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Regulation of Amino Acid Transporter Trafficking by mTORC1 in Primary Human Trophoblast cells is Mediated by the Ubiquitin Ligase Nedd4-2

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Abstract

Changes in placental amino acid transfer directly contribute to altered fetal growth, which increases the risk for perinatal complications and predisposes for the development of obesity, diabetes and cardiovascular disease later in life. Placental amino acid transfer is critically dependent on the expression of specific transporters in the plasma membrane of the trophoblast, the transporting epithelium of the human placenta. However, the molecular mechanisms regulating this process are largely unknown. Nedd4-2 is an ubiquitin ligase that catalyzes the ubiquitination of proteins, resulting in proteasomal degradation. We hypothesized that inhibition of mTORC1 decreases amino acid uptake in primary human trophoblast cells (PHT) by activation of Nedd4-2, which increases transporter ubiquitination resulting in decreased transporter expression in the plasma membrane. mTORC 1 inhibition increased the expression of Nedd4-2, promoted ubiquitination and decreased the plasma membrane expression of SNAT2 (an isoform of the System A amino acid transporter) and LAT1 (a System L amino acid transporter isoform), resulting in decreased cellular amino acid uptake. Nedd4-2 silencing markedly increased the trafficking of SNAT2 and LAT1 to the plasma membrane, which stimulated cellular amino acid uptake. mTORC1 inhibition by silencing of raptor failed to decrease amino acid transport

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Disclosure

The authors have nothing to disclose.

Author contribution

F.J.R., T.J. and T.L.P made contribution to conception and design of the experiments. F.J.R., T.J. and T.L.P performed collection, analysis and interpretation of data: KGD performed confocal analysis: F.J.R., T.J. Y.K. and T.L.P wrote the manuscript. All authors approved the final version of the manuscript.

following Nedd4-2 silencing. In conclusion, we have identified a novel link between mTORC1 signaling and ubiquitination, a common posttranslational modification. Because placental mTORC1 is inhibited in fetal growth restriction and activated in fetal overgrowth, we propose that regulation of placental amino acid transporter ubiquitination by mTORC1 and Nedd4-2 constitutes a molecular mechanisms underlying abnormal fetal growth.

Keywords

placenta; SNAT2; LAT1 and ubiquitination

Introduction

Abnormal fetal growth increases the risk for perinatal complications and predisposes the infant for the development of obesity, diabetes and cardiovascular disease later in life [1, 2]. Fetal growth is strongly dependent on nutrient availability, which is determined by placental nutrient transport [3, 4]. The activity of key placental amino acid transporters is decreased in intrauterine growth restriction (IUGR) [5–8] and up regulated in fetal overgrowth [9, 10], suggesting that changes in the activity of placental nutrient transporters may directly contribute to abnormal fetal growth [11–13].

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that responds to nutrient availability and growth factor signaling to control cell growth and metabolism mediated by effects on gene transcription and protein translation [14, 15]. mTOR resides in two distinct multiprotein complexes referred to as mTORC1 and mTORC2. mTORC1 phosphorylates S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which mediates many of the down-stream effects of mTOR [14, 15]. mTORC2 phosphorylates Akt, Protein Kinase C- α (PKC α) and Serum and Glucocorticoid-regulated Kinase (SGK) and influences the actin skeleton and regulates cell metabolism [16, 17]. DEPTOR, a protein containing two DEP (Dishevelled, Egl-10, Pleckstrin) domains, is an endogenous inhibitor of both mTORC1 and 2 signaling [18].

We have recently shown that silencing of mTORC1 and/or mTORC2 markedly inhibits trophoblast System A and L amino acid transport activity by affecting transporter trafficking to the plasma membrane [19]. Both placental mTORC1 and mTORC2 activity is decreased in human IUGR [20, 21] as well as in animal models of IUGR such as low protein diet in the rat [22]. In addition, maternal nutrient restriction in the baboon inhibits placental mTOR signaling [23]. These observations are consistent with an important role for placental mTOR signaling in regulating placental function and fetal growth and provide a possible mechanistic link between inhibition of placental mTOR signaling, down-regulation of placental nutrient transport and IUGR. However, the molecular mechanism by which mTOR regulates the cell surface expression of trophoblast amino acid transporters are largely unknown.

System A is a sodium-dependent transporter mediating the uptake of non-essential neutral amino acids into the cell [24]. All three known isoforms of System A, SNAT1 (SLC38A1), SNAT2 (SLC38A2), and SNAT4 (SLC38A4) are expressed in the placenta [25]. System L is

a sodium-independent amino acid exchanger mediating cellular uptake of essential amino acids including leucine [26]. The System L amino acid transporter is a heterodimer, consisting of a light chain, typically LAT1 (*SLC7A5*) or LAT2 (*SLC7A8*), and a heavy chain, 4F2hc/CD98 (*SLC3A2*). Amino acid uptake via all SLC38 transporters is coupled to the inward movement of sodium down its electrochemical gradient, which helps develop an outwardly-directed concentration gradient for System A substrates that can be utilized to drive the exchange uptake of a range of essential amino acids (*e.g.* leucine) through transporters (such as System L) that function in parallel with SLC38 in the plasma membrane.

