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Research Report

Trophoblast-specific overexpression of the LAT1 increases transplacental transport of essential amino acids and fetal growth in mice

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Abstract

Placental System L amino acid transporter activity is decreased in pregnancies complicated by intrauterine growth restriction (IUGR) and increased in fetal overgrowth. However, it is unknown if changes in the expression/activity of placental Large Neutral Amino Acid Transporter Small Subunit 1 (Slc7a5/LAT1) are mechanistically linked to placental function and fetal growth. We hypothesized that trophoblast-specific *Slc7a5* overexpression increases placental transport of essential amino acids, activates the placental mechanistic target of rapamycin (mTOR) signaling, and promotes fetal growth in mice. Using lentiviral transduction of blastocysts with a *Slc7a5* transgene, we achieved trophoblast-specific overexpression of *Slc7a5* (*Slc7a5* OX) with increased fetal (+27%) and placental weights (+10%). Trophoblast-specific *Slc7a5* overexpression increased trophoblast plasma membrane (TPM) LAT1 protein abundance and TPM System L transporter (+53%) and System A transporter activity (+ 21%). *Slc7a5* overexpression also increased transplacental transport of leucine (+ 85%) but not of the System A tracer, 14C-methylamino isobutyric acid, in vivo. Trophoblast-specific overexpression of *Slc7a5* activated placental mTORC1, as assessed by increased (+44%) phosphorylation of S6 ribosomal protein (Ser 235/236), and mTORC2 as indicated by phosphorylation of PKCα-Tyr-657 (+47%) and Akt-Ser 473 (+96%). This is the first demonstration that placental transport of essential amino acids is mechanistically linked to fetal growth. The decreased placental System L activity in human IUGR and the increased placental activity of this transporter in some cases of fetal overgrowth may directly contribute to the development of these pregnancy complications.

Keywords: placenta, maternal–fetal exchange, leucine, mechanistic target of rapamycin, fetal development

Significance Statement

Intrauterine growth restriction (IUGR) and fetal overgrowth are complications affecting 15–20% of pregnancies worldwide. Placental transport of essential amino acids is decreased in human IUGR and increased in fetal overgrowth; however, if these associations reflect cause-and-effect, it is unknown. We developed a mouse with trophoblast-specific overexpression of *Slc7a5*, encoding the L-type amino acid transporter LAT1 that mediates the transport of essential amino acids. Trophoblast-specific *Slc7a5* overexpression increased placental transport of essential amino acids, activated placental mTOR signaling, and caused fetal overgrowth. We show that placental transport of essential amino acids mediated by LAT1 is mechanistically linked to fetal growth. This information will help us better understand the causes of abnormal fetal growth and may lead to novel treatments for pregnancy complications.

Introduction

Intrauterine growth restriction (IUGR) and fetal overgrowth are common pregnancy complications associated with elevated perinatal morbidity and mortality and increased risk for the child to develop cardiovascular and metabolic disease later in life (1–3). No specific intervention is available to treat abnormal fetal growth, and the underlying mechanisms remain poorly understood. Normal fetal growth critically depends on the optimal supply of oxygen and nutrients, mediated by placental transport from mother to fetus (4). Of particular importance is the transplacental

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transfer of amino acids, which the fetus requires for protein accretion, metabolic processes, and biosynthetic pathways (5, 6). Altered fetal growth in women is associated with specific changes in placental amino acid transport capacity with decreased activity of several placental amino acid transport systems in IUGR (7–11), and increased placental amino acid transport capacity in some cases of fetal overgrowth (12, 13). However, whether these changes in placental amino acid transport cause abnormal fetal growth or are a secondary consequence of altered fetal growth remains largely unknown. One exception is SNAT2 (*Slc38a2*), mediating the transport of non-essential amino acids. We recently reported that placenta-specific knockdown of *Slc38a2* causes fetal growth restriction in mice (14), suggesting that changes in the activity of this particular amino acid transporter in the placental barrier are mechanistically linked to altered fetal growth.

The Na^+-independent System L amino acid transporters are believed to be vital for the transfer of essential amino acids, including leucine, across the human placenta (15). Several studies have reported decreased activity of placental System L transport in IUGR (7, 8) and increased System L activity in the placenta of large for gestational age (LGA) infants (12). System L transporters are heterodimeric exchangers, which consist of the light chain L-type amino acid transporter LAT1 (*SLC7A5*) or LAT2 (*SLC7A8*) covalently linked to the 4F2hc (*SLC3A2*) via a disulfide bridge. LAT1 transports neutral amino acids with branched or aromatic side chains (16). In the human placenta, LAT1 is predominately colocalized with 4F2hc in the maternal-facing microvillous plasma membrane (MVM) and basal plasma membrane of the syncytiotrophoblast.

LAT1 mRNA is expressed in all stages of the pre- and post-implantation embryos of the mouse (17). Placental MVM System L amino acid transporter activity was higher in early human gestation than in late pregnancy (18). Several pieces of evidence suggest that System L is critical in mediating trophoblast essential amino acid uptake and that placental system L amino acid activity is correlated to fetal growth. For example, reduction in vivo placental system L amino acid transport precedes the development of fetal growth restriction in diet-restricted rats and nonhuman primates (19, 20). Targeting the LAT1 transporter with small molecule inhibitors reduced System L-mediated leucine transfer in primary human trophoblast (PHT) cells and BeWo cells cultured in a Trans well system, a model of the maternal–fetal placental barrier in women (21). Fetal overgrowth is associated with increased placental System L amino acid transport activity in a variety of animal models (22). In addition, hormones that are known to influence fetal growth in pregnant animals frequently tend to alter the LAT1 abundance and the activity of System L in the placenta (23, 24). However, if changes in placental LAT1 expression are mechanistically linked to placental function and fetal growth is unknown.

Mechanistic target of rapamycin (mTOR) signaling is a conserved serine–threonine kinase, that is present in the cell in two discrete protein complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 promotes protein translation and activates anabolic pathways, supporting cellular growth and proliferation (25). mTORC2 regulates cellular cytoskeletal remodeling, cell migration, glycolysis and autophagy (26, 27). mTOR function as a master regulator of placental function, including amino acid transport (28). Specifically, we reported that inhibition of mTOR signaling via siRNA-mediated knockdown of raptor (inhibiting mTORC1) or rictor (inhibiting mTORC2) in cultured PHT cells resulted in decreased LAT1 transporter trafficking to MVM and inhibited the uptake of leucine (28, 29). mTOR responds to an array of diverse nutritional and metabolic signals, particularly essential amino acids, including those transported by LAT1 (30). Amino acid dependent activation of the mTOR signaling is well known in many cell types. However, if the function of trophoblast LAT1 is mechanistically linked to placental mTOR signaling remains to be established.

