target of rapamycin (TOR) is a large protein of about 290 kDa, which was discovered in *Saccharomyces cerevisiae* as the target gene of the growth arresting effects of the immunosuppressant drug rapamycin. It is a serine/threonine protein kinase which has been shown to regulate cell growth (i.e. accumulation of cell mass) by regulating transcription and translation (reviewed in (73, 175)). In mammalian cells, inhibition of TOR with rapamycin decreases the phosphorylation of Thr-389 of the ribosomal protein S6K1 and induces hypophosphorylation of the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) at Thr-37/46, Ser-65, and Thr-70, resulting in inhibition of the cap-binding protein eIF4E (Reviewed in (65)). Exactly how S6K1 regulates cell growth is unclear, but it has been proposed that the translational regulators eEF2 kinase and eIF-4B are S6K1 substrates (143, 172).

mTOR signals through two complexes, mTORC1 and mTORC2 (Fig. 2). mTORC1 consists of raptor (rapamycin-sensitive adaptor protein of mTOR), the G protein β-subunit-like protein (GβL), the proline-rich protein kinase B substrate 40 kDa (PRAS40), and mTOR (36), and mTORC1 is inhibited by the drug rapamycin and mediates temporal control of cell growth. Temporal control refers to mTOR-regulated processes that determine cell mass accumulation. mTORC2, which consists of rictor (rapamycin-insensitive companion of mTOR), GβL, mammalian stress-activated protein kinase-interacting protein-1 (mSIN1), and mTOR (36), has been suggested to be insensitive to rapamycin (148) and regulate spatial aspects of growth. Spatial control refers to the cell-cycle-dependent regulation of the actin cytoskeleton. Recent findings have however showed that long-term treatment with rapamycin also inhibits mTORC2 (149) and rictor may also be regulated by rapamycin (2). mTORC2 does not phosphorylate S6K1 or 4E-BP1, but has instead been shown to activate Akt (150) and control the actin cytoskeleton (148).

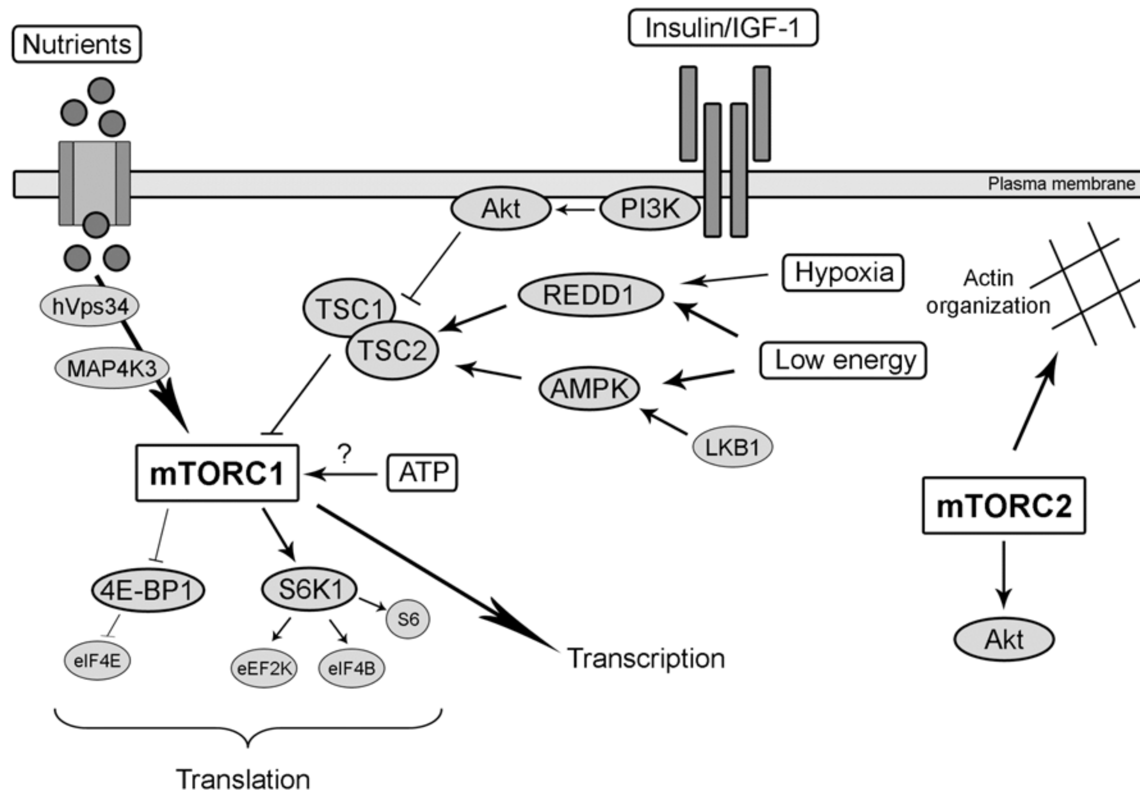

Fig. 2. mTOR Signaling. mTOR integrates nutrient and growth factor signaling (and possibly a number of other upstream signals) to control cell growth and metabolism. mTOR forms two complexes in the cell, mTORC1 and mTORC2. In response to growth factors, Akt phosphorylates and inactivates TSC1/TSC2, allowing for mTORC1 activation. Low energy inhibits mTORC1 by activating AMPK or REDD1, which phosphorylates and activates TSC1/TSC2. The pathway by which nutrients, such as amino acids, activate mTOR remain poorly understood, but has been proposed to involve hVps34 and MAP4K3. mTORC1 controls protein synthesis by phosphorylating and activating S6K1 and by phosphorylating and inactivating the translational inhibitor 4E-BP1. mTORC2 phosphorylates Akt and controls actin organization. The upstream regulators of mTORC2 are currently unknown. Arrows and bars represent activation and inhibition respectively.

Upstream regulators of mTOR

mTOR is a central integrator of various signals such as growth factors, amino acids, glucose, energy status, and many forms of stress. Activation of mTOR by growth factor signaling is perhaps the best studied and it is mediated by the activation of PI3K and Akt by receptor phosphorylation of IRS-1. Akt then phosphorylates the TSC1/2 complex leading to its inactivation (175). Activated mTORC1 controls protein synthesis by phosphorylating 4E-BP1 and S6K1 (65), transporter trafficking and thereby nutrient uptake (44), and the transcription of many genes (134).

Nutrient levels, especially amino acids, represent another major mTOR signaling input through mechanisms that still have not been completely identified. Recent evidence suggests that hVps34, a class III PI3K, and a MAP4 kinase may be involved in the regulation of mTOR by nutrient availability (21, 50, 127). The Rag proteins have also recently been shown to mediate the amino acid signal to mTOR (97, 147). The relationship between the Rag proteins and

hVps34 remains unclear, whereas MAP4K has been suggested to be either upstream or downstream of hVps34. Glucose may regulate mTOR either through hVps34 (21) or through energy production in the form of ATP. Low levels of ATP activate AMPK, which leads to inactivation of mTOR. Energy depletion phosphorylates TSC2 at sites distinct from those phosphorylated by Akt (72). mTOR has also been proposed to be a homeostatic ATP sensor (37), which is directly regulated by intracellular ATP levels. There is also evidence for AMPK independent inhibition of mTORC1 after energy depletion, through the hypoxia-inducible gene REDD1 (regulated in development and damage responses 1), which also seems to require TSC2 (159). Oxygen has also been shown to modulate mTOR pathway activity, as hypoxia causes a decrease in mTOR signaling, independent of both HIF1-α and AMPK (5), but requiring TSC1/TSC2 and REDD1 (20).

mTOR and amino acid transporters

The regulation of nutrient transporters by mTOR has recently been summarized in an excellent review by Edinger (42). For example, it has been shown that treating human BJAB B-lymphoma cells with rapamycin decreases the mRNA expression of five amino acid transporters (134) and the PDGF stimulated expression of LAT1 mRNA in rat vascular smooth muscle cells is dependent on mTOR (109). Cell surface expression of 4F2hc in the FL5.12 cell line is dependent on mTOR (44, 45), but may be insensitive to rapamycin (43) suggesting involvement of not only mTORC1 but also mTORC2. This effect seems to be conserved from *Drosophila* to humans, because it was recently shown that TOR stimulation results in accumulation of a cationic amino acid transporters at the cell surface in the fat body of *Drosophila* (66). Little is known of the effect of mTOR on amino acid transporter activity, but leucine-stimulated increase in system A activity in L6 myotubes is inhibited by rapamycin, although there was no effect on basal system A activity (137). In a leucine-dependent yeast strain, rapamycin inhibits both growth and leucine uptake (173) and in budding yeast rapamycin inhibits the import of tryptophan (9).

mTOR and the placenta

There are some studies that have investigated the role of mTOR in the placenta, and it has been shown that mTOR is critical for early growth and proliferation, because deletion of the mTOR gene leads to lethality (52, 121). Likewise, the development of trophoblast cell motility and initiation of implantation is stimulated by amino acid signaling through mTOR (116). Studies in immortalized cell lines originating from human trophoblast suggest that glucose and growth factor induced trophoblast cell proliferation is mediated through mTOR activation (174). In 2002 it was shown that mTOR is present at the mRNA level in the mature placenta (96), but there is no information of its cellular localization and/or functional role.