Ubiquitination is a post-translational modification that has emerged as an important mechanism regulating the plasma membrane expression of transporters. Ubiquitination requires three sequential enzymatic steps (E1, E2, and E3), through which ubiquitin, a highly conserved 76-aa polypeptide, is attached to proteins. It was originally described as a signal that targets cellular proteins for rapid degradation, but was later found also to regulate numerous other processes in the cell, including protein trafficking [27]. Specificity of ubiquitination in mammals is provided by hundreds of different E3 ubiquitin–protein ligases that recognize relevant target proteins and promote the conjugation of ubiquitin. There are two major E3 classes—RING (really interesting new gene) and HECT (homologous to the E6-AP, carboxyl terminus)—the latter including the neuronal precursor cell-expressed developmentally downregulated (Nedd) 4-like proteins Nedd4-1 and Nedd4-2, which interact with target proteins through a specific motif, PY (L/PPxY) [28].

The molecular mechanisms regulating intracellular trafficking of membrane transporters have been established in detail for only two mammalian transporters, the epithelial sodium channel (ENaC) and glucose transporter 4 (GLUT4). Aldosterone and insulin regulates ENaC cell surface expression mediated by phosphorylation of SGK1 and Nedd4-2 [29, 30]. Recent studies in mice with tissue specific Nedd4-2 knockout have revealed a critical role for this E3 ubiquitin ligase in regulating salt and fluid transport in the lung and kidney epithelia and in maintaining homeostasis of systemic blood pressure [31]. Phosphorylation of Nedd4-2 interferes with the ability of the ubiquitin ligase to associate to ENaC, resulting in a decrease in ENaC internalization and, consequently increased cell surface expression of the transporter and increased sodium uptake [29, 30, 32, 33].

Although ubiquitination is well established to control trafficking of amino acid permeases in yeast [34], little is known about the mechanisms involved in regulating the trafficking of amino acid transport in mammalian cells. We have previously reported that both mTORC1 and mTORC2 regulate amino acid uptake in cultured primary human trophoblast (PHT) cells by influencing the plasma membrane trafficking of specific transporter isoforms: the System A isoform SNAT 2 and the System L transporter isoform LAT1 [19]. Membrane SNAT2 stability is regulated by the ubiquitin proteasome system (UPS) [35, 36]. The aim of this study was to explore the mechanism by which mTOR regulates plasma membrane trafficking of SNAT 2 and LAT1 in PHT cells, with special focus on mTORC1 and Nedd4-2 mediated ubiquitination. We hypothesized that inhibition of mTORC1 decreases amino acid uptake in PHT cells by activation of Nedd4-2, which increases transporter ubiquitination resulting in decreased transporter expression in the plasma membrane.

Materials and Methods

Isolation and culture of primary human trophoblast (PHT) cells

Cytotrophoblast cells were isolated from normal term placentas and cultured *in vitro* [37, 38]. Tissue collection was approved by the Institutional Review Board of the University of Colorado Anschutz Medical Campus. Cells were plated in either 60 mm culture dishes ($\sim 7.5 \times 10^6$ cells/dish for Western blot analysis) or 6-well plates (for amino acid uptake experiments; $\sim 3.75 \times 10^6$ cells/well for RNAi mediated gene silencing) and cultured in 5% CO₂, 95% atmosphere air at 37°C for 90 h. Cell culture media (DMEM/Hams F-12, supplemented with L-glutamine, penicillin, streptomycin, gentamycin, and 10 % fetal bovine serum) was changed daily.

Assessment of biochemical differentiation and viability—To confirm that trophoblast cells were undergoing biochemical differentiation, and to assess their viability with time in culture, the release of human Chorion Gonadotropin (hCG) by trophoblast cells into the culture medium 18, 42, 66 and 90 hours after plating was measured using a commercial ELISA kit, which detects the β -subunit of hCG (*Immuno Biological Labs, Minneapolis, MN*).

RNA Interference-mediated silencing—Dharmafect 2 transfection reagent (Thermo Scientific, Rockford, IL) and small interference RNAs (siRNAs) (Sigma-Aldrich, St. Louis, MO), targeting raptor (100 nM; sense, 5' CAGUUCACCGCCAUCUACA), rictor (100nM; sense, 5' CGAUC AUGGGCAGGUAUUA), DEPTOR (SASI_1297010-H/5582, 1297011-H) or Nedd4-2 (100 nM; 5' CCCUAUACAUUUAAGGACU) were used. Control cells were transfected with a non-coding scrambled sequence (100nM; sense: 5' GAUCAUACGUGCGAUCAGATT). siRNAs were added to cultured PHT cells ($\sim 3.75 \times 10^6$ cells/well in 6 well plate; $\sim 7.5 \times 10^6$ cells in 60 mm dish) after 18 h in culture, incubated for 24 h [39] and subsequently removed and replaced by fresh medium. At 90 hours in culture, efficiency of target silencing was determined at the protein and functional levels using Western blot.

System A and System L amino acid uptake assay

Amino acid uptake in PHT cells was determined at 90 hours in culture. The activity of System A and System L amino acid transporters was assessed by measuring the Na⁺-dependent uptake of [¹⁴C] methyl-aminoisobutyric acid (MeAIB) and the 2-amino-2-norbornane-carboxylic acid (BCH)-inhibitable uptake of [³H] leucine, respectively, as described in detail previously [19, 40].