The aim of the current study was to determine the mechanistic role of trophoblast LAT1 in mediating the in vivo placental transport of essential amino acids and in regulating fetal growth. We hypothesized that trophoblast-specific overexpression of *Slc7a5* increases placental transport of essential amino acids, activates placental mTOR signaling, and promotes fetal growth in mice. We used a lentiviral vector to deliver a *Slc7a5* overexpression (*Slc7a5* OX) construct to the blastocyst trophectoderm in early gestation (31–33) and subsequently determined placental and fetal weight, placental mTOR signaling, in vivo placental amino acid transport capacity by unidirectional maternal–fetal clearance of the tracer leucine and placental amino acid transporter abundance near term.

Results

Validation of trophoblast-specific overexpression of *Slc7a5* in mice

On E18.5, implantation and viability rates were similar in NSS and *Slc7a5* overexpression conceptuses (Table S1). Placental (includes labyrinth and junctional zone) *Slc7a5* mRNA expression was 8.8-fold higher ($P = 0.01$) in *Slc7a5* overexpression placentas than in NSS placentas (Fig. 1a), whereas *Slc7a8, Slc3a2, Slc38a1, Slc38a2*, and *Slc38a4* mRNA expression were comparable in the two groups, as expected (Fig.1b–f). Furthermore, *Slc7a5*

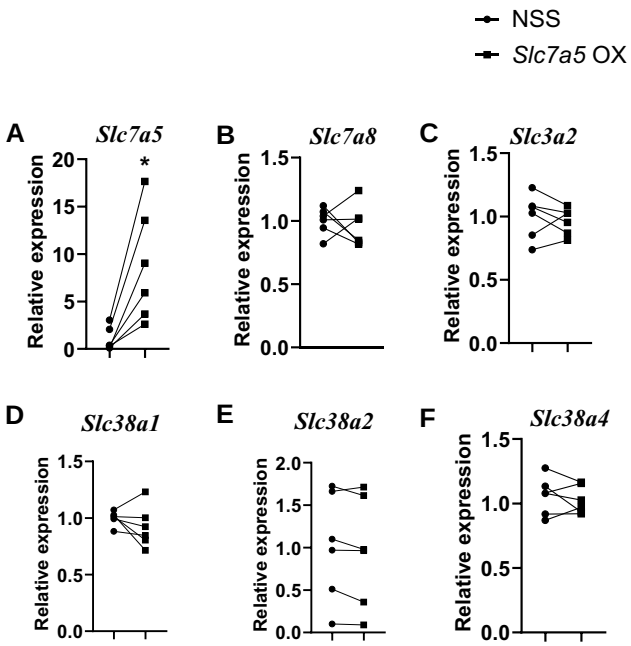

Fig. 1. Trophoblast-specific overexpression of *Slc7a5* increases placental *Slc7a5* mRNA expression at E18.5. a–f) Gene expression of *Slc7a5* (LAT1), *Slc7a8* (LAT2), *Slc3a2* (4F2hc), *Slc38a1* (SNAT1), *Slc38a2* (SNAT2), *Slc38a4* (SNAT4) mRNA in NSS and *Slc7a5* overexpression placentas, pooled from $n = 6$ litters/group. Each dot represents individual value; *$P < 0.05$ vs. NSS; paired Student's t test. All placentas of each genotype in a litter were pooled and homogenized prior to analysis.

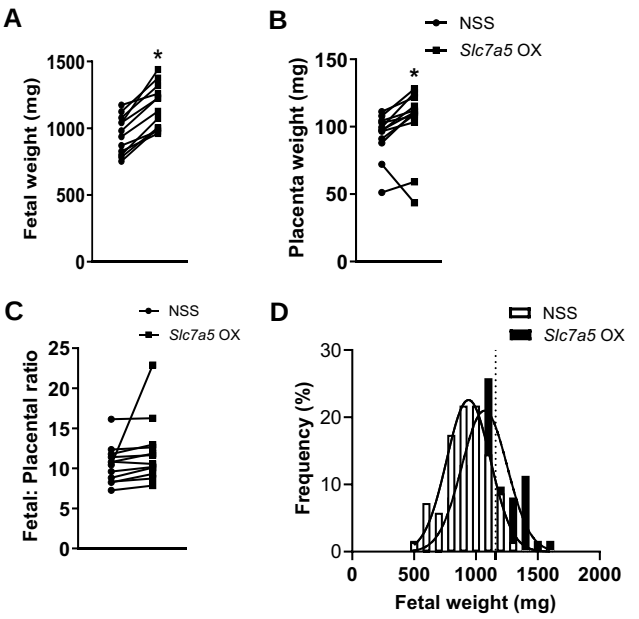

Fig. 2. Trophoblast-specific overexpression of *Slc7a5* increases fetal and placental weights at E18.5. a–c) Fetal weight, placental weight, fetal/placental ratio in NSS and *Slc7a5* overexpression group, $n = 12$ litters/group. For fetal and placental data, the means of each genotype in a litter were calculated and used in statistical analysis. Each dot represents individual value; *$P < 0.05$ vs. NSS; paired Student's t test. d) Frequency distribution of individual fetal weights in NSS ($n = 69$) and *Slc7a5* OX ($n = 62$) groups. Gaussian curves fitted by least-squares nonlinear regression and compared by extra sum-of-squares F-test ($P = 0.001$): NSS amplitude 22.57%, mean 940 mg, SD 176; *Slc7a5* OX amplitude 20.9%, mean 1069 mg, SD 183 mg. The dotted vertical line indicates the 90th percentile of NSS fetal weights (1159 mg).

mRNA expression in the fetal liver did not differ between NSS and *Slc7a5* OX group (Fig. S1), supporting trophoblast-specific gene targeting.