RATIONALE

Optimal growth is of critical importance for the developing fetus. Today, as many as 15% of all pregnancies result in abnormal growth of the fetus, either IUGR or fetal overgrowth. These pregnancy complications are important as they increase the risk of perinatal morbidity and make the baby susceptible to develop metabolic abnormalities such as obesity, Type 2 diabetes, and cardiovascular disease in childhood and adult life. There is currently no specific treatment for abnormal fetal growth. Recent research has implicated placental amino acid transporters as key regulators of fetal growth. In order to understand the mechanisms underlying altered fetal growth, information on the factors regulating placental amino acid transporters is needed. These factors are largely unknown, in particular with regard to transporters for essential amino acids.

AIMS

Overall aim and central hypothesis

The overall aim of the present study was to investigate the mechanisms regulating placental amino acid transporters. The central hypothesis was that placental mTOR acts as an integrator of maternal nutrient signals and growth factors and regulates amino acid transporters in the human placenta in response to changes in nutrient/growth factor availability to coordinate fetal growth with maternal nutrient supply.

Specific aims and hypotheses

1. To investigate the regulation of the taurine transporter (TAUT) in the human placenta.
   - *Hypothesis*: Placental TAUT is regulated by mTOR and by hormones, which have been reported to be altered in fetal and/or maternal plasma in IUGR.

2. To determine the effect of inhibition of placental mTOR on amino acid transport.
   - *Hypothesis*: Placental System A, system L, and TAUT activity is positively regulated by the mTOR signaling pathway.

3. To establish the effect of long-term inhibition of placental mTOR on amino acid transport.
   - *Hypothesis*: The placental mTOR pathway alters system A, system L, and TAUT transport by inducing changes in the protein and mRNA expression of the transporters.

4. To identify the upstream regulators of placental mTOR.
   - *Hypothesis*: Glucose, insulin, and IGF-I regulate placental system A, system L, and TAUT activity by inducing changes in mTOR signaling.

METHODOLOGICAL CONSIDERATIONS

Patient selection and tissue collection

Placental tissue was collected at the Sahlgrenska University Hospital with informed consent and was approved by the Committee for Research Ethics at the University of Gothenburg. Placentas were obtained after either vaginal or cesarean delivery from healthy women delivering babies of normal birth weight (appropriate-for-gestational-age, AGA), from pregnancies complicated by fetal overgrowth (resulting in a large-for-gestational-age, LGA, baby) or intrauterine growth restriction (IUGR). Early-second-trimester tissue was obtained at terminations. AGA was defined as a birth weight between -2 SD and +2 SD using intrauterine growth curves for a Scandinavian population based on ultrasonically estimated fetal weight (115). IUGR was defined as a birth weight more than 2 SD below the mean for gestational age. In order to decrease the risk that genetically or constitutionally small babies were included in the IUGR group, the presence of one or more signs of fetal compromise (such as increased umbilical artery pulsatility index, oligohydramnios, low ponderal index, and an intrauterine growth deviation observed with serial ultrasound) were used as additional criteria. Pregnancies with other complications than IUGR (such as preeclampsia), cases with chromosomal abnormalities, and other IUGR pregnancies with an identifiable etiology to the growth restriction were excluded. Thus, the IUGR cases under study may be characterized as "idiopathic", i.e. IUGR without known cause (53), and were assumed to primarily be due to uteroplacental insufficiency. LGA was defined as a birth weight more than 2 SD above the mean for gestational age (115) and LGA placentas were only obtained from pregnancies without Type 1 diabetes or gestational diabetes.

Immunohistochemistry

Immunohistochemistry is a technique that localizes antigens in a tissue section. The basic principle is the use of enzyme-linked antibodies to detect the antigens. The colorless substrate is converted by cleavage of the enzyme into a colored product that precipitates on the slide at the site of the reaction.

Placental tissue samples were either rinsed in ice-cold physiological saline and fixed in a zinc solution or freshly frozen in liquid nitrogen. After zinc-fixation, the tissue was embedded in paraffin, cut into 4-μm sections, and mounted on positively charged slides. Fresh-frozen tissue samples were cut into 7-μm sections, mounted on positively charged slides, and stored at -20°C. Immunohistochemistry was then performed as described in detail in Papers I and II. Immunoreactivity was visualized using 3,5-diaminobenzidine (DAB) (paraffin-embedded sections) or Qdot® secondary antibody conjugates (fresh-frozen sections). Qdot® nanocrystals are fluorophores and when conjugated to a

secondary antibody, they enable multicolor analysis of immunochemical applications, such as co-localization studies. Antibodies used in immunohistochemistry are listed in Table 3.

Table 3. *Antibodies used in immunohistochemistry*

Primary antibody	Dilution	Ref/Company	Secondary antibody	Dilution
taurine transporter	1:100, 1:200	(59)	goat anti-rabbit IgG	1:300
mTOR	1:25	Abcam, Cambridge, UK	goat anti-rabbit IgG	1:300, 1:100
cytokeratin 7	1:200	Dako Sweden AB, Stockholm, Sweden	goat anti-mouse IgG	1:100

Isolation of plasma membrane vesicles

The method used for isolation of syncytiotrophoblast microvillous and basal plasma membranes from human placenta was first described by Illsley and co-workers (71) and is described in detail in Paper I. Briefly, the method uses low-speed centrifugation to remove tissue debris and then high-speed centrifugation to pellet the plasma membranes. To separate MVM from other membranes, $MgCl_2$ is used, because Mg^{2+} forms an aggregate with all membranes except brush-border membranes. BM is then further purified by means of a sucrose step gradient centrifugation.

This method allows for the separation of MVM and BM from the same placenta and protein expression of amino acid transporters in these plasma membrane fractions can subsequently be investigated independently.

Amino acid transporter activity in primary villous fragments

Using primary villous fragments as an experimental model when studying placental amino acid transport has the advantages that there is no need for prior culture of the fragments, which could alter the characteristics of nutrient transporters, and the polarization of the syncytiotrophoblast and cell-to-cell contacts are likely to resemble the *in vivo* situation.

System A, system L, and taurine transporter activity was measured according to a method developed for the system A amino acid transporter (75) and detailed information can be found in Papers I and II. Hormonal and mTOR regulation of system A, system L, and taurine transporters were assessed by incubating fragments with the effectors stated in Table 4.

Table 4. *Effectors used in transporter activity assays in placental villous fragments*

Effectors under study	Working concentration
Chelerythrine	1,920 ng/ml
EGF	600 ng/ml
GH	500 ng/ml
Glucose	5.4 mg/ml
IGF-I	250 ng/ml
IGF-II	250 ng/ml
IL-1β	20 ng/ml
IL-6	30 ng/ml
TNF-α	20 ng/ml
Leptin	500 ng/ml
PMA	617 ng/ml
Rapamycin	20 and 91 ng/ml
SIN-1	620 μg/ml

Trophoblast cell culture

Placental tissue is readily available, making it fairly easy to isolate cytotrophoblast cells. These cells serve as precursor cells for the syncytiotrophoblast and are located basally of the syncytiotrophoblast. With the addition of growth factors, these cells undergo differentiation and form syncytiotrophoblast-like monolayers when cultured. Trophoblast cells can be very useful for studying placental function, such as the transport of amino acids.

Trophoblast cells were isolated from human placentas using a method developed by Kliman et al. (98), which has been modified further by Greenwood and co-workers (55), and established in our lab (111). About 50 g of starting tissue was minced and then transferred to Hank's balanced salt solution (HBSS) containing the digestion enzymes trypsin (0.25%) and DNase I (0.2 mg/ml). Trypsin in this case is not purified trypsin, but a crude mixture of proteases, polysaccharidases, nucleases, and lipases extracted from porcine pancreas. DNase is weakly digestive; its main function is rather to avoid cell clumping caused by DNA released from ruptured cells. Tissue digestion was carried out by incubating the suspension in a shaking water bath at 37°C. To neutralize the trypsin, the cell suspension was carefully layered over newborn calf serum, and

centrifuged at 2,200 rpm for 10 minutes. All pellets were pooled and spun down and the cytotrophoblast cells were then separated out using a discontinuous Percoll density gradient and the cells banding between 35% and 55% Percoll were collected and plated at a density of approximately 1.5×10^6 cells in 6-well plates or 10×10^6 in 25 cm^2 flasks. Cells were maintained in a 1:1 mixture of DMEM and Ham's F12 culture medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 50 μg/ml gentamicin, 60 μg/ml benzylpenicillin, and 100 μg/ml streptomycin. The cells were cultured in a humidified incubator at 37°C in 5% CO_2-95% air. On the day after isolation, the cytotrophoblast cells were washed two times with 37°C Dulbecco's PBS (DPBS) with Mg^{2+} and Ca^{2+} and fresh medium was added. Thereafter the medium was changed daily and cells were cultured until 90 h. A media sample was collected each day and assessed for hCG content, which is a syncytial biochemical marker. After 90 h in culture, total cell lysates were analyzed by Western blot for the expression of the trophoblast marker cytokeratin 7 (clone OVTL 12/30) and the mesenchyme-marker vimentin. To study the presence of apoptosis in cells, the expression of active caspase-3 and cleaved PARP was measured. Lactate dehydrogenase (LDH) release into the medium after 90 h of culture was assessed using an LDH-based in vitro toxicology assay. LDH release measured after cells had been subjected to sonication was used as a positive control.