Isolation of MVM from trophoblast cells

Microvillous plasma membranes (MVM) were isolated from total homogenates of cultured PHT cells using differential centrifugation and Mg²⁺ precipitation as described previously [19]. In brief, cells were lysed, scraped off the plate, homogenized and centrifuged. The pelleted crude membrane fraction was resuspended and 12 mM MgCl₂ was added. The mixture was stirred slowly for 20 min on ice and then centrifuged. The supernatant containing MVM was centrifuged at 125,000 g for 30 min and the final pellet was

resuspended. Protein concentration was determined using the Bradford assay. MVM enrichment was assessed using the MVM/homogenate ratio of alkaline phosphatase protein expression as determined using Western blot after loading equal amounts of protein of MVM and cell lysates.

Western blotting

For immunoblotting, cells were lysed in buffer containing phosphatase and protease inhibitors. Subsequently, cells were scraped, collected, and sonicated. Proteins in cell lysates and MVM were separated by electrophoresis. Western blotting was carried out as described [40]. Protein expression of Nedd4-2, ubiquitin and total and phosphorylated S6K (T-389), S6 (S-235/236), Akt (S-473), SGK (S-422) and PKC α (S-657), was determined in cell lysates using commercial antibodies (Cell Signaling Technology, Boston, MA). Protein expression of the System A amino acid transporter isoforms (SNAT2) and the System L amino acid transporter isoform (LAT1) was analyzed in total cell lysates and MVM preparations. A polyclonal SNAT2 antibody generated in rabbits [41], was received as a generous gift from Dr. Prasad at University of Georgia, Augusta. SNAT2 antibody specificity was validated by SNAT2 blocking peptide. An antibody targeting the LAT1 was produced in rabbits as described previously [42]. LAT1 antibody specificity was validated using LAT1 siRNA [43]. Anti- β actin was from Sigma-Aldrich, St. Louis, MO. The expression of actin was used as loading control. For each protein target the mean density of the control sample bands was assigned an arbitrary value of 1. All individual densitometry values were expressed relative to this mean.

Proximity ligation assay (PLA) and confocal microscopy

The principle for proximity ligation assay is described in Supplementary Figure 2. Isolated cytotrophoblast cells were grown on chamber slides (Lab-Tek) for 90 hours. At 66 hours, cells were treated with rapamycin (100 nM), a specific mTORC1 inhibitor to induce SNAT2/Ubiquitin interaction. The cells were fixed in ice-cold methanol at -20°C for 20 min and were blocked using 5% new born calf (NCS) serum in PBS for 1 hour followed by anti-SNAT2 (anti-rabbit) and anti-Ubiquitin (anti-mouse) incubation for 2 hours. PLA probes anti-rabbit PLUS and anti-mouse MINUS were diluted in Duolink dilution buffer and incubated in a pre-heated humidity chamber for 1 hour. This was followed by ligation, amplification and detection according to the Duolink In Situ Orange kit (Sigma-Aldrich) manufacturer's protocol. Confocal microscopy was performed using Zeiss LSM 780 microscope at 63x magnification using oil immersion. Images were captured in the same laser settings with four Z-step of 0.4 μm .

Immunoprecipitation—PHT cell lysates were incubated with SNAT2 or LAT1 antibody overnight and antibodies were precipitated with protein G-Sepharose. Immunoprecipitates and aliquots of cell lysates were denatured in sample buffer at 95°C , resolved by electrophoresis, and probed with ubiquitin antibody.

Data presentation and statistics—The number of experiments (n) represents the number of placentas studied. Data are presented as means $\pm$ S.E.M or + S.E.M. Differences between two independent groups were tested using unpaired Student's t-test. The repeated

measures analysis of variance (RMANOVA) with Tukey's post-hoc test was used to assess statistical differences between scramble siRNA, raptor/ricor siRNA, rictor + DEPTOR siRNA or raptor/ricor + Nedd4-2 siRNA groups. P value <0.05 was considered significant.

Results

DEPTOR silencing activates mTORC1 and mTORC2 signaling in PHT cells

We have previously reported that inhibition of mTORC1 or mTORC2 decreases amino acid uptake in PHT cell [19]. To further establish mTOR regulation of PHT amino acid transport we tested the hypothesis that activation of mTOR stimulates cellular amino acid uptake. DEPTOR is a constituent of both mTOR complexes and is considered a negative regulator of mTOR function [18]. Thus, to activate mTORC1 and mTORC2 signaling, we silenced the endogenous mTOR inhibitor DEPTOR in PHT cells. DEPTOR silencing in PHT cells decreased the protein expression of DEPTOR by 55% ($P<0.001$; $n=5$, Suppl Fig 1a, b). We also measured the activity of the mTOR pathway in cells with RNAi-mediated DEPTOR silencing. Importantly, DEPTOR silencing significantly increased the phosphorylation of S6 kinase (Thr-389), 4E-BP1 (Thr-37/46), S6 ribosomal protein (Ser-235/236), functional readouts for mTORC1, and Akt (Ser-473), reflecting the activity of mTORC2 signaling (Suppl Fig 1c, d). These results confirm that our DEPTOR RNAi approach efficiently activated the both mTORC1 and mTORC2 signaling pathways in PHT cells.