Trophoblast-specific overexpression of *Slc7a5* increases fetal weight in mice

As show in Fig. 2, trophoblast-specific overexpression *Slc7a5* increased the fetal weight by 27% ($P = 0.0001$, $n = 12$ dams/each group, Fig. 2a) as compared to NSS conceptuses. Moreover, there was an increase in placental weight by 10% ($P = 0.02$, $n = 12$ dams/each group) in the *Slc7a5* OX group compared to NSS (Fig. 2b). There was no statistically significant difference in fetal/placental weight-ratio between groups ($P = 0.141$, Fig. 2c). The frequency distribution of fetal weights in *Slc7a5* OX group was right-shifted ($P = 0.001$, $n = 62$–67 fetuses/group), compared to NSS (Fig. 2d).

Trophoblast-specific *Slc7a5* overexpression increases placental trophoblast plasma membrane LAT1 expression

To mediate cellular uptake and transplacental transport, the LAT1 protein must be translocated to the trophoblast plasma membrane (TPM). In addition, the plasma membrane trafficking of LAT1 is highly regulated in trophoblasts (9, 28, 29, 34). It is therefore important to confirm that the marked increase in *Slc7a5* gene expression in *Slc7a5* OX placentas also results in an increase of LAT1 protein in the TPM. We used western blotting to determine LAT1 protein abundance in TPM isolated from E18.5 NSS and *Slc7a5* OX placentas. As shown in Fig. 3a and b, TPM LAT1 was 73% higher in *Slc7a5* OX than in NSS placentas ($P = 0.0004$, $n = 6$/group).

Trophoblast-specific *Slc7a5* overexpression increases placental TPM System L amino acid transport

We previously demonstrated that the mediated uptake of ^{3}H-leucine, corresponding to system L activity, is linear up to 30 s (24, 35). Based on time-course experiments (24, 35), an incubation time of 15 s was chosen to measure the System L amino acid transport activity in the current study. In TPM isolated from *Slc7a5* overexpression placentas, the capacity to transport leucine was markedly increased (+53%, $P = 0.001$, $n = 6$/each group; Fig. 3c) as compared to TPM isolated from NSS placentas.

Trophoblast-specific *Slc7a5* overexpression increases placental TPM System A amino acid transport

We recently demonstrated that microvillus plasma membrane System L activity positively correlated with System A activity in nonhuman primates (19). System A is a ubiquitous Na^+-dependent transporter that is responsible for active transport of small, neutral and nonessential amino acids with short, unbranched side chains, including alanine, serine, and glutamine. As shown in Fig. 3d, overexpression of *Slc7a5* in the placenta increased TPM System A amino acid transport by + 21% ($P = 0.03$, $n = 6$/each group) as compared to TPM isolated from NSS placentas.

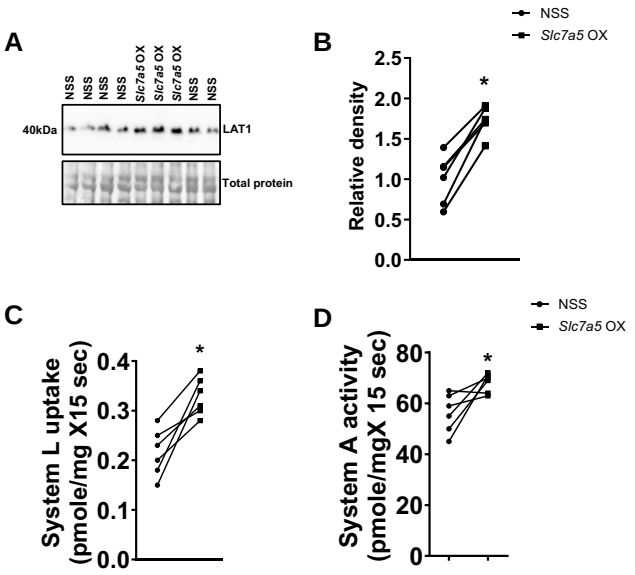

Fig. 3. Protein expression of system L amino acid transporter isoform LAT1. a, b) and activity of system L c) and system A d) amino acid transporters in TPM isolated from NSS and *Slc7a5* overexpression placentas. a) Representative western blot is shown for L-type amino acid transporter (LAT1, 42 kDa) in TPM isolated from NSS and *Slc7a5* OX mice placenta at E 18.5. b) The histogram summarizes the western blotting data (NSS, $n = 6$; *Slc7a5*, $n = 6$). After normalization to total protein, the mean density of NSS samples was assigned an arbitrary value of 1. Subsequently, individual NSS and *Slc7a5* group density values were expressed relative to this mean. c) System L and A d) System transporter activities were determined using isotope-labeled substrates and rapid filtration techniques in TPM isolated from NSS and *Slc7a5 OX* mice placenta at E 18.5. (NSS, $n = 6$; *Slc7a5 OX*, $n = 6$). Each dot represents individual value; *$P < 0.05$ vs. NSS; paired Student's t test. All placentas of each genotype in a litter were pooled and homogenized prior to analysis.

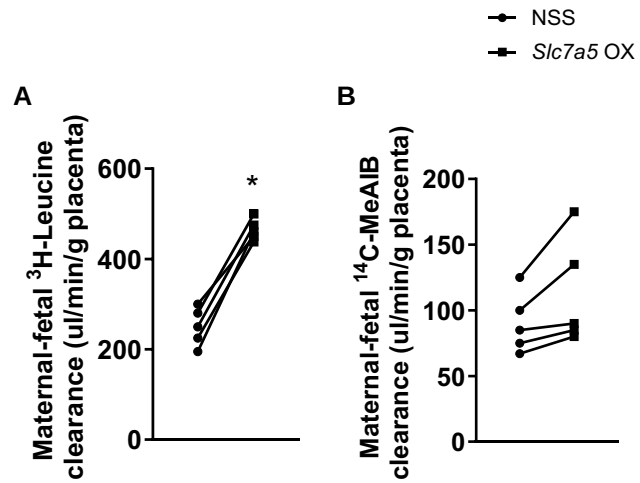

Fig. 4. Trophoblast-specific overexpression of *Slc7a5* increases transplacental ^{3}H-Leucine transport. Unidirectional maternal–fetal clearances for a) ^{3}H-Leucine and b) ^{14}C-MeAIB were measured in anesthetized dams. Each dot represents individual value. *$P < 0.05$ vs. NSS, paired Student's t test, $n = 5$/each group.