When using cell culture as an experimental system, one has the advantage that the milieu, such as temperature, pH, oxygen, and media, can be controlled for. Using a single cell type makes it possible to test the direct effect of, for example, a drug, without the potential secondary effects of other cell types, hormones, and plasma metabolites. Trophoblast cell culture also allows the study of molecular mechanisms.

Amino acid transporter activity in trophoblast cells

Amino acid transporter activity studies were performed according to the previously developed protocol for amino acid uptake by system A in primary villous fragments (75), which we modified for use in cultured trophoblast cells. Cells were plated in 6-well plates (~1.5×10^6 cells/well) and cultured for 66 h. At this time point, cells were washed once with DPBS, and media was changed to media containing the specific mTOR inhibitor rapamycin (100 nM), various glucose concentrations (0.5 mM, 4.5 mM, or 16 mM), insulin (60 ng/ml) or IGF-I (300 ng/ml). After another 24 h in culture, cells were washed twice with 3 ml 37°C Tyrode's solution with or without Na^+, and then incubated for variable times up to 10 minutes with 1.5 ml Tyrode's solution (with or without Na^+ and 1 mM BCH) containing ^{14}C-MeAIB and ^{3}H-leucine or ^{3}H-taurine, in final concentrations of 10 μM, 50 nM, and 25 nM, respectively. Uptake was terminated by washing the cells three times with ice-cold sodium free Tyrode's solution. Cells were lysed with distilled water for 1 h, and then denaturated with 0.3 M NaOH for 2 h or over night. Protein concentrations were determined

using the Bio-Rad protein assay or a BCA protein assay kit. System A and taurine transporter activity was measured as Na^{+}-dependent ^{14}C-MeAIB or ^{3}H-taurine uptake, respectively, and system L amino acid transporter activity was determined as the BCH-inhibitable uptake of ^{3}H-leucine. Time courses of taurine, leucine, and MeAIB uptake in cytotrophoblast cells were performed to define the linear portion of the uptake, subsequently an 8 min incubation time was chosen.

Western blotting

Western blotting is an immunodetection method that can be used to determine a number of important characteristics of protein antigens, including the presence and relative quantity of an antigen and the molecular weight of the antigen. The first step of the blotting procedure is a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates individual proteins by size, shape and charge. The separated proteins are then transferred to a nitrocellulose paper (blotting) for detection (probing) with specific antibodies.

Tissue/cell preparation

Placental tissue was homogenized using a Polytron (15,000 rpm for 2 minutes), and then centrifuged (12,000 rpm, 15 minutes, +4°C) to enrich cytosolic components in the supernatant (Paper II). Isolated trophoblast cells were harvested using a cell scraper in buffer D containing protease inhibitors, EDTA, and freshly added phosphatase inhibitor cocktails in Paper III. The cell lysate was then homogenized by passing the lysate several times through a 20-gauge needle fitted to a syringe. In Paper IV, cells were washed twice with ice-cold PBS, and then collected using a cell scraper in an ice-cold cell lysis buffer containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 1% Triton X-100, and 1 mM dithiothreitol (DTT), pH 7.2, with freshly added phosphatase inhibitor cocktails 1 and 2 and a protease inhibitor cocktail. After homogenization by passing the lysate five times through a 20-gauge needle fitted to a syringe, total cell lysate was sonicated four times for 10 seconds.

Protein concentrations were determined using the Bradford assay and samples were prepared in 3× sample buffer (mTOR) containing DTT (SNAT4 and TAUT), Laemmli buffer (SNAT2 and TAUT), or the NuPAGE® 4× LDS sample buffer and NuPAGE® sample reducing agent (S6K1, 4E-BP1, 4F2hc, LAT2, cytokeratin, vimentin, AMPKα, LKB1, caspase-3, cleaved PARP, and REDD1). Fifteen (P-Thr-70-4E-BP1 and TAUT), 20 (P-Thr-389-S6K1, S6K1, P-Thr-37/46-4E-BP1, 4E-BP1, SNAT2, SNAT4, TAUT, 4F2hc, LAT2, mTOR, cytokeratin, vimentin, caspase-3, cleaved PARP, and REDD1), or 30 (P-AMPKα) μg of protein was separated on pre-cast 4-12% Bis-Tris gels (S6K1, 4E-BP1, SNAT2, SNAT4, TAUT, 4F2hc, LAT2, cytokeratin, vimentin, AMPKα, LKB1, caspase-3, cleaved PARP, and REDD1) with the use of NuPAGE® MES or MOPS buffer as appropriate (the MES buffer is

recommended for resolving small proteins, while the MOPS buffer is used for resolving medium to large size proteins) or on 7% (TAUT, mTOR) SDS-polyacrylamide gels with the use of a Tris/glycine electrophoresis buffer and then transferred onto a nitrocellulose membrane. The membranes were blocked in 5% milk-TBS/PBS and then incubated with the antibodies listed in Table 5. Proteins were detected by incubating membranes with either Amersham (GE Healthcare, Uppsala, Sweden) ECL chemicals or the Super Signal Western Dura Substrate (Pierce, Rockford, IL). Relative densities of the bands were established by densitometry using the Multi Gauge Analyses Software (version 3.0, Fuji Film).

Table 5. *Antibodies used in Western blotting*

Primary antibody	Dilution	Reference/Company	Secondary antibody	Dilution
TAUT	1:4,000	Chemicon, Temecula, UK	goat anti-rabbit IgG	1:1,000
mTOR	1:2,000	ab2732, Abcam	goat anti-rabbit IgG	1:1,000
Cytokeratin 7 (clone OVTL 12/30)	1:150	ab9098, Abcam	horse anti-mouse IgG	1:5,000
Vimentin	1:500	ab20346, Abcam	horse anti-mouse IgG	1:5,000
Caspase-3 (active)	1:2,000	AF-605-NA, R&D Systems, Minneapolis, MN, USA	horse anti-goat IgG	1:2,000
Cleaved PARP	1:200	PA1-26430, Affinity BioReagents, Golden, CO, USA	goat anti-rabbit IgG	1:2,000
P-Thr-389-S6K1	1:500, 1:1,000	Cell Signalling Technology (CST), Beverly, MA, USA	goat anti-rabbit IgG	1:1,000
S6K1	1:1,000	CST	goat anti-rabbit IgG	1:3,000
P-Thr-70-4E-BP1	1:1,000	CST	goat anti-rabbit IgG	1:1,000
P-Thr-37/46-4E-BP1	1:1,000	CST	goat anti-rabbit IgG	1:1,000
4E-BP1	1:1,000	CST	goat anti-rabbit IgG	1:3,000
GLUT1	1:10,000	Chemicon	goat anti-rabbit IgG	1:7,000
SNAT2	1:4,000	(108)	goat anti-rabbit IgG	1:1,000
SNAT4	1:4,000	Raised against: YGEVEDELLHAYSKV	goat anti-rabbit IgG	1:3,000
4F2hc	1:400	Santa Cruz Biotechnology, Santa Cruz, CA, USA	donkey anti-goat IgG	1:3,000
LAT2	1:2,000	(133)	goat anti-rabbit IgG	1:3,000
P-Thr-172-AMPKα	1:500	CST	goat anti-rabbit IgG	1:2,000
AMPKα	1:1,000	CST	goat anti-rabbit IgG	1:2,000
P-Ser-428-LKB1	1:500	CST	goat anti-rabbit IgG	1:2,000
REDD1	1:50	Ab 63059, Abcam	goat anti-rabbit IgG	1:3,000
β-actin (clone AC-74)	1:2,000	Sigma-Aldrich, Schnelldorf, Germany	horse anti-mouse IgG	1:5,000

Real-time reverse transcriptase quantitative PCR

PCR is a technique for amplifying specific regions of DNA present in a tissue or cells. Quantitative real-time RT-PCR refers to a PCR that is quantifiable, run in real time on cDNA converted from RNA.

mRNA isolation

Total RNA was extracted from trophoblast cells using the RNA STAT-60 protocol provided by the manufacturer (Nordic BioSite AB), with an extra RNA wash step. RNA concentration was calculated by determining absorbance at 260 nm and purity was monitored by the A260/A280 ratio. All samples ranged in concentration from 0.60 μg/μl to 2.44 μg/μl and had A260/A280 ratio of > 1.9 when diluted in water, which means that the samples are pure. A lower ratio indicates protein or DNA contamination of samples. The quality of the RNA samples was determined by electrophoresis through 1% agarose gels containing ethidium bromide, and the 18S and 28S RNA bands were visualized under UV light. Samples were diluted further to 0.5 μg/μl.

RT-PCR

Synthesis of first strand cDNA was performed with 2 μg of total RNA using a SuperScript™ II reverse transcriptase kit, random primers, and a deoxynucleoside triphosphate set (dNTP) in a final volume of 40 μl as described in detail elsewhere (10). The cDNA was then diluted to 100 μl with water and stored at -20°C.