DEPTOR silencing does not affect PHT cell viability or differentiation

PHT cells form syncytial islands in culture, and this is used as a model for the syncytiotrophoblast, the transporting epithelium of the human placenta. After 66 h in culture, there was a marked increase in hCG production by trophoblast cells, and the levels remained high until at least 90 h after plating (Suppl Fig 1e). Because hCG is produced predominantly by syncytialized cells, these data provide evidence of cell differentiation. Furthermore, hCG secretion profiles were similar in cells in which DEPTOR had been silenced as compared to cells incubated in scrambled siRNA (Suppl Fig 1e). Collectively, these data indicate that key components of the trophoblast mTOR signaling pathway can be activated without adversely affecting trophoblast cell viability and differentiation.

DEPTOR silencing increases System A and L amino acid uptake

To determine if the activation of mTORC1 and 2 signaling resulted in increased amino acid transport, we measured amino acid uptake in DEPTOR deficient cells. DEPTOR silencing caused a pronounced increase in System A (+379%, $P<0.04$; $n=3$, Fig 1a) and System L (+175%, $P<0.05$; $n=4$, Fig 1b) mediated amino acid uptake as compared to control. This data confirms that both mTORC1 and mTORC2 signaling pathways are positive regulators of trophoblast amino acid uptake.

DEPTOR silencing increases MVM System A and L amino acid isoform expression

To study changes in cell surface transporter expression we isolated a microvillous plasma membrane (MVM) fraction from cultured primary trophoblast cells and determined isoform protein expression in response to activation of mTORC1 and 2 signaling. The justification for determining protein expression of transporters in MVM rather than in cell lysates is that

trophoblast nutrient transporters mediate cellular uptake and transfer across the placental barrier only if localized in the syncytiotrophoblast plasma membranes. Thus, data on amino acid transporter protein expression in MVM is much more informative than determination of protein expression in cell lysates. Furthermore, we demonstrated that mTOR regulates placental system A and L amino acid transporter isoform expression in MVM [19]. The enrichment of alkaline phosphatase, a MVM marker, was determined by the ratio of alkaline phosphatase expression in MVM over total cell lysates. Alkaline phosphatase enrichment in MVM isolated from control cells was 6.7 ± 0.5 , $n=3$, which was not different from MVM isolated from DEPTOR silenced cells (7.0 ± 0.6 , $n=3$). These data confirm significant MVM enrichment. We found that silencing of DEPTOR caused a marked increase in the expression of the System A transporter isoform SNAT2 (+ 73 %, $P<0.0005$; $n=6-7$, Fig 1c, e) and the System L transporter isoform LAT1 (+ 69 %, $P<0.005$; $n=6-7$, Fig. 1d, f,) in the MVM fraction. This data indicates that mTOR signaling regulates system A and L transport by post-translational mechanisms.

mTORC 1 inhibition increases SNAT2 and LAT1 ubiquitination

Ubiquitination is a post-translational modification that has been shown to be involved in regulating the plasma membrane expression of mammalian transporters, in particular ENaC [44]. To study the molecular mechanisms linking mTORC1 signaling to system A and L amino acid transporter intracellular trafficking, we measured system A (SNAT2) and system L (LAT1) transporter isoform ubiquitination in PHT cell lysates following mTORC1 inhibition. To this effect, we immunoprecipitated proteins in the cell lysate using an anti-SNAT2 or LAT1 antibody. The proteins in the immunoprecipitates were then separated by SDS-PAGE and subsequently immunoblotted with an anti-ubiquitin antibody. As shown in Fig 2a, b, raptor silencing (mTORC1 inhibition) resulted in increased ubiquitination of SNAT2 and LAT1 as compared to control.

To further confirm that inhibition of mTORC1 increases the interaction between SNAT2 and ubiquitin, we used proximity ligation assay and confocal microscopy, which allows the in situ detection of interacting endogenous proteins. A few SNAT2/ubiquitin complexes (Yellow) were co-localized in the control/scramble cells (Fig. 3). After rapamycin (a mTORC1 inhibitor) treatment and raptor siRNA silencing the number of SNAT2/ubiquitin complexes increased markedly (Fig. 3), suggesting increased ubiquitination of SNAT2.

mTORC1 but not mTORC2 silencing increases Nedd4-2 expression

Nedd4-2 is one of the E3 ubiquitin ligase that catalyzes ubiquitination of plasma membrane transporter and controls cell surface expression of transporters [44]. Membrane trafficking of the System A amino acid transporter isoform SNAT 2 in 3T3-L1 adipocytes is regulated by Nedd4-2 dependent ubiquitination [36]. Moreover, aldosterone and insulin regulates ENaC cell surface expression mediated by phosphorylation of SGK1 and Nedd4-2 [29, 30]. Because SGK1 is a downstream target of mTORC2, this data implicates mTORC2 in Nedd4-2 mediated transporter ubiquitination. This provides the rationale for studying the regulation of Nedd4-2 by mTORC1 and mTORC2 in PHT cells. We found that silencing of raptor, but not rictor silencing (specifically inhibiting mTORC2), increased the expression of Nedd4-2 (+81%, $P<0.05$; $n=3$, Suppl Fig 3a, b) as compared to PHT cells transfected with

scramble siRNA. Because SGK1 has been shown to regulate Nedd4-2 we explored the possibility that mTORC1 signals through SGK1 in PHT cells. However, mTORC1 inhibition by raptor silencing did not alter the phosphorylation of SGK1 in PHT cells (Suppl Fig 3c, d). Similarly, silencing of raptor did not alter the phosphorylation of PKC α , another mTORC2 target (Suppl Fig 3c, d).