Trophoblast-specific *Slc7a5* overexpression increases transplacental System L amino acid transport

To examine whether the increased fetal growth in response to trophoblast-specific *Slc7a5* overexpression was associated with increased transplacental amino acid transport, unidirectional maternofetal clearance of ^{3}H-leucine (System L-mediated transport) and 14C-methylamino isobutyric acid (^{14}C-MeAIB) (System A amino acid transport) was measured. Placental specific *Slc7a5* OX increased the transplacental transport of ^{3}H-Leucine (+85%, $P = 0.0003$, $n = 5$) as compared to NSS group (Fig. 4a). However, there was no significant difference in transplacental in ^{14}C-MeAIB transport between groups (Fig. 4b).

Trophoblast-specific *Slc7a5* overexpression activates placental mTORC1 and mTORC2 signaling

To further explore the mechanisms linking trophoblast-specific overexpression of Slc7a5 to the increased placental and fetal growth, we used western blot to determine functional readouts of the mTOR signaling pathways in NSS and *Slc7a5* OX placentas. Overexpression of *Slc7a5* significantly increased (+44%, $P = 0.02$, Fig. 5a) S6 ribosomal protein phosphorylation at Serine 235/236, which is an mTORC1 downstream target. Total S6 ribosomal protein expression level was comparable between groups (Fig. 5b). Akt, PKCα, and SGK1 are key downstream target of mTORC2 (36), and we used phosphorylation of these proteins as a read-out for mTORC2 activity. Trophoblast-specific overexpression of *Slc7a5* increased the phosphorylation of PKCα (Tyr-657, +47%, $P = 0.04$, $n = 6$; Fig. 6a) and protein kinase B (Akt-Ser 473, +96%, $P = 0.04$, $n = 5$; Fig. 6c), as compared to NSS. Total PKCα expression level was comparable between NSS and *Slc7a5* OX groups (Fig. 6b). Moreover, total expression and phosphorylation of SGK (Ser-422) and phosphorylation of Akt (Thr-308) was not different in *Slc7a5* and NSS placentas (Fig. 6d–g).

Trophoblast-specific overexpression of *Slc7a5* did not alter placental AMP kinase phosphorylation

Amino acids may modulate the cellular energy sensor AMP kinase (AMPK) directly (36) or indirectly by altered ATP levels following changes in the use amino acids in oxidative phosphorylation. Placental AMPK activity, as determined by Thr-172 phosphorylation, was comparable between *Slc7a5* overexpression and NSS placentas (Fig. S2a). Similarly, placental total AMPK expression was comparable in *Slc7a5* overexpression and NSS groups (Fig. S2b).

Trophoblast-specific *Slc7a5* overexpression increases the association of plasma membrane 4F2hc with LAT1

We examined whether *Slc7a5* overexpression increases the association of plasma membrane 4F2hc with LAT1. We immunoprecipitated proteins in the TPM using an anti-LAT1 antibody. Proteins in the TPM immunoprecipitate were separated by SDS-PAGE and subsequently immunoblotted with anti-4F2hc antibody. Trophoblast-specific overexpression resulted in increased association between 4F2hc and LAT1 in the TPM (+93%, $n = 6$, $P = 0.007$, Fig. S3).

Discussion

The present study demonstrates for the first time a direct mechanistic link between transplacental transport of essential amino acids and fetal growth. Specifically, we show that trophoblast-specific overexpression of System L amino acid transporter *Slc7a5* increases the abundance of System L amino acid transporter isoform LAT1 in the TPM, which promotes the transplacental transport of essential amino acids and increases fetal growth (Fig. 7). These data suggest that the decreased placental System L activity in human IUGR and the increased System L activity

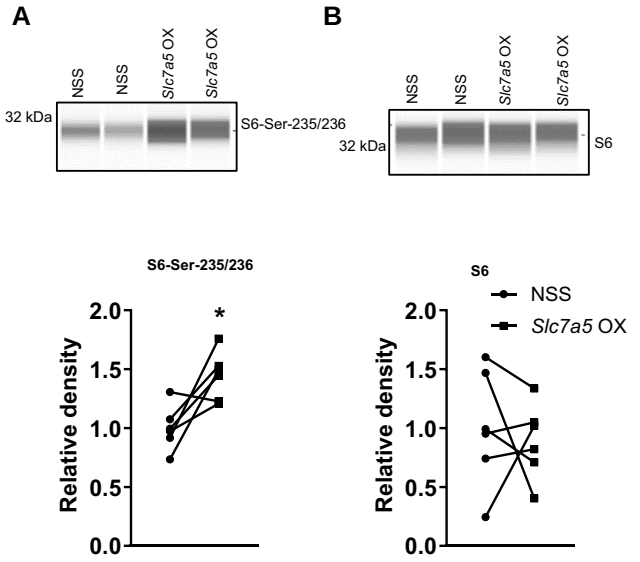

Fig. 5. Trophoblast-specific overexpression of *Slc7a5* activates ribosomal S6 phosphorylation at serine 235/236. a) Effect of trophoblast-specific overexpression of Slc7a5 on placental a) S6Serine-235/236 and b) total S6 protein expression at E 18.5. Representative western blots of S6Serine-235/236 and total S6 expression in placental homogenates of NSS and Slc7a5 overexpression placentas. Protein abundance was normalized to total capillary protein measured using the total protein detection module (AM-PN01, Protein Simple, San Jose, CA, USA). Equal loading was performed. Summary of the western blot data. Each dot represents individual value, $n = 6$/each group. *$P < 0.05$ vs. NSS, paired Student's t test. All placentas of each genotype in a litter were pooled and homogenized prior to analysis.