Quantitative real-time RT-PCR

The PCR reaction is performed by temperature cycling. Each cycle starts with an incubation at high temperature (95°C) to separate the strands, the temperature is then lowered to allow the primers to anneal to the template, and then set at 72°C, at which temperature the primers are extended by dNTP incorporation, i.e. the product is formed. The generation of the product is determined by measuring the SYBR Green I fluorescence signal. Unbound SYBR Green I exhibits very little fluorescence, but during elongation SYBR Green I dye molecules bind to the newly synthesized DNA, enhancing the fluorescence. The increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded product formed. After the PCR has been completed, a melting curve analysis can be performed to prove that only the desired PCR product has been amplified. This is done by increasing the temperature gradually to 95°C, which is the temperature when the double stranded DNA separates, making the dye come off and fluorescence drops rapidly. The PCR product can also be run on a 1.5% agarose/0.5X TBE gel containing ethidium bromide to confirm that the product is of expected size. SYBR Green I is a fairly inexpensive assay to run, which is an advantage when wanting to test the expression of a number of

genes. However, SYBR Green I binds to all double-stranded DNA, including genomic DNA, making primer design important. If primers are designed to span over long introns, genomic DNA cannot result in a PCR product.

Oligonucleotide primers for SDHA and TBP were designed using the Primer3 program (v0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). SNAT1, SNAT2, SNAT4, SDHA, LAT1, LAT2, 4F2hc, and TBP primers were synthesized by Cybergene AB (Huddinge, Sweden) and are summarized in Table 6. For detection of TAUT (NM_003043) mRNA, the Hs_SLC6A6 QuantiTect Primer Assay from Qiagen was used. For Cybergene synthesized primers, real-time PCR reactions were performed in 20 μl mixtures, containing 2 μl cDNA (diluted 1:4) and 18 μl FastStart SYBR Green I PCR Mix. For the detection of TAUT, real-time PCR was performed in 20 μl using the QuantiFast SYBR Green PCR Kit. The mitochondrial protein succinate dehydrogenase complex, subunit A (SDHA) and the TATA box binding protein (TBP) served as internal controls (119). All samples were assayed in duplicate and water was used as a negative template control. A standard curve for each gene product was generated using a dilution series of cDNA (1:2-1:32).

The amplification transcripts for each gene were quantified using the relative standard curve. The standard curve was obtained by plotting the threshold cycle (C_T) on the y-axis and the log concentration on the x-axis. The slope and the intercept were then used to calculate the relative amount of each gene according to the formula: $10^{(Intercept - C_T)/slope}$. The R^2 values for the standard curves were between 0.95 and 1 and the amplification efficiencies (given by the slope of each standard curve) were between -3.3 and -3.8. The relative amounts of target genes were normalized against SDHA and TBP by calculating a normalization factor by averaging SDHA and TBP using the geometric mean. Reporting the amount of a particular gene of interest relative to a housekeeping gene helps to avoid sample-to-sample variation.

Table 6. *Primers used in PCR amplification*

Gene name	Oligonucleotide sequence	Accession no	Reference
Slc38a1 (SNAT1)	forward: 5'-GTGTATGCTTTACCCACCATTGC-3' backward: 5'-GCACGTTGTCATAGAATGTCAAGT-3'	NM_030674	(38)
Slc38a2 (SNAT2)	forward: 5'-ACGAAACAATAAACACCACCTTAA-3' backward: 5'-AGATCAGAATTGGCACAGCATA-3'	NM_018976	(38)
Slc38a4 (SNAT4)	forward: 5'-TTGCCGCCCTCTTTGGTTAC-3' backward: 5'-GAGGACAATGGGCACAGTTAGT-3'	NM_018018	(38)
SDHA	forward: 5'-TACAAGGTGCGGATTGATGA-3' backward: 5'-CACAGTCAGCCTCGTTCAAA-3'	NM_004168	
Slc7a5 (LAT1)	forward: 5'-TGTGCTGGCATTATACAGCG-3' backward: 5'-AGGTGATAGTTCCCGAAGTC-3'	NM_003486	(101)
Slc7a8 (LAT2)	forward: 5'-TGTGCTGGCATTATACAGCG-3' backward: 5'-AGGTGATAGTTCCCGAAGTC-3'	NM_182728	(101)
Slc3a2 (4F2hc)	forward: 5'-TTGGCTGAGTGGCAAAATATCA-3' backward: 5'-GATTCGAGTAGGCTCAGGATCTG-3'	NM_001012662	(6)
TBP	forward: 5'-GTTCTGGGAAAATGGTGTGC-3' backward: 5'-GCTGGAAAACCCAACTTCTG-3'	NM_003194	

Statistics

Results are presented as mean ± SEM. The number of experiments (*n*) represents the number of placentas studied. In studies of amino acid uptake, each condition was performed in triplicate. In Paper I, differences between groups were evaluated using a *t*-test or repeated-measures ANOVA followed by Dunnett's *post hoc* test. Statistical differences between groups in Western blot experiments were assessed by the Mann-Whitney *U*-test (Paper II), the Wilcoxon Signed Ranks Test (Paper III and IV), or the Friedman test followed by the Wilcoxon Signed Ranks Test (Paper IV). In Paper II, differences between groups in amino acid uptake experiments were tested using one-way ANOVA followed by Dunnett´s *post hoc* test. hCG-measurement differences were evaluated using the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney *U*-test (Paper III and IV). In Paper III and IV, differences between groups in amino acid transport experiments were assessed by the Wilcoxon Signed Ranks Test.

SUMMARY OF RESULTS

Placental taurine transport

TAUT localization in the human placenta

Functional studies have suggested that the taurine transporter is polarized to MVM (128). To determine the cellular localization of TAUT, villous tissue from early-second-trimester (13 wk of gestation) and term placentas was screened for the expression of the taurine transporter (TAUT) using immunohistochemistry. Expression of TAUT was detected primarily in MVM of the syncytiotrophoblast, regardless of gestational age (see Fig. 1 in Paper I), which is in agreement with previously published functional studies.

TAUT expression is unaltered in IUGR

The activity of the taurine transporter has previously been shown to be decreased in MVM preparations isolated from IUGR placentas (128). To determine whether this decrease in activity is associated with a decrease in protein expression of TAUT, vesicle preparations of syncytiotrophoblast MVM from AGA and IUGR placentas were analyzed for the expression of TAUT by Western blot. TAUT was expressed at 70 kDa (see Fig. 2A in Paper I). Densitometry analyses showed that the relative expression of TAUT was not different between placentas from AGA (1.0 ± 0.1, $n = 8$) and IUGR (1.1 ± 0.1, $n = 8$) subjects, indicating that the reduction in TAUT activity is caused by posttranslational modifications of the transporters rather than a reduction in the number of transporter proteins.

Regulation of TAUT

TAUT has previously been reported to be regulated by cytokines, NO, glucose, and PKC in cell culture models (27, 95, 102, 155, 161). IUGR is associated with changes in the endocrine environment since both fetal and maternal plasma hormone concentrations are altered (Reviewed in (144)). Therefore, we investigated the regulation of TAUT by hormones which have been reported to be altered in fetal and/or maternal plasma in IUGR (GH, leptin, IGF-I, IGF-II, and EGF) or factors shown to regulate TAUT in other tissues (PMA, chelerythrine (CHT), TNF-α, IL-1β, IL-6, 3-morpho-linosydnonimine (SIN-1), and glucose). We also assessed the effect of the specific mTOR inhibitor rapamycin. The incubation time was set to 1 h, because the Western blotting data showed that MVM TAUT expression is unaltered in IUGR, suggesting that the reduction in MVM taurine transport in this pregnancy complication is not due to changes in translation of the transporter, but rather to changes in activity.

The Na^+-dependent uptake of taurine was inhibited when placental villous fragments were incubated for 1 h with either the NO-donor SIN-1 (-35%, $n = 7$, $P < 0.05$, see Fig. 4B in Paper I) or the PKC activator PMA (-21%, $n = 15$, $P < 0.05$, see Fig. 4C in Paper I). However, none of the cytokines, hormones, CHT, nor rapamycin altered TAUT activity significantly.

In summary, we demonstrate that TAUT is polarized to the MVM of the syncytiotrophoblast in the human placenta. We further show that there are no differences in the protein expression of TAUT between healthy placentas and placentas obtained from pregnancies complicated by IUGR. We have also demonstrated that PKC and NO inhibit taurine transporter activity. Because IUGR has been suggested to be associated with increased NO levels, NO inhibition of TAUT may decrease placental taurine transport and contribute to the reduced fetal taurine levels in this pregnancy complication.

Placental mTOR and nutrient transport

mTOR localization in human placenta

In the mature placenta, mTOR is expressed at the mRNA level (96). To determine the cellular localization of the mTOR protein in the placenta, mTOR expression was investigated by immunohistochemistry. Expression of mTOR was primarily detected in the cytoplasm of the syncytiotrophoblast (see Fig. 1A in Paper II). The cellular localization of mTOR suggests that it may be involved in regulating syncytiotrophoblast function.

Effect of inhibition of mTOR on the activity of placental amino acid transporters

To assess the effect of the mTOR signaling pathway on amino acid transporter activity, primary villous fragments were incubated for 4 hours with two different concentrations (22 and 100 nM) of rapamycin. 100 nM rapamycin completely inhibited system L transport activity (-100%, $n = 8$; controls $n = 15$; 22 nM $n = 9$, $P < 0.05$, see Fig. 3A in Paper II), but it did not have any significant effect on system A or taurine transporters (see Fig. 3B and C in Paper II).