Nedd4-2 silencing increases system A and L amino acid transport

Next, we determined the effect of Nedd4-2 silencing on amino acid transport activity. To confirm efficient silencing, we performed western blots on siRNA-transfected cells at 90 h of culture (Suppl Fig 4a, b). Nedd4-2 siRNA decreased the Nedd4-2 protein expression by 70 % ($P<0.0005$; $n=3$) as compared to control cells. Furthermore, hCG secretion profiles were similar in cells in which Nedd4-2 had been silenced as compared to cells incubated in scrambled siRNA (Suppl Fig 4c). As shown in Fig 4, Nedd4-2 silencing resulted in a marked increase in System A (+77%, $P<0.05$; $n=5$, Fig 4a) and System L (+126%, $P<0.001$; $n=5$, Fig 4b) amino acid uptake as compared to control, suggesting that Nedd4-2 is involved in the regulation of amino acid transporter activity.

Nedd4-2 silencing promotes trafficking of SNAT2 and LAT1 to the plasma membrane

We found that Nedd4-2 silencing caused a marked increase in the expression of the System A transporter isoform SNAT2 (+120%, $P<0.05$; $n=3$, Fig 4c, d) and System L transporter isoform LAT1 (+135%, $P<0.005$; $n=3$, Fig 4c, d) in the microvillous plasma membrane fraction. Collectively, these data support the hypothesis that Nedd4-2 regulates System L and A amino acid transporter activity by modulating transporter trafficking through ubiquitination of specific transporter isoforms.

Rictor + DEPTOR silencing efficiency

Our data suggest that mTORC1 inhibition (raptor silencing) increased the Nedd4-2 expression (Supl Fig 2a,b) and increased the ubiquitination of SNAT2 and LAT1 isoforms (Fig 2). As expected, silencing of raptor decreased the phosphorylation of mTORC1 functional readouts S6K and S6 but had no effect on the phosphorylation of Akt at Ser-473, a functional readout for mTORC2 (Suppl Fig 5). This observation suggests that there is no significant cross talk between mTORC1 and mTORC2 in PHT cells. To further confirm that Nedd4-2 is involved in mTORC1 mediated membrane trafficking of transporters, we studied Nedd4-2 expression, amino acid transporter activity and expression in PHT cells with activation of mTORC1. To selectively activate mTORC1, we silenced DEPTOR (activates mTORC1 and mTORC2) and rictor (inhibits mTORC2). Silencing of PHT cells with rictor + DEPTOR siRNA increased the phosphorylation of S6K (Thr-389) and S6 (Ser-235/236) but did not affect the phosphorylation of Akt at Ser-473 (Suppl Fig 5), consistent with specific activation of mTORC1.

mTORC1 activation increases System A and L amino acid transport activity and isoform expression

Confirming our previous studies [19], inhibition of mTORC1 by silencing of raptor decreased System A (−69 %, $P<0.05$; $n=4$, Fig 5a) and System L amino acid transporter

activity (−67%, $P<0.05$; $n=4$, Fig 5b) as compared to cells transfected with scramble siRNA. Activation of mTORC1 by silencing rictor + DEPTOR decreased Nedd4-2 protein expression (data not shown) and increased System A (+64%, $P<0.05$; $n=4$, Fig 5a) and System L activity (+48%, $P<0.05$; $n=4$, Fig 5b). Furthermore, inhibition of mTORC1 decreased the expression of the System A transporter isoform SNAT2 (−56%, $P<0.05$; $n=3$, Fig 5c, d) and the System L transporter isoform LAT1 (−76%, $P<0.05$; $n=4$, Fig 5c, e) in the microvillous plasma membrane fraction as compared to cells transfected with scramble siRNA. In contrast, activation of mTORC1 by silencing rictor + DEPTOR increased SNAT2 (+64%, $P<0.05$; $n=3$, Fig 5c, d) and LAT1 expression in MVM (+107%, $P<0.01$; $n=3$, Fig 5c, e). This data is consistent with the model that mTORC1 activation stimulates PHT amino acid uptake and cell surface expression of key amino acid transporter isoforms by down-regulation of Nedd4-2.