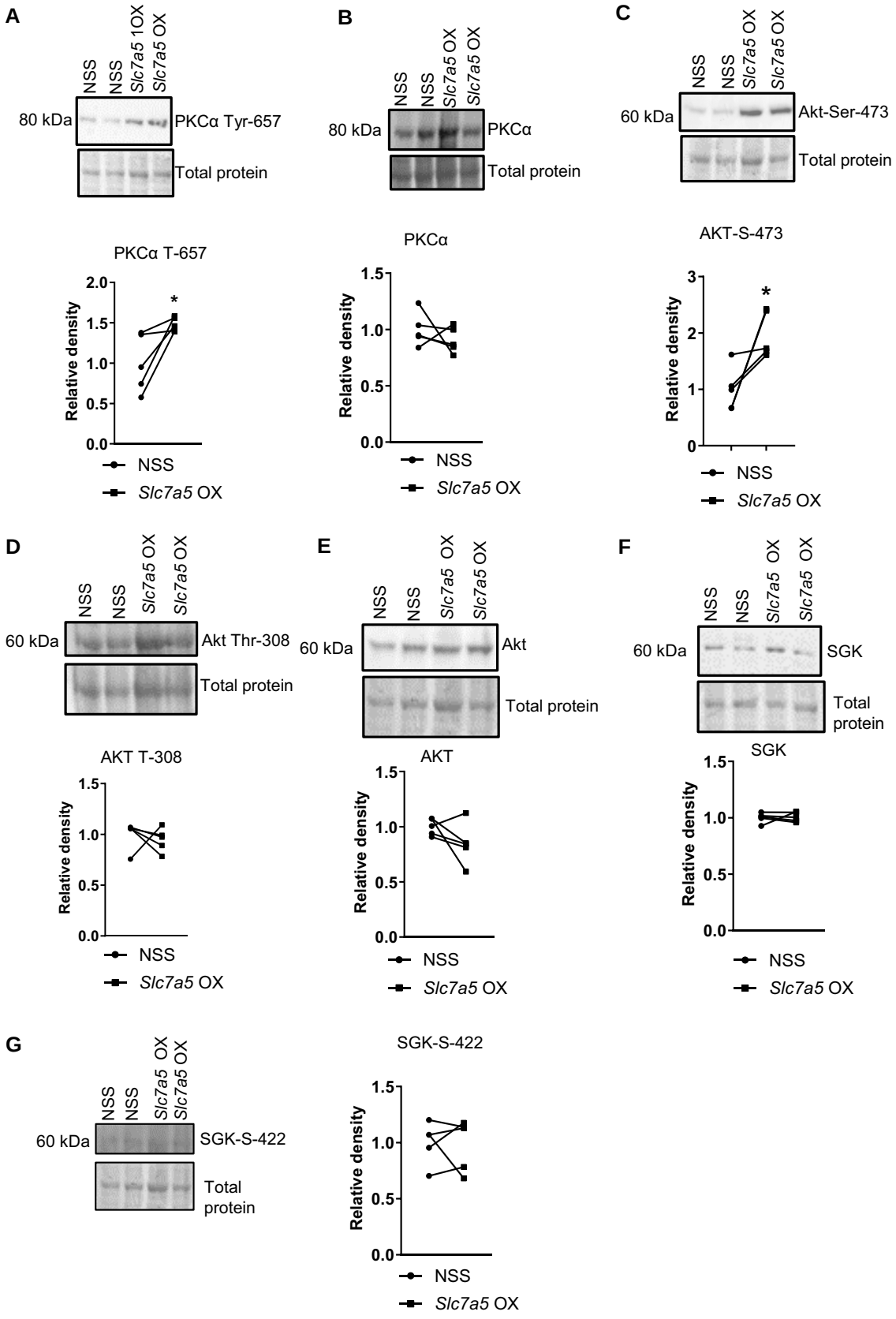

Fig. 6. Trophoblast-specific overexpression of *Slc7a5* activates phosphorylation of placental PKCα at tyrosine-657 and Akt at serine-473. a) Effect of trophoblast-specific overexpression of Slc7a5 on placental a) PKCαTyrosine-657, b) total PKCα, c) AktSerine-473, d) AktThreonine-308, e) total Akt, f) SGKSerine-422, and g) total SGK protein expression at E 18.5. Representative western blots of PKCαTyrosine-657, total PKCα, AktSerine-473, AktThreonine-308, total Akt, SGKSerine-422, and total SGK expression in placental homogenates of NSS and Slc7a5 overexpressed placentas. Equal loading was performed. Summary of the western blot data. Each dot represents individual value, $n = 5–6$/each group. *$P < 0.05$ vs. NSS, paired Student's t test. All placentas of each genotype in a litter were pooled and homogenized prior to analysis.

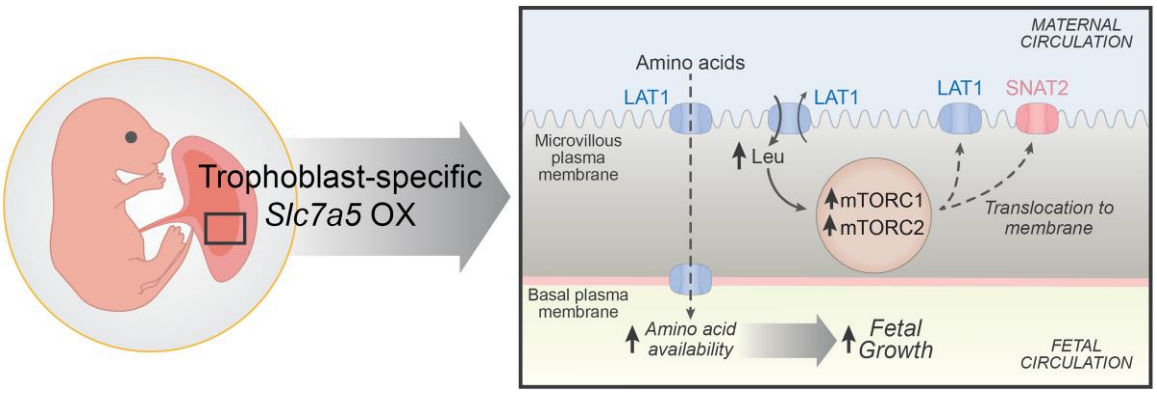

Fig. 7. Direct mechanistic link between transplacental transport of essential amino acids and fetal growth. Trophoblast-specific overexpression of system L amino acid transporter *Slc7a5* increases the abundance of system L amino acid transporter isoform LAT1 in the TPM, which promotes the transplacental transport of essential amino acids and increases fetal growth.

reported in some women with LGA babies directly contribute to the abnormal fetal growth.

The 27% increase in fetal weight as a result of trophoblast-specific *Slc7a5* overexpression was similar to what was observed in a mouse model of maternal obesity with fetal overgrowth accompanied with upregulation of TPM LAT1 isoform expression and increased transplacental System L amino acid transport (22, 23). Similarly, intraamniotic IGF-1 treatment in ewes increases fetal weight and System L amino acid transport in the same magnitude (37). Fetal overgrowth in our study was likely attributable to an increased abundance of TPM LAT1 and increase in transplacental System L-mediated delivery of essential amino acids to the fetal circulation, as transplacental leucine transport was 85% higher in *Slc7a5* overexpression placentas as compared to NSS. Similarly, gastrocnemius muscle-specific knockout of the *Slc7a5* gene in mice results in reductions of *Slc7a5* mRNA expression and leucine transport, and intracellular leucine concentration in the skeletal muscle (38).