Placental mTOR activity in relation to fetal growth

To further investigate the role of mTOR signaling in the placenta, we studied the activity of the mTOR signaling pathway in relation to fetal growth. This can be done by investigating the phosphorylation state of S6K1 at Thr-389, which is unique to mTOR and commonly used to measure the activity of the mTOR signaling pathway (19). Expression of phosphorylated S6K1 at Thr-389 was down-regulated by 45% in IUGR placentas ($n = 8$; AGA $n = 6$; $P = 0.05$, see Fig. 4H in Paper II). This decrease in placental mTOR activity may represent a mechanism for the previously demonstrated decrease in placental leucine transport in IUGR (82, 132).

In summary, we show that the mTOR protein is expressed in the transporting epithelium of the human placenta. We also demonstrate that inhibition of placental mTOR signaling markedly decreased leucine uptake via the system L transporter in primary villous fragments incubated for 4 hours, but had no effects on system A or taurine transporters. In addition, the protein expression of the key downstream signaling component of mTOR, phospho-Thr-389-S6K1, was markedly down-regulated in placentas from IUGR pregnancies. Therefore, a decreased placental mTOR activity may represent the mechanism for the previously demonstrated decrease in placental leucine transport in IUGR.

mTOR is a positive regulator of amino acid transport in cultured trophoblast cells

Validation of cell culture

To confirm the purity of our trophoblast isolation and to demonstrate that the cells differentiated into syncytial islands in culture, hCG produced by the cells was measured. After 66 h in culture, there was a marked increase in hCG production as compared with 18 h and these levels remained high until at least 90 h (see Fig. 1A in Paper III). Cultured cells were also cytokeratin-positive, confirming that cells were trophoblast, and vimentin-negative suggesting no contamination with cells of mesenchymal origin (see Fig. 1B in Paper III). Furthermore, the release of lactate dehydrogenase (LDH), a marker of cell integrity, was low in cells maintained for 90 h in culture. LDH release in cells subjected to sonication was 10 times greater. During apoptosis, PARP (Poly ADP-Ribose Polymerase) is cleaved into 85- and 29-kDa fragments (129). Cultured trophoblast cells were PARP negative, demonstrating that apoptotic activity in these cells is low and that rapamycin does not increase apoptosis (see Fig. 1C in Paper III). Rapamycin treatment did not alter cell characteristics, because there were no differences in hCG production or LDH release between cells incubated with rapamycin for 24 h when compared with cells incubated with vehicle.

mTOR is a positive regulator of amino acid transport

To further elucidate the function of mTOR in the human placenta, we studied the effect of prolonged (24 h) inhibition of mTOR on amino acid transport. This was done to be able to observe contingent regulation of amino acid transporters mediated by effects on transcription and/or translation. To this effect, trophoblast cells were isolated from healthy term placentas, cultured for 66 h, incubated with rapamycin (100 nM) or vehicle (0.02% DMSO) for 24 h, and then amino acid transporter activity was measured. Long-term mTOR inhibition significantly decreased the activity of system A, system L, and taurine transporters (see Fig. 3 in Paper III). To confirm that rapamycin inhibits mTOR activity in isolated trophoblasts, the phosphorylation state of S6K1, a key kinase

downstream of mTOR, was measured. Rapamycin completely abolished the phosphorylation of S6K1 at Thr-389 (see Fig. 4A in Paper III).

Effect of mTOR on amino acid transporter mRNA and protein expression

For further examination of the mechanisms underlying the altered amino acid transporter activity after treating cells with rapamycin for 24 h, the quantity of mRNA and protein of isoforms of the system A, system L, and the taurine transporter was measured. Even though LAT1 and TAUT mRNA expression was down-regulated after 24 h incubation with rapamycin, there was no effect on transporter protein expression (see Fig. 5 and 6 in Paper III). These findings illustrate that a change in mRNA expression is not necessarily paralleled by a similar change in protein expression. These results also suggest that mTOR regulates placental amino acid transporters by posttranslational modifications or by affecting transporter translocation to the plasma membrane.

Herein, we show for the first time that mTOR inhibition decreases the activity of system L, system A, and taurine amino acid transporters in primary trophoblast cells. Despite decreased mRNA expression of TAUT in rapamycin-treated cells, protein expression of the transporter was not significantly changed after mTOR inhibition. We propose that mTOR regulates placental amino acid transporters by posttranslational modifications of the protein or by affecting membrane trafficking of the transporters.

Glucose, IGF-I, and insulin are upstream regulators of placental mTOR

Effect of decreasing glucose concentrations on intracellular signaling pathways

mTOR pathway

The upstream regulators of placental mTOR are currently unknown, but glucose has been shown to regulate mTOR activity in, among others, HEK-293 cells (37) and mouse embryonic fibroblasts (49). The placental mTOR signaling pathway activity after glucose deprivation was studied by measuring the protein expression of the key downstream target of mTOR, phospho-Thr-389-S6K1. The protein expression of phospho-Thr-389-S6K1 is an indication of the activity of the mTOR signaling pathway, and it was down-regulated in response to decreasing glucose concentrations in trophoblast cells (see Fig. 4 in Paper IV).

AMPK pathway

Glucose has been suggested to regulate mTOR signaling through AMPK activation (72). To investigate whether the AMPK pathway was activated in

glucose-deprived trophoblast cells, the protein expression of phospho-Thr-172-AMPKα, total AMPK, and phospho-Ser-428-LKB1 was studied in control and glucose-starved cells. The data demonstrate that the LKB1-AMPK pathway was not affected by glucose deprivation (see Fig. 5 in Paper IV).

REDD1

It was recently shown that REDD1 is induced by chronic energy depletion in mouse embryonic fibroblasts, and that this in turn leads to inactivation of mTORC1, independent of AMPK (159). To examine the involvement of REDD1 in the inactivation of mTORC1 in glucose-deprived trophoblast cells, the protein expression of REDD1 was studied in cells incubated with control or glucose deprived media. Our results show that REDD1 is not activated in glucose deprived trophoblast cells (see Fig. 6 in Paper IV).

Amino acid transporter activity in response to glucose and growth factors

IUGR fetuses may be hypoglycemic (40) and have reduced plasma concentrations of insulin and IGF-I (105, 126). Maternal concentrations of IGF-I and glucose are also reduced in this condition (40, 120), suggesting that the IUGR placenta is exposed to decreased levels of glucose and growth factors, which are known to regulate mTOR in cell lines. Our data show that in cultured primary trophoblast cells, glucose deprivation decreased system L activity in an mTOR dependent manner. We also demonstrate that insulin and IGF-I stimulated amino acid transporter activity by activating mTOR signaling (see Fig. 2 and 3 in Paper IV).

In this study, we show that glucose and insulin/IGF-I regulate amino acid transporter activity by altering mTOR signaling. The exact mechanisms underlying these effects remain however to be established.

GENERAL DISCUSSION

Abnormal fetal growth, which is associated with both perinatal morbidity as well as metabolic diseases in adulthood, is an important clinical problem as it affects as many as 15% of all pregnancies. To this date, there is no specific treatment of pathological fetal growth. Fetal growth is intimately linked to the transport functions of the placenta and placental amino acid transporter activity is known to be altered in cases of abnormal fetal growth. Therefore, detailed information on the mechanisms regulating placental amino acid transport will increase our understanding of how abnormal fetal growth develops and may provide new targets for intervention. The focus of this study was to identify factors regulating three key amino acid transporters in the human placenta. The novel findings of the study are the demonstration that the mammalian target of rapamycin signaling pathway constitutes an important regulator of placental amino acid transporters and that trophoblast mTOR is regulated by nutrient levels and growth factor signaling. We propose a model in which trophoblast mTOR functions as a placental nutrient sensor regulating placental amino acid transport and fetal growth in response to nutritional and hormonal cues in the maternal compartment.

Placental TAUT expression, regulation, and relation to fetal growth

Taurine is a β amino acid, which is not incorporated into proteins. Fetuses lack the ability to synthesize taurine and are therefore dependent on placental transport for an adequate supply. As a consequence, taurine is regarded as an essential amino acid for the fetus and maternal taurine deficiency results in IUGR and neurological disturbances in the fetus (1). We have previously shown that IUGR is associated with a decrease in the MVM activity of the taurine transporter (128), which perhaps can explain the lower fetal plasma levels of taurine in this pregnancy complication (24, 25, 41) in this pregnancy complication. In this study, we show for the first time that TAUT is expressed in the MVM, which is in accordance with the functional data reported previously (128), and has been verified by others (26). The MVM protein expression of TAUT was however not altered in IUGR, suggesting that posttranslational modifications of the transporter may be involved in the down-regulation of taurine transporter activity seen in MVM vesicles isolated from pregnancies complicated by IUGR. Using an in vitro model of placental villous fragments, we found that TAUT was insensitive to short-term hormonal regulation, but was down-regulated by SIN-1, which releases NO and O_2^-. There are contradictory reports regarding maternal NO levels in mothers carrying small for gestational age (SGA) babies. For example, Xiao & Li (176) found that maternal NO levels were decreased in pregnancies complicated by IUGR, whereas a recent report

showed increased NO and peroxynitrate ($ONOO^-$) levels in platelets obtained from mothers carrying SGA babies (124). Placental nitrite (NO_2^-) concentrations have also been shown recently to be elevated in IUGR (167). It has been suggested that the taurine transporter, at least in isolated MVM vesicles, is down-regulated by NO through formation of peroxynitrate (95). The production of peroxynitrate can be indirectly localized by the presence of nitrotyrosine residues. In contrast to the hypothesis that NO regulates TAUT in IUGR placentas, no differences in nitrotyrosine immunostaining were observed when comparing villous tissue samples from IUGR and control placentas (122). To our knowledge, nitrotyrosine residues in MVM isolated from IUGR pregnancies have not been investigated. Based on our experiments in villous fragments, we suggest that NO may down-regulate taurine transporters in MVM in pregnancies complicated by IUGR, and that this down-regulation is one causative factor leading to the lower taurine concentrations in fetal plasma. Further studies are however needed to investigate the mechanism of NO-induced inhibition of taurine uptake.