Nedd4-2 is required for mTORC1 but not mTORC2 regulation of System A and L amino acid transport activity and isoform plasma membrane trafficking

To mechanistically link mTOR, Nedd4-2 and amino acid transport activity, PHT cells were transfected with raptor or rictor siRNA or raptor + Nedd4-2 or rictor + Nedd4-2 siRNA, and System A and L amino acid transport activity were measured at 90 hours. As shown in Fig 6, silencing of raptor (System A, −57%, $P<0.01$; System L, −55%, $P<0.01$; $n=5$, Fig 6a, b) or rictor (System A, −59%, $P<0.01$; System L, −58%, $P<0.02$; $n=5$, Fig 6c, d) markedly inhibited basal levels of System A and System L amino acid transporter activity in cultured primary human trophoblast cells as compared to cells transfected with scramble siRNA. These data confirm our previous observations that mTORC1 and mTORC2 independently regulate trophoblast amino acid transport [19]. Raptor + Nedd4-2 silencing prevented the inhibitory effect of mTORC1 silencing on System A ($n=5$, Fig 6a) and System L ($n=5$, Fig 6b) amino acid transport activity. In contrast, Nedd4-2 silencing did not influence the ability of mTORC2 silencing to inhibit System A (Fig 6c) and System L (Fig 6d) amino acid transport activity.

Inhibition of mTORC1 or mTORC2 caused a marked decrease in the expression of the System A transporter isoform SNAT2 (raptor siRNA: −67 %, $P<0.05$; $n=3$, Fig 6e, f; rictor siRNA: −71 %, $P<0.01$; $n=3$, Fig 6h, i) and System L transporter isoform LAT1 (raptor siRNA: −65 %, $P<0.05$; $n=3$, Fig 6e, g; rictor siRNA: −47 %, $P<0.01$; $n=3$, Fig 6h, j) in the MVM, as compared to cells treated with scramble siRNA. Nedd4-2 silencing prevented the decrease in SNAT2 (Fig 6e, f) and LAT1 (Fig 6e, g) MVM expression in response to mTORC1 inhibition. In contrast, Nedd4-2 silencing did not influence the decrease in SNAT2 (Fig 6h, i) and LAT1 (Fig 6h, j) MVM expression following mTORC2 inhibition. This data demonstrates that Nedd4-2 is required for the regulation of plasma membrane trafficking of amino acid transporter isoforms by mTORC1 but not mTORC2.

Discussion

The novel finding in this report is that Nedd4-2 is required for the regulation of plasma membrane trafficking of amino acid transporter isoforms by mTORC1. First, silencing of mTORC1 increased Nedd4-2 expression and caused ubiquitination of the System A and L

isoforms SNAT2 and LAT1. Second, Nedd4-2 inhibition stimulated system A and L amino acid transporter activity by increasing SNAT2 and LAT1 expression in MVM. Third, Nedd4-2 silencing prevented the decrease in System A and L activity in response to mTORC1 inhibition. These findings are consistent with the model that inhibition of placental mTORC1 in human IUGR [20, 21] increases the ubiquitination of specific System A and L amino acid transporter isoforms mediated by activation of Nedd4-2 (Fig 7), resulting in decreased placental amino acid transport and fetal amino acid availability [5–8].

DEPTOR (DEP-domain containing mTOR-interacting protein) binds to both mTORC1 and mTORC2, as evident from co-immunoprecipitation experiments [18]. Its precise function is not fully elucidated, but Peterson et al. [18] have shown in a series of elegant experiments that knocking down DEPTOR leads to activation of signaling through mTORC1 and mTORC2. We have previously shown that inhibition of mTORC1 by raptor silencing or inhibition of mTORC2 using silencing of rictor cause a marked decrease in System A and System L amino acid transport, mediated by altered plasma membrane trafficking of SNAT2 and LAT1 [19]. Here, we extend these studies and demonstrate that activation of mTORC1 and mTORC2 by DEPTOR silencing promotes trafficking of SNAT2 and LAT1 to the plasma membrane, which increases System A and System L amino acid transporter activity. This is the first report showing up regulation of amino acid transport by mTORC1 and 2 activation, in any cell type, and confirms an important role of mTOR in regulating trophoblast amino acid transport.

The ubiquitin-proteasome pathway is involved in the degradation of plasma membrane transporters [45]. Nedd4–2 belongs to the family of homologous to E6-AP COOH-terminus (HECT) E3 ligases that catalyze the final step in the ubiquitination cascade, the conjugation of ubiquitin to lysine residues of their target proteins, thus targeting them for degradation [27, 46]. Nedd4–2 ubiquitinates the epithelial sodium channel ENaC [47], the chloride channel CIC-5, voltage-gated potassium and the cardiac potassium channel hERG1 [48]. In the present study we found that mTORC1 silencing increased Nedd4-2 expression and SNAT2 ubiquitination, which is associated with a decreased translocation of amino acid transporter SNAT2 from an intracellular pool to the MVM. Furthermore, silencing Nedd4-2 increases SNAT2 and LAT1 expression in MVM and prevented mTORC1 inhibition of System A and System L activity. Taken together, this data is consistent with an important role in Nedd4-2 mediated ubiquitination in mediating the effect of mTORC1 on transporter isoform plasma membrane trafficking and transport activity.