Leucine is a potent regulator of the mTOR signaling (39). Leucine stimulates the translocation of mTORC1 to the lysosomal surface, which is the site for mTORC1 activation (40). Wang et al. (41) demonstrated that leucine supplementation during late gestation increased the protein expression of System L and A isoforms and activated mTOR signaling in the placenta and stimulated fetal growth. Loss of *Slc7a5* expression resulted in impaired activation of the mTORC1 signaling pathway in osteoclasts (42). In the current study, trophoblast-specific *Slc7a5* OX not only increased mTORC1 activity in the placenta, it also activated the mTORC2 pathway. Using siRNA-mediated targeting of the two mTOR complexes in cultured PHT cells, we have previously shown that both mTORC1 and 2 are positive regulators of placental System L amino acid transport by regulating the plasma membrane trafficking of LAT1 (34). Thus, it is possible that the activation of mTOR signaling in *Slc7a5* OX placentas contributed to activation of System L transport activity (29, 34).

Poncet et al. (38) reported that LAT1 heterozygous knockout (*Slc7a5*$^{+/-}$) mice have no overt phenotype. However, global homozygous *Slc7a5* (*Slc7a5*$^{-/-}$) knockout is embryonically lethal, demonstrating that LAT1 is vital for early embryogenesis (38). Kanai and co-workers found that global LAT1 knockout confers defective syncytiotrophoblast formation, which disrupts the integrity of the placental exchange surface and that the LAT1$^{-/-}$ embryos at the time of embryonic death were smaller than LAT1$^{+/-}$ siblings (43). LAT1 is also important for implantation and placentation in mice by affecting trophoblast differentiation and invasion (44, 45) In the human term placenta, LAT1 transporters are abundantly expressed in the syncytiotrophoblast MVM, which faces the maternal blood in the intervillous space (15). Using cultured PHT cells, we previously demonstrated that LAT1 and LAT2 isoforms contribute to trophoblast System L-mediated amino acid uptake (15). The present study shows that overexpressing *Slc7a5* specifically in the trophoblast is sufficient to increase placental System L amino acid transport and fetal weight, mechanistically linking placental transport of essential amino acids to fetal growth.

It is well established that LAT1 forms a heterodimer with 4F2hc in the plasma membrane and 4F2hc contributes to LAT1 protein stability and promotes the translocation of LAT1 to the cell membrane (46). LAT1 transporters have been colocalized with 4F2hc in the syncytiotrophoblast at the maternally facing microvillous plasma membrane and the basal plasma membrane adjacent to fetal capillaries (15, 47, 48). Mice lacking 4F2hc did not survive early embryogenesis (49). Furthermore, LAT1 silencing decreases membrane presentation of 4F2hc in the labyrinth and inhibits fusion of trophoblast cells, contributing to impaired placental development (43). Importantly, the increased plasma membrane expression of LAT1 in trophoblast-specific *Slc7a5* OX was accompanied with an increased abundance of TPM LAT1-4F2hc complexes in the membrane, likely reflecting the requirement of heterodimerization with 4F2hc for LAT1 function. However, placental 4F2hc mRNA levels in the LAT1 OX group were not changed, suggesting that the increased 4F2hc and LAT1 colocalization in the TPM that we observed is due to posttranscriptional regulation. Campbell and Thomason (50) demonstrated that overexpression of both LAT1 and 4F2hc increased system L-specific transport activity relative to LAT1 or 4F2hc overexpression. However the mechanisms involved remain to be fully established (51).

System A activity was increased in TPM isolated from *Slc7a5* overexpression placentas. We have previously reported that mTOR signaling is a positive regulator of trophoblast System A transporter activity by promoting the plasma membrane trafficking of SNAT2 (29, 34). Thus, the increased TPM System A activity in vitro following trophoblast overexpression of *Slc7a5* in the current study is likely to be due to the activation of mTOR signaling in response to increased trophoblast uptake of essential amino acids (28, 29). System A and L amino acid transporters are functionally linked because System L transporters exchange extracellular essential amino acids with non-essential neutral amino acids that are present in high concentrations in the cytosol due to

System A activity (52). Thus, the increased in plasma membrane System A activity following overexpression of *Slc7a5* is an important example of coordination of trophoblast amino transporter expression and activity. Moreover, the lack of increase of transplacental transport of System A substrates, represented by MeAIB, despite increased TPM System A activity is likely to be explained by rapid efflux of nonessential amino acids back into the maternal circulation to drive the increased TPM System L Activity.

Changes in transplacental transport of amino acids and the activity of individual placental amino acid transporters are strongly associated with altered fetal growth (53). For example, lower placental LAT1 expression and/or decreased System L activity have been reported in IUGR (54) and in preeclampsia (55) and placental amino acid transport capacity is increased in some cases of fetal overgrowth (12, 13) Similarly, MVM LAT1 expression is increased in women with type-2 diabetes and correlated with birth weight and neonatal fat mass (56). Increased MVM LAT1 expression (56) and system L amino acid transport activity (12) in the placenta may directly contribute to LGA fetuses. In the present study, we provide evidence that mechanistically links increased TPM LAT1 expression and System L activity, and the resulting increase in transplacental transport of essential amino acids, to fetal overgrowth.

Compelling epidemiological and animal experimental data demonstrate that a suboptimal intrauterine environment increases the risk to develop cardiovascular disease and diabetes/obesity later in life. In the current study, we demonstrate a direct mechanistic link between transplacental transport of essential amino acids and fetal growth. These data suggest that the decreased placental System L activity in human IUGR and the increased System L activity reported in some women with LGA babies directly contribute to the abnormal fetal growth. Abnormal fetal growth is strongly associated with an increased risk for the development of a range of diseases, including obesity, diabetes, and cardiovascular disease, in childhood and in adult life (57–65). Thus, targeting placental LAT1, or mTOR signaling, in IUGR and fetal overgrowth may not only alleviate important pregnancy complications but may also improve the health of the next generation.

In conclusion, this is the first study to establish a cause-and-effect relationship between placental *Slc7a5* expression and activity and fetal growth in mice. Placental System L amino acid transport activity has been reported to be decreased in human pregnancies complicated by IUGR and increased in women delivering large babies. Based on the mechanistic link demonstrated in the current study between changes in placental LAT1 expression/placental transport of essential amino acids and fetal growth, we speculate that placental changes in System L activity and transplacental transport of essential amino acids directly contributes to altered fetal growth.