Previous studies investigating placental taurine transporter activity have been performed in isolated plasma membrane vesicles. This method has several advantages. First, the individual transport mechanisms in the two plasma membranes of the syncytiotrophoblast can be defined without the complication of placental metabolism. Second, the experimental conditions on each side of the membrane can be precisely controlled. However, these isolated plasma membrane vesicles lack intracellular components and are not very useful when studying regulation of individual transporters, because these processes most certainly involve intracellular signaling cascades. To study the regulation of the taurine transporter, we have used primary villous fragments as an experimental model. Villous fragments do not need to be cultured before experiments can be performed, and are therefore quick to prepare and study. The method also allows for taurine transporter activity to be compared in a large number of placentas fairly easy. The polarization of the syncytiotrophoblast and cell-to-cell contacts in the fragments are also likely to resemble the *in vivo* situation. Even though the fragments contain a mixture of cell types in addition to syncytiotrophoblasts, such as macrophages and endothelial cells, the syncytiotrophoblast microvillous membrane is likely to be the major contributor to the measured amino acid uptake, because it has the greatest surface area. However, only MVM uptake can be measured in primary villous fragments and the sample-to-sample variation is fairly large, increasing the number of placentas needed to be studied in each experimental condition. A recent study (153) performed in primary villous fragments confirmed previously published amino acid transporter activity measurements in MVM isolated from IUGR placentas (39, 54, 86, 113), indicating that the two methods are in concordance and information obtained in one model can be used when designing experiments in the other.

The role of placental mTOR in nutrient sensing and regulation of nutrient transporters

mTOR is expressed in the trophoblast and regulates system L amino acid transporter activity

The serine/threonine protein kinase mTOR has been shown to regulate cell growth in response to nutrient and growth factors in cell lines (73, 175). This study shows for the first time that mTOR is present at the protein level in the cytoplasm of the transporting epithelium of the human placenta. To study the short-term (4 h) regulation of amino acid transporters by the mTOR signaling pathway, the primary villous fragment model was used. Under these conditions, mTOR inhibition markedly decreased system L amino acid transporter activity. This suggests that mTOR is involved in the regulation of syncytiotrophoblast function, such as the transport of essential amino acids. Previous studies have shown some involvement of mTOR in the regulation of the system L transporter in other cell types. For example, LAT1 mRNA expression in rat vascular smooth muscle cells is stimulated by PDGF in an mTOR-dependent manner (109). The authors did however not investigate the effect of mTOR-inhibition on basal or PDGF-stimulated leucine transporter activity. Leucine transport has been studied in a fission yeast strain dependent on leucine (173), where it was found that rapamycin inhibited both growth and leucine uptake. The current investigation is however the first study showing mTOR regulation of system L in a human tissue.

Decreased placental mTOR activity in IUGR may explain the down-regulation of amino acid transporters

In IUGR, the activity of the system L transporter is reduced (82, 132), and we hypothesized that this may be due to changes in mTOR signaling. S6K1 is a key kinase downstream of mTOR, and the phosphorylation of S6K1 at Thr-389 is unique for mTOR, and thus a decrease in the expression of this phospho-site is equivalent to a decreased activity in the mTOR signal transduction pathway (19). In the current study we show that the expression of phospho-Thr-389-S6K1 is decreased in homogenates from IUGR placentas, suggesting a decreased placental mTOR signaling activity in this pregnancy complication. These results have been corroborated by a subsequent report (178). These findings, together with the demonstration that mTOR inhibition markedly inhibits the system L amino acid transporter in the human placenta, are consistent with the possibility that the decreased placental system L activity in the IUGR placenta (82, 132) is caused by the inhibition of the mTOR signaling pathway.

mTOR is a positive regulator of amino acid transporter activity

To elucidate possible mechanisms of mTOR regulation of amino acid transporters in the human placenta, we designed a series of experiments to allow effects on transporter activity mediated by transcriptional and translational regulation to be studied. Since the use of primary villous fragments has been thoroughly validated only for experiments lasting 4-6 hours (75) we performed these experiments in cultured primary human trophoblast cells. These cells were incubated with rapamycin for 24 hours and subsequently amino acid transporter mRNA and protein expression and transporter activity were measured. This length of incubation is commonly used in the literature (2, 149) for these types of studies, which allow for comprehensive investigation of transcriptional and translational regulation. Furthermore, cultured trophoblast cells have been extensively validated for prolonged exposures of factors regulating amino acid transporter activity (see for example (125)). We also argue that studies on long-term effects of mTOR inhibition on amino acid transporters may be more physiologically relevant since fetal size is determined by the amino acid delivery over a longer time period rather than just a few hours. The concentration of rapamycin used was based on previously published reports (2, 149), as well as on the fact that 100 nM rapamycin resulted in a more or less complete inhibition of mTOR, as measured by the phosphorylation of S6K1 at Thr-389.

We show that 24 h mTOR inhibition decreased the activity of system L, system A, and TAUT transport in cultured primary human trophoblast cells. Thus, the effects of mTOR inhibition on amino acid transporter activity in cultured primary trophoblast cells were somewhat different as compared with the previous studies in primary villous fragments, in which only system L activity was decreased after mTOR inhibition. These differences may be a result of the distinct character of the two experimental systems used. However, villous fragments were incubated with rapamycin for 4 hours only and an alternative explanation is that the longer rapamycin incubation time in the cultured cells also affects mTORC2 (2, 149), which then down-regulates system A and TAUT activity. Interestingly, the degree of inhibition (-17 to -40%) in response to mTOR inhibition differed between the three amino acid transport systems. The differential effects on the transporters under study suggest that mTOR does not induce a total and global down-regulation of amino acid transporter activity. Instead these results are consistent with the possibility that the three transporters are regulated separately, perhaps in part by different mechanisms.

Even though the effects on transporter down-regulation in our cell culture experiments may, at a first glance, seem modest, we believe that they are physiologically relevant. For example, the taurine transporter in the MVM of the syncytiotrophoblast is the only transporter mediating taurine uptake, and is likely to be the rate-limiting step for transplacental taurine transport. Therefore, a 40% decrease in taurine uptake into the placenta *in vivo* is likely to markedly affect fetal taurine supply, which has the potential to alter fetal growth. Similarly,

system L is the only pathway for placental uptake of key essential amino acids such as leucine, and a 28% decrease in the activity of this transporter is likely to have significant effects on the fetal supply of essential amino acids. Along these lines, the effects of mTOR inhibition on amino acid transport activity in the present study are of similar magnitude as reported for changes in placental transporter activity in human intrauterine growth restriction. For example, we have shown previously that IUGR is associated with a 34% decrease in taurine transporter activity in MVM (128) and a 38 and 46% decrease in system L amino acid transporter activity in BM and MVM, respectively (82). Effects on amino acid transporter activity may have been even more marked after a few hours, and perhaps after 24 hours secondary homeostatic mechanisms may have limited the initial changes. Furthermore, if we would have added physiological concentrations of growth factors such as insulin and IGF-I to the cell culture medium, we might have observed more marked inhibition of amino acid transporter activity following incubation with rapamycin. In the current experiments, the only growth factors present in the medium in our cell culture originate from fetal bovine serum. Since this is used at a concentration of 10%, insulin and IGF-I concentrations are likely to be lower than physiological.

The mechanism(s) underlying mTOR regulation of amino acid transporters are far from clear. Peng and co-workers have shown that mTOR regulates transcription of some amino acid transporters (134) and Edinger and Thompson have reported that nutrient transporter trafficking is under the control of mTOR (44, 45). Despite the decreased activity of the three amino acid transport systems under study following mTOR inhibition for 24 hours, the protein expression of the relevant transporter isoforms was not altered in whole cell lysates. These results indicate that mTOR regulates some placental amino acid transporters by posttranslational modifications or by affecting transporter translocation to the plasma membrane rather than exclusively by transcriptional or translational regulation. mTOR is best known as a regulator of protein translation, but we believe that the finding that mTOR influences amino acid transporter activity, without having an effect on protein expression, contributes to the novelty of these data. The placental SNAT2 protein has been shown to be redistributed to the plasma membrane of BeWo cells after amino acid deprivation and cortisol treatment, and this correlates with an increase in system A transporter activity in these cells (91, 92). These results indicate that mTOR inhibition may down-regulate at least system A activity by translocating system A transporters from the plasma membrane to the perinuclear region. Since mTORC2 is known to regulate the actin cytoskeleton (74), it is tempting to speculate that these effects could be mediated via mTORC2. However, further studies are needed to explore the effect of mTOR on amino acid transporter trafficking in the syncytiotrophoblast.