Our data in primary human trophoblast cells are in general agreement with the observation by Hatanaka [36] and coworkers that membrane trafficking of the System A amino acid transporter isoform SNAT 2 in 3T3-L1 adipocytes is regulated by Nedd4-2 dependent ubiquitination. It is therefore possible that Nedd4-2 dependent ubiquitination is a mechanism by which key amino acid transporters are regulated in many cell types. The current study extends previous knowledge by demonstrating that mTORC1 inhibition increases Nedd4-2 protein expression and that Nedd4-2 is required for mTORC1 regulation of System A and L activity. The mechanism involved in mTORC1 modulation of Nedd4-2 remains to be established. Although it was reported recently that mTORC2 regulates ENaC via SGK1 phosphorylation [49], it is unknown whether mTORC1 regulates amino acid transporter

trafficking mediated by this signaling pathway. However, our data show that mTORC1 does not signal through SGK in PHT cells.

Although both mTORC1 and mTORC2 signaling regulates System A and L amino acid uptake by governing the cell surface expression of SNAT2 and LAT1, the current study demonstrates that distinct molecular mechanisms are involved. Specifically, whereas Nedd4-2 is required for mTORC1-mediated regulation of plasma membrane trafficking of amino acid transporters, mTORC2 regulation of amino acid transport in PHT cells is independent of Nedd4-2. Given the involvement of SGK1, a mTORC2 target, and Nedd4-2 in ENaC trafficking [50] this finding was unexpected. However, this may reflect that primary human cells behave differently than transformed cell lines. Alternatively, the biology of human trophoblast cells may differ from primary cells isolated from other tissues. This possibility is supported by the observation that full length adiponectin, which is well known to promote insulin sensitivity in, for example, muscle and liver, has the opposite effect in trophoblast *in vitro* [51, 52] and the placenta *in vivo* [53]. Nevertheless, the mechanisms linking mTORC2 to increased cell surface expression of amino acid transporter remains to be established.

Abnormal fetal growth affects 15% of all babies and increases the risk for injuries at delivery and to develop obesity, diabetes, and cardiovascular disease in childhood and later in life [1, 2]. No specific strategies to treat these conditions are currently available. Altered placental nutrient transport is believed to directly contribute to changes in fetal growth [11–13] and in order to better understand the underlying causes of these conditions identification of key mechanisms regulating the transfer of nutrients in the human placenta is critically needed. Placental mTORC1 signaling is inhibited in human IUGR [20, 54] and activated in association to fetal overgrowth [10]. Furthermore, preliminary observations from our laboratory show that placental Nedd4-2 protein expression and SNAT2 ubiquitination is increased and SNAT2 expression in the syncytiotrophoblast plasma membranes is decreased in IUGR [55]. These findings together with the data reported in the current study suggest that placental amino acid transporter ubiquitination by mTORC1 and Nedd4-2 constitutes a molecular mechanism underlying abnormal fetal growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

BCH	2-amino-2-norbornane-carboxylic acid
DEPTOR	DEP domain-containing mTOR-interacting protein

4EBP-1	4E-eukaryotic initiation factor binding protein-1
ENaC	epithelial sodium channel
4F2hc	4F2 cell-surface antigen heavy chain
hCG	human chorion gonadotropin
HECT	homologous to the E6-AP, carboxyl terminus
GLUT4	glucose transporter 4
IGF-I	insulin-like growth factor I
IUGR	intrauterine growth restriction
LAT	large neutral amino acid transporter
MeAIB	methyl-aminoisobutyric acid
mTOR	mechanistic target of rapamycin
mTORC1 and 2	mechanistic target of rapamycin complex 1 and 2
MVM	microvillous plasma membrane
NCS	newborn calf serum
Nedd4-2	neuronal precursor cell-expressed, developmentally downregulated gene 4 isoform 2
PKCα	protein kinase C α
PLA	proximity ligation assay
Raptor	regulatory associated protein of mTOR
Rictor	rapamycin-insensitive companion of mTOR
RING	really interesting new gene
SGK1	serum and glucocorticoid-regulated kinase 1
siRNA	small interfering RNA
S6K1	p70 S6 kinase
SNAT	sodium-dependent neutral amino acid transporter
UPS	ubiquitin proteasome system

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Summary statement

We demonstrate that mTOR Complex 1 modulates amino acid transport in primary human trophoblast cells by regulating Nedd4-2 mediated ubiquitination and plasma membrane trafficking of specific transporter isoforms, which may constitute a molecular mechanisms underlying abnormal human fetal growth.

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Clinical perspectives

- Changes in placental amino acid transport directly contribute to altered fetal growth, which increases the risk for perinatal complications and predisposes for the development of obesity, diabetes and cardiovascular disease later in life. However, the molecular mechanisms regulating placental amino acid transporters are not well established.
- We demonstrate that mTOR Complex 1 modulates amino acid transport in primary human trophoblast cells by regulating Nedd4-2 mediated ubiquitination and plasma membrane trafficking of specific transporter isoforms.
- Because placental mTORC1 is inhibited in fetal growth restriction and activated in fetal overgrowth, we propose that regulation of placental amino acid transporter ubiquitination by mTORC1 and Nedd4-2 constitutes a molecular mechanisms underlying abnormal fetal growth. This data advance our mechanistic understanding of common pregnancy complications and may provide a foundation for the development of intervention strategies for fetal growth restriction and overgrowth.