Materials and methods

Lentiviral vectors: A third-generation Lentivector pLV[Exp]-CMV > mSlc7a5[NM_011404.3]/E2A/mCherry was sourced from Vectorbuilder (VectorBuilder Inc., Chicago, IL, USA). The bicistronic transgene, Slc7a5 ORF (NM_011404.3)-E2A-mCherry, was located downstream of the cytomegalovirus (CMV) promoter. The plasmid backbone also contained an ampicillin resistance gene and mCherry. A control Lentivector pLV-EGFP-U6 > NSS (NSS), containing an enhanced green fluorescent protein (EGFP) open reading frame and a nonsense sequence, was sourced from Vectorbuilder (VectorBuilder Inc., Chicago, IL, USA). The nonsense sequence (NSS) group represents the control. To amplify the plasmid, *Escherichia coli* glycerol stocks of both plasmids were cultured overnight in LB broth containing 100 g mL^{-1} ampicillin, and DNA was extracted using a commercial kit (Plasmid Maxi kit, Qiagen) as previously described (14, 66). Expression and control plasmids were packaged into vesicular stomatitis virus G pseudo typed lentiviral particles by co-transfecting 293FT cells with the isolated DNA (440 ng mL^{-1}, with Qiagen Polyfect 1% v/v in culture medium), together with packaging (330 ng mL^{-1} pCMV delta 8.9) and envelope plasmids (140 ng mL^{-1} pCMV-VSV-G) (67), purchased from the Functional Genomics Shared Resource at the University of Colorado Cancer Centre. Human female embryonic kidney epithelial cells (293FT) were obtained from Thermo Fisher Scientific and cultured at 37°C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 6 mM glutamine, 1 mM sodium pyruvate, and 1% pen–strep. Seventy-two hours after transfection, lentiviral particles were isolated from the filtered conditioned medium by ultracentrifugation over 20% sucrose (22,000 rpm, 2 h), then pellets were resuspended in PBS. The functional efficiency of each batch of lentivirus was determined in terms of transforming units per milliliter by transducing 293FT cells with serial dilutions of the viral suspension and then measuring the proportion of EGFP and mCherry fluorescent cells using flow cytometry.

Lentiviral transduction of mouse blastocysts and surgical embryo transfer

All animal procedures were performed at the University of Colorado with approval from the Institutional Animal Care and Use Committee (Protocol #344). B6D2F1 strain code #100006 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and CD-1 IGS (Strain code 022) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All animals were maintained under standard 14-h:10-h light:dark cycle with ad libitum access to food and water. To induce superovulation, female B6D2F1 mice, aged 6 weeks, were injected with pregnant mare serum gonadotrophin (5 I.U., i.p., Prospec, East Brunswick, NJ, USA) and human chorionic gonadotrophin (5 I.U., i.p., Sigma–Aldrich, St Louis, MO, USA) and subsequently mated overnight with stud B6D2F1 males. Superovulated females that had successfully mated were identified by the presence of a copulatory plug the following morning, which was assigned embryonic day (E) 0.5 (term ~E19.5). On day E3.5, pregnant females were euthanized by carbon dioxide asphyxiation and cervical dislocation, their uteri were removed, and each horn was flushed with 5 mL of prewarmed M2 medium (M7167, Sigma–Aldrich, St. Louis, MO, USA). All in vitro procedures were carried out at 37°C and in an environment with a low light intensity. The zona pellucida were removed from the flushed blastocysts by serially incubating blastocysts in three drops of acidic Tyrodes solution for less than 10 s each. Subsequently, blastocysts were washed and incubated in embryo culture media (Embryo Max Advanced KSOM Embryo medium, MR-101-D, Millipore). The trophectoderm was transduced with either *Slc7a5* overexpression or NSS plasmid by incubating batches of 5–10 blastocysts with either 5×10^5 or 5×10^6 transforming units of lentivirus in a total volume of 20 µL, for 4 h. After lentiviral transduction, blastocysts were washed in 12 drops of embryo culture medium and then surgically transferred to pseudopregnant female CD-1 recipients as described below (Fig. S4).

A total of 13 female CD-1 mice were used as recipients for embryo transfer. Female CD-1 mice were mated with vasectomized

B6D2F1 males overnight to induce pseudopregnancy. Surgical embryo transfer was performed 2.5 days after copulation. Pseudopregnant CD-1 mice were administered preoperative analgesia (meloxicam, 1 mg/kg, intraperitoneally) followed by anesthesia with isoflurane (2%, inhalation). Under aseptic conditions, a 0.5-cm incision was performed in the skin of the flank over the right ovary, with the animal in sternal recumbency. Specifically, the incision was made 1 cm caudal to the rib cage. The ovary, oviduct, and distal uterine horn were extruded through the body wall, and a 26-gauge needle was employed to puncture the uterus below the junction of the oviduct. The blastocysts that had been transduced with lentivirus were subsequently introduced into the uterine horn using a small amount of embryo culture medium. Subsequently, the uterus and oviducts were reinserted into the body cavity, followed by closure of the body wall using a solitary absorbable suture (5.0 vicryl), and the skin was closed using a 9-mm wound clip. Subsequently, the aforementioned protocol was replicated for the left ovary. Approximately, 10 blastocysts (5 Blastocyst/group, [*Slc7a5* OX and NSS]) were transferred to each horn. Blastocysts that were transduced with *Slc7a5* OX (mCherry reporter gene) and NSS (GFP reporter gene) were mixed and transferred in both uterine horns. For transplacental transport study, blastocysts transduced with *Slc7a5* OX and NSS, respectively were transferred to the contralateral uterine horns. The allocation of blastocysts to the left and right sides of each recipient was randomized. Following the surgical procedure, female recipients were allowed to recover from anesthesia on a heated mat. They were then housed in pairs.

At E18.5, dams were euthanized for collection of fetal and placental samples. After laparotomy, fetuses and placentas were removed and weighed. For the molecular analysis, all the placentas of each genotype in a litter were pooled and homogenized. Specifically, whole placentas were used for homogenization which includes both the junctional and labyrinthine zones.