Despite a decreased TAUT mRNA expression following 24 hours incubation with rapamycin, we did not observe any significant effects on protein expression

of TAUT. These findings illustrate that a change in mRNA expression is not necessarily paralleled by a similar change in protein expression (57). For example, in the BeWo choriocarcinoma cell line, decreased concentrations of non-essential amino acids in the medium for 3 h increased SNAT2 mRNA expression, but had no effect on SNAT2 protein expression (92). Furthermore, the expression of SNAT4 mRNA in the placenta is significantly higher in first trimester tissue as compared with term tissue. In contrast, there was a gestational increase in SNAT4 protein expression (38).

Due to the previous lack of antibodies directed to the amino acid transporters under study, there are few, if any, previous studies investigating both activity and protein expression of system A, system L, and TAUT transporters. Therefore, one novel aspect of our study is that we have been able to study mRNA and protein expression in conjunction with the activity of these transporters. We have had the privilege to obtain a SNAT2 antibody from the Ganapathy lab (108) and a LAT2 antibody from the lab of Dr. Kanai (133). The SNAT4 antibody used has been raised to the amino acid sequence YGEVEDELLHAYSKV of human SNAT4. The specificities of these antibodies were verified by incubating the primary antibodies with their respective blocking peptides prior to use in Western blotting analyses. Preincubation with the blocking peptides prevented the detection of the bands.

Hypoglycemia inhibits placental mTOR signaling and regulates amino acid transporters

The factors down-regulating placental mTOR signaling in pregnancies complicated by IUGR remain to be fully established, but IUGR fetuses are more likely to be hypoglycemic (40) and have reduced plasma concentrations of insulin and IGF-I (105, 126). Maternal concentrations of IGF-I and glucose are also reduced in this pregnancy complication (40, 120), suggesting that the IUGR placenta is exposed to decreased levels of glucose and growth factors, which have previously been shown to be upstream regulators of mTOR in cell lines. Studies in immortalized cell lines originating from human trophoblast suggest that glucose and growth factor induced trophoblast cell proliferation is mediated through mTOR activation (174).

In this study, we demonstrate that hypoglycemia inhibits mTOR signaling in cultured primary human trophoblast cells. Furthermore, we show for the first time that glucose deprivation down-regulates placental system L in an mTOR dependent manner, by showing that glucose deprivation and rapamycin use a common mechanism (inhibition of mTOR) to down-regulate leucine transport. In contrast to the results for system L, incubation of cells with media with low glucose concentrations did not have any effects on system A activity. However, in the presence of rapamycin (24 h), glucose deprivation resulted in an increase in system A activity. One interpretation of these results are that two mechanisms regulating system A activity are present: (i) an adaptive up-regulation mediated

by an mTOR independent mechanism and (ii) down-regulation of system A activity by an mTOR dependent mechanism. Adaptive regulation refers to the up-regulation of system A activity in response to amino acid deprivation, which has been demonstrated in, among others, trophoblast cells (92).

The distinct effects on system A and system L activity in response to glucose deprivation in cultured trophoblast cells suggest that these amino acid transporters are regulated differently. There is ample support for this conclusion in the literature. For example, amino acid deprivation in the BeWo cell line does not alter LAT2 mRNA expression, but it has a profound effect on SNAT2 mRNA expression (92). Similarly, hypoxia (24 h exposure) inhibits system A transport by 55% in cultured trophoblast cells, but has no effect on system L amino acid transporter activity (125). Likewise, we previously presented data indicating that short-term inhibition (4 h) of mTOR only decreased system L transporter activity. 24 h of rapamycin treatment of trophoblast cells decreased the activity of both system A and system L, but only LAT1 mRNA expression was down-regulated.

TAUT activity was stimulated by glucose-deprivation, which is in accordance with previously published reports demonstrating a decrease in TAUT activity after incubation of human retinal pigment epithelial cells with media containing high glucose concentrations (161). This does not seem to be controlled by the mTOR pathway, since mTOR inhibition had no effect on the stimulation of TAUT by glucose-deprivation.

Hypoglycemia does not inhibit mTOR signaling through AMPK activation

Glucose deprivation in cell lines has previously been shown to inhibit mTORC1 via the activation of the AMP-activated protein kinase (AMPK), either through activation of TSC2 (72) or independent of TSC2 via the hVps34 pathway (21). Our studies demonstrate that the reduction in mTOR pathway activity by glucose deprivation in trophoblast cells does not involve the AMPK pathway, because we found no changes in the expression of phosphorylated AMPK or phosphorylated LKB1 in glucose deprived trophoblast cells.

There are several possible alternative mechanisms for mTOR inhibition in the absence of AMPK activation in response to low glucose concentrations in the cell culture medium. First, it is possible that the AMPK pathway does not respond to energy depletion in trophoblast cells. For example, it was recently shown in immortal mouse embryonic fibroblasts that REDD1 is induced by energy starvation, and this in turn leads to inactivation of mTORC1 measured as phosphorylation of S6K1 at Thr-389, independent of AMPK (159). On the contrary, we show that REDD1 is not involved in the mTORC1 inactivation in primary trophoblast cells after glucose deprivation, since we found no differences in the protein expression of REDD1. Second, intracellular ATP concentrations may be maintained by intracellular mobilization of glucose from

glycogen stores since isolated trophoblast cells contain glycogen (152), which could serve as an energy source for the placenta. ATP could also be produced from other energy substrates such as amino acids or fatty acids. Trophoblast cells have been shown to maintain ATP production in the complete absence of extracellular glucose for at least 8 hours (170), suggesting that they indeed can produce ATP from other sources than glucose. Rapamycin-treatment (24 and 48 h) has been shown to increase fatty acid oxidation in L6 myotubes (158). The placenta has the capacity to oxidize fatty acids (Reviewed in (177)), and perhaps switches from glucose metabolism to fatty acid metabolism during glucose deprivation. The capacity of placental mitochondria to generate oxidative energy has been suggested to be low (64), but this has later been questioned and it has been proposed that the mitochondria in the placenta are capable of regular ATP production (67), implicating amino acids as another source of ATP during glucose starvation. Third, it is possible that low glucose levels inhibit mTORC1 through the hVps34 pathway without involvement of AMPK, in analogy with the signaling pathway linking amino acid deprivation to mTORC1 inhibition (21, 127). One limitation to our study is that we did not measure AMP or ATP levels in glucose deprived trophoblast cells, which may help in determining which signaling events occur after glucose deprivation. Placental metabolism has not been fully elucidated, and further studies are needed to clarify signaling pathways leading to mTOR inactivation in glucose-deprived trophoblast cells.

Growth factors stimulate amino acid transport through mTOR

We demonstrate that system A activity is stimulated by insulin and IGF-I, which is consistent with previous studies in cultured trophoblast cells (93, 94), the BeWo cell line (48), as well as in primary villous fragments (75). We also found that insulin stimulates system L activity, which to our knowledge has not been shown before. We now extend previous studies by showing that insulin and IGF-I stimulate amino acid transporters through the activation of mTOR, because treating cells with rapamycin abolishes the effects of insulin and IGF-I. There are some reports in the literature that have investigated the involvement of mTOR in insulin/growth factor stimulation of system A activity (48, 117, 137). In these studies, insulin was shown to stimulate system A transport in L6 myotubes (117, 137) and IGF-I was shown to stimulate system A transport in the BeWo choriocarcinoma cell line (48). These studies did however not find any effects of rapamycin on system A transporter activity. The discrepancy between our study and the previously published reports may be due to the incubation time with rapamycin, which was less than one hour in the previous studies as compared with 24 hours in this study. Our previous data show that mTOR inhibition for 4 hours had an inhibitory effect on system L activity in primary villous fragments, but system A was unaffected. It may be that system A is primarily regulated by mTORC2, which is inhibited by prolonged rapamycin-treatment (149).

CONCLUDING REMARKS

In summary, this study shows that NO down-regulates the taurine transporter, which may provide a mechanism for the decreased taurine transporter activity in the IUGR placenta. We further demonstrate the presence of the mTOR protein in the transporting epithelium of the placenta and we show that the activity of placental mTOR signaling is reduced in IUGR. mTOR is a positive regulator of amino acid transport, and our data suggest that this effect is not mediated by an increased expression of transporters. The exact mechanism for mTOR regulation of amino acid transporters in trophoblast cells remains to be established, but we speculate that mTOR signaling influences transporter trafficking, possibly through mTORC2. Furthermore, glucose and growth factors stimulate amino acid transport through mTOR.

These data are in line with our hypothesis that the mTOR-signaling pathway in the placenta functions as a nutrient sensing pathway. For example, in situations of compromised nutrient supply due to reduced blood flow or maternal malnutrition, a number of upstream signals feed into the mTOR signaling pathway. We have shown low levels of growth factors (insulin and IGF-I) and glucose are possible inhibitors of mTOR pathway activity in trophoblast cells. Other stimuli, such as energy deprivation, low oxygen, and low leptin levels may also inhibit mTORC1 during situations of low nutrient supply. A summation of these upstream signals then down-regulate mTORC1 (and possibly mTORC2), which in turn leads to the down-regulation of amino acid transporter activity previously demonstrated in the IUGR placenta. The down-regulation of placental amino acid transporters then leads to a decreased amino acid supply to the fetus, and in the end to restricted fetal growth.