Figure 1. Activation of mTORC1 and 2 stimulates PHT amino acid transport

a–b) Silencing of DEPTOR activates System A and System L amino acid uptake in PHT cells.

System A activity (a) was determined as the Na^+ -dependent uptake of [^{14}C] MeAIB, and System L activity (b) was measured as the BCH-inhibitable uptake of [^3H] leucine. Values are means + SEM; n = 3–4 for System A and System L. * P < 0.05 versus control; unpaired Student's t test.

c–d) Protein expression of System A (SNAT2) and System L (LAT1) amino acid transporter isoforms in MVMs isolated from control and DEPTOR silenced PHT cells.

Representative western blots are shown for sodium-coupled neutral amino acid transporter (SNAT2) and L-type amino acid transporter (LAT1) in MVMs of PHT cells transfected with scramble siRNA or siRNA targeting DEPTOR. Equal loading was performed. Histograms

(e–f) summarize the data. Expression in control cells for each isoform was arbitrarily assigned a value of one. Values are means + SEM; n=6–7. *P < 0.05 versus control; unpaired Student's t test.

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Figure 2. Increased ubiquitination of SNAT2 and LAT1 in response to mTORC1 inhibition
PHT cell lysates were used for immunoprecipitation (IP) with an anti-SNAT2 (a) or LAT1 antibody (b). Immunoprecipitated proteins were separated by SDS-PAGE and blotted (IB) with an anti-ubiquitin (Ub) antibody. The data are from a representative experiment, and similar results were obtained from two other experiments.

Figure 3. Interaction of SNAT2 and ubiquitin following mTORC1 inhibition

Immunofluorescence confocal microscopy in combination with *in situ* PLA, which detects protein-protein complexes, was used to explore interactions between SNAT2 to ubiquitin following mTORC1 inhibition with rapamycin or raptor siRNA. Each detected complex is represented by a yellow dot. DNA was counterstained by DAPI (blue). Scale bar represents 20 μ m.

Figure 4. Nedd4-2 silencing stimulates PHT amino acid transport

System A activity (a) was determined as the Na⁺-dependent uptake of [¹⁴C] MeAIB, and System L activity (b) was measured as the BCH-inhibitable uptake of [³H] leucine. Values are means + SEM; n = 5 for System L and System A. * P < 0.05 versus control; unpaired Student's t test. Representative western blots are shown for L-type amino acid transporter [LAT1] and sodium-coupled neutral amino acid transporter [SNAT2] in MVM of control and Nedd4-2 silenced cells (c). Equal loading was performed. Histogram (d) summarize the data. Expression in control cells for each isoform was arbitrarily assigned a value of one. Values are means + SEM; n=6–7. *P < 0.05 versus control; unpaired Student's t test.

Figure 5. Activation of mTORC1 increases System A and System L amino acid uptake in PHT cells

System A activity (a) was determined as the Na^+ -dependent uptake of [^{14}C] MeAIB, and System L activity (b) was measured as the BCH-inhibitable uptake of [^3H] leucine. Values are means + SEM; $n = 3-4$ for System L and System A. Means without a common letter differ significantly ($P < 0.05$) by RMANOVA with Tukey–Kramer multiple comparisons *post hoc* test.

Representative western blots are shown for L-type amino acid transporter (LAT1) and sodium-coupled neutral amino acid transporter (SNAT2) in MVMs of scramble siRNA, raptor siRNA and rictor + DEPTOR silenced PHT cells (c). Equal loading was performed. Histograms (d–e) summarize the results. Expression in control cells for each isoform was arbitrarily assigned a value of one. Values are means + SEM; $n=4$. Means without a common letter differ significantly ($P < 0.05$) by RMANOVA with Tukey–Kramer multiple comparisons *post hoc* test.

Figure 6. Nedd4-2 is required for mTORC1 but not mTORC2 regulation of System A and L amino acid transport activity and isoform plasma membrane trafficking

System A activity (a, c) was determined as the Na^+ -dependent uptake of [^{14}C] MeAIB, and System L activity (b, d) was measured as the BCH-inhibitable uptake of [^3H] leucine. Values are means + SEM; $n = 5$ for System L and System A. Means without a common letter differ significantly ($P < 0.05$) by RMANOVA with Tukey–Kramer multiple comparisons *post hoc* test.

Representative western blots are shown for sodium-coupled neutral amino acid transporter [SNAT2] and L-type amino acid transporter [LAT1] in MVM of scramble, raptor/ricitor and raptor or rictor +Nedd4-2 silenced cells (e & h). Equal loading was performed. Histograms (f, g, i & j) summarize the data. Expression in control cells for each isoform was arbitrarily assigned a value of one. Values are means + SEM; $n=3$. Means without a common letter differ significantly ($P < 0.05$) by RMANOVA with Tukey–Kramer multiple comparisons *post hoc* test.

Figure 7. A proposed model linking mTORC1 inhibition to decreased placental amino acid transport in human IUGR

The findings of the present study are consistent with the model that inhibition of placental mTORC1 in human IUGR increases the ubiquitination of specific System A and L amino acid transporter isoforms mediated by activation of Nedd4-2, resulting in decreased placental amino acid transport and fetal amino acid availability.