All the placentas were observed under a fluorescent microscope to visualize placenta-specific mCherry (or) GFP reporter expression and to separate the two placental genotypes (NSS/*Slc7a5* OX) (Fig. S5). Ninety percent of viable conceptuses exhibited placenta-specific GFP (NSS) or mCherry reporter (*Slc7a5* OX) expression at term and GFP/mCherry negative conceptuses (10%, 16/159 implantation resulting in viable fetus) were excluded from further analyses. Placentas were frozen in Tissue-Tek OCT and sectioned (10 µm) in a cryotome and sections were mounted onto slides. Fluorescence (GFP/mCherry) was observed in sectioned slides using a Leica DM600B Slide Scanner microscope (Objective: 10× w/0.4 aperture. Filter: Texas Red Filter Cube Set/Fluorescein isothiocyanate). As shown in Fig. S5, histology revealed uniform and ubiquitous expression of GFP/mCherry in the placenta.

TPM isolation

TPMs were isolated to measure System L and System A amino acid transporter activity in vitro and determine the protein expression of LAT1. In brief, *Slc7a5* overexpression and NSS placentas, respectively, were pooled from each litter. Pooled frozen placental tissue was homogenized in ice cold buffer D (250 mM sucrose, 10 mM Hepes-Tris, and 1 mM EDTA pH 7.4) with protease and phosphatase inhibitors (1:1,000 dilution). Using differential ultracentrifugation and Mg^{2+} precipitation, TPM vesicles were isolated from the placental homogenate, as previously described (22, 68). Briefly, to remove tissue debris and nuclei, placental homogenates were centrifuged at 10,000×g (10 min, 4°C) and 125,000×g (30 min, 4°C). Subsequently, pellets were resuspended and precipitated by addition of $MgCl_2$ (12 mM) with stirring, on ice. Finally, TPM was isolated by ultracentrifugation of the supernatant at 125,000×g for 30 min at 4°C. Pellets were resuspended and vesiculated using a Dounce homogenizer. Protein content in homogenate and TPM were determined by micro-BCA assay and the enrichment of the TPM preparation determined by the TPM/homogenate ratio of alkaline phosphatase activity per unit protein. Average TPM enrichment ratios were comparable between NSS (10.8 ± 1.9, $n = 5$ dams) and *Slc7a5* overexpression group (10.4 ± 1.5, $n = 5$ dams).

TPM amino acid transporter activity measurements

Using radiolabeled amino acids and rapid filtration techniques, the TPM System A and L amino acid transporter activity was measured as described previously (24, 69). TPM vesicles from NSS and *Slc7a5* overexpression group were preloaded by incubation in 300 mM mannitol and 10 mM Hepes-Tris, pH 7.4 overnight at 4°C. Subsequently, TPM vesicles were resuspended in a small volume of the same buffer after being pelleted (final protein concentration: 5–10 mg mL^{-1}). Membrane vesicles were kept on ice until transport activity measurements were performed. Immediately prior to transport activity measurements, TPM vesicles were warmed to 37°C. At time zero, 30 µL TPM vesicles were quickly mixed (1:2) with the incubation buffer containing [^{14}C] methyl-aminoisobutyric acid (MeAIB, 150 µM) with or without Na^+ or L-[^{3}H] leucine (0.375 µM). Based on previous time-course studies (24), uptake at 15 s was used in all subsequent experiments. The uptake of radiolabeled substrate was terminated by adding 2 mL of ice-cold PBS. Subsequently, vesicles were rapidly separated from the substrate medium by filtration on mixed ester filters (0.45 µm pore size, Millipore Corporation, Bedford, MA, USA) and washed with 3 × 2 mL of PBS. In all uptake experiments, each condition was studied in duplicate for each membrane vesicle preparation. Filters were dissolved in 2 mL liquid scintillation fluid (Filter Count, PerkinElmer, Waltham, MA, USA) and counted. Appropriate blanks were subtracted from counts and uptakes expressed as pmol (mg protein)$^{-1}$. Na^+-dependent uptake of MeAIB (corresponding to system A activity) was calculated by subtracting Na^+-independent from total uptakes. For leucine, mediated uptake was calculated by subtracting nonmediated transport, as determined in the presence of 20 mM unlabeled leucine, from total uptake.

In vivo placental transport

At embryonic day 18.5, dams were anesthetized using ketamine (60 mg/kg) and xylazine (6 mg/kg, both i.p.) and placed on a heated mat. The lateral tail vein was cannulated and flushed with heparinized saline using a 28 gauge needle attached to a 0.5 mL insulin syringe, via polyethylene (PE20) tubing (0.38 mm I.D., ∼12 cm length, B.D. Intramedic 427405, Becton Dickinson, NJ, USA). A combined bolus of ^{14}C-MeAIB (50µCi/kg, specific activity 58.7mCi/mmol) and ^{3}H-leucine (250µCi/kg, specific activity 60,000mCi/mmol) was delivered to the tail vein, with flushing. At exactly 4.5 min later, the dam was euthanized with sodium pentobarbital (390 mg/mL, 100 µL, i.v.) and a cardiac blood sample rapidly collected into a heparinized syringe, and laparotomy was performed to remove fetuses and placentas. Individual fetuses and placentas were blotted briefly and weighed. Fetuses and placentas belonging to NSS and *Slc7a5* overexpression groups, respectively from the same litters were pooled and minced.

Finally, they were solubilized in Biosol (National Diagnostics, Atlanta, GA, USA) overnight at 55°C. Radioactivity in maternal serum and in solubilized fetuses and placentas was determined by β-counting. The unidirectional maternofetal clearance (K_{mf}, measured as microliters per minute per gram [$\mu L \cdot min^{-1} \cdot g^{-1}$]) was calculated as follows:

$$K_{mf} = N_x / AUC_{0-x} \times W.$$

N_x is counts in the fetus taken at time × when the mother was euthanized (measured as disintegrations per minute). AUC_{0-x} is area under the maternal radiolabel concentration curve from time 0 to the time the mother was killed (measured as dpm per microliter [$dpm \cdot min \cdot \mu L^{-1}$]). W is wet weight of the placenta (measured in grams).

Gene expression analysis of System A and L amino acid transporter isoforms

RNA was extracted from frozen placentas and reverse transcribed using commercially available kits (RNeasy Plus Mini kit, Qiagen and High-Capacity cDNA RT kit, Invitrogen). The expression of System L amino acid transporter isoforms (*Slc7a5, Slc7a8,* and *Slc3a2*) *and* System A amino acid transporter isoform (*Slc38a1*, *Slc38a2*, and *Slc