Since the mTOR pathway regulates amino acid transporters, and amino acid transporters are important regulators of fetal growth, this suggests that mTOR de facto determines fetal growth and that placental mTOR constitutes a mechanistic link between maternal nutrient availability and fetal growth (Fig. 3).

The concept that the placenta functions as a nutrient sensor, which matches fetal growth to the ability of the maternal supply line to deliver nutrients to the placenta by regulating placental nutrient transport, is novel and may have evolutionary implications. We speculate that in situations of maternal starvation a homeostatic mechanism decreasing fetal growth, thereby conserving energy for maternal survival over fetal survival, will constitute an evolutionary advantage since maternal survival will allow new opportunities for reproduction later on when food supply may be more abundant. However, in the absence of a placental nutrient sensor, maternal survival may be jeopardized before reproduction is completed, which from an evolutionary perspective is highly unfavorable. Clearly, the concept of a placental nutrient sensor is somewhat at odds with the widely held view that the fetus is a 'parasite', which always extracts the necessary nutrients from the maternal circulation.

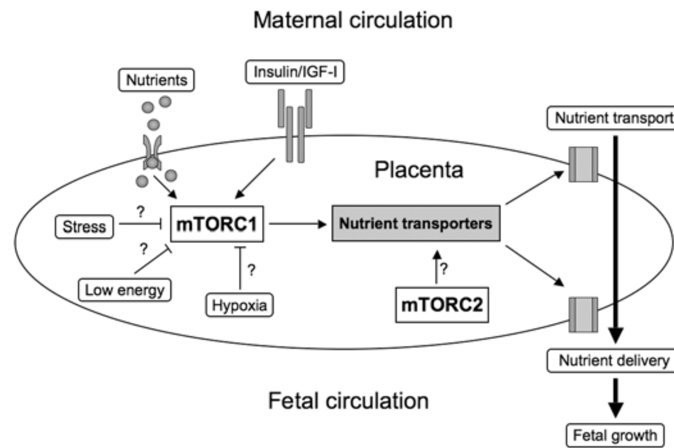

Fig. 3. A model of mTOR signaling as a placental nutrient-sensing pathway. mTOR integrates nutrient and growth factor signaling (and possibly a number of other upstream signals) to control nutrient transport from the mother to the fetus. mTOR forms two complexes in the cell, mTORC1 and mTORC2. The exact mechanism for mTOR regulation of nutrient transport in the placenta remains to be established, but it may involve both mTOR complexes. Since placental amino acid transporters are important regulators of fetal growth, we propose that placental mTOR constitutes a mechanistic link between maternal nutrient availability and fetal growth. Arrows and bars represent activation and inhibition, respectively.

Our studies may have implications beyond placental physiology and the regulation of fetal growth. Our data clearly shows that mTOR signaling is a positive regulator of amino acid transport. Since stimulation of amino acid uptake will result in increased intracellular concentrations of amino acids, which will further activate mTOR signaling and amino acid transporters, this will result in a positive feedback loop that must be limited by cellular homeostatic mechanisms. Recently, it was shown that S6K1 activation results in inhibitory phosphorylation of IRS-1, providing a link between mTOR activation and insulin resistance (168, 169). The physiological relevance of this feedback loop is that it will limit further mTOR activation through the growth factor signaling pathway in response to increased nutrient availability. This homeostatic regulation is of physiological importance in the short-term perspective. However, in situations of chronic nutrient overload, such as obesity, this mechanism causes insulin resistance. Another area that our studies may have implications for is tumor growth, which is dependent on up-regulation of amino acid transporters such as system L (51, 60). Furthermore, the mTOR signaling pathway is activated in many cancer cells. Targeting the mTOR pathway and amino acid transporters are emerging areas in cancer treatment, and a better understanding of the molecular mechanisms regulating amino acid transporters in human cells will facilitate this development.

FUTURE PERSPECTIVES

This thesis has started to examine the role of mTOR in the regulation of placental nutrient transport, but being in the early stages of investigation it raises further questions to resolve. For example:

- **Which molecular mechanisms are involved in mTOR regulation of amino acid transporters?** There are a few reports in the literature implicating mTOR regulation of transporters in cell lines via effects on membrane trafficking, and some of our results in primary human trophoblast cells are consistent with this hypothesis. Future studies in this area will for example include monitoring of transporter trafficking using confocal microscopy subsequent to incubation with rapamycin or knockdown of mTORC1 (raptor) and mTORC2 (rictor) by siRNA.

- **Which is the primary molecular amino acid sensor?** Despite major progress in identifying key components of the signaling pathway linking amino acid availability to mTOR, the primary molecular amino acid sensor remains elusive. Clearly this sensor has to bind or interact with amino acids, in particular those amino acids that are the most potent to elicit an mTOR response.

- **Is the observed effect on amino acid transporters mediated through S6K1?** This important question can be addressed by studying the effects of mTOR inhibition on amino acid transport activity in trophoblast cells transfected with S6K1 siRNA.

- **Does mTOR affect any other nutrient or ion transporters in the human placenta?** Of particular interest to study are glucose transporters and lipoprotein lipase, representing key components of the placental transport systems for glucose and lipids, respectively.

- **What is upstream of mTORC2?** The factors regulating mTORC2 are currently unknown. Since mTORC2 influences the actin cytoskeleton, which is involved in membrane trafficking, this is a particularly pertinent question with respect to the regulation of nutrient transporters. One initial approach to address this question is to perturb the cells by for example altering nutrient and/or growth factor concentrations and measure mTORC2 activation by phosphorylation of Ser-473 of Akt.

SUMMARY IN SWEDISH (POPULÄRVETENSKAPLIG SAMMANFATTNING)

Idag är ca 15 % av alla förlossningar förknippade med avvikande fostertillväxt, antingen onormalt liten fostertillväxt (intrauterin tillväxthämning) eller onormalt stor fostertillväxt (accelererad fostertillväxt). Dessa tillstånd är förknippade med en ökad risk att drabbas av sjukdomar och handikapp före, under och strax efter förlossningen. Tillväxthämmade foster har t ex ökad risk att drabbas av syrebrist under födseln, vilket kan ge hjärnskador som kvarstår i vuxen ålder. Att föda ett barn som haft en accelererad fostertillväxt ökar risken för en svår förlossning, är barnet alltför stort krävs kejsarsnitt. Barn med hög eller låg födelsevikt har vidare en ökad risk att drabbas av ögon- och hörselskador, fetma, högt blodtryck samt tidigt utvecklad typ 2-diabetes, vilket gör att vissa av dessa barn kräver mycket avancerad vård tiden efter förlossningen samt medicinsk uppföljning under hela barndomen. Dessutom är avvikande fostertillväxt förenat med en rad folksjukdomar i vuxen ålder, såsom diabetes, hjärt-kärlsjukdomar och fetma. Även om genetiska faktorer är av betydelse så bestäms fostrets tillväxt i livmodern huvudsakligen av tillförseln av näringsämnen och syre, vilket är starkt beroende av moderkakans (placentans) transportkapacitet. Senare tids forskning har visat att det vid avvikande fostertillväxt föreligger specifika förändringar i de transportsystem som ansvarar för leverans av näringsämnen till fostret, i synnerhet aminosyretransportörer. Idag finns ingen specifik behandling för avvikande fostertillväxt. För att förstå hur dessa graviditetskomplikationer uppstår och i syfte att identifiera nya behandlingsstrategier är information om hur placentans transportörer regleras av stor vikt.

Syftet med avhandlingen har varit att undersöka om mammalian target of rapamycin (mTOR)-signaleringssystemet utgör en molekylär mekanism i placentan som styr näringstillförsel till fostret genom att reglera transportörer för aminosyror som svar på förändrade koncentrationer av näringsämnen och tillväxtfaktorer hos modern. Detta har gjorts genom att studera hur aminosyretransport regleras i celler, små vävnadsbitar samt homogenat, allt från human placenta.

Vi fann att mTOR-signaleringskaskaden stimulerar aminosyretransport och att aktiviteten av mTOR är reducerad vid intrauterin tillväxthämning. Vidare fann vi att mTOR i placentan stimuleras av näringsnivåer och tillväxtfaktorer. Sammanfattningsvis har vi visat att mTOR-signaleringskaskaden utgör en viktig länk mellan nivåer av näringsämnen/tillväxtfaktorer och aminosyretransport i den humana placentan. Eftersom placentans transport av aminosyror är avgörande för fostertillväxt spekulerar vi att mTOR fungerar som en placentär näringssensor som anpassar fostertillväxten efter tillgängligheten av näringsämnen och tillväxtfaktorer i moderns blodcirkulation genom att reglera placentans transportkapacitet för näringsämnen. Dessa resultat kommer

förhoppningsvis att bidra till att öka förståelsen kring hur förändrad fostertillväxt uppstår. Det är möjligt att mTOR-kaskaden i placentan skulle kunna utgöra en måltavla för interventionsåtgärder mot onormal fostertillväxt i framtiden.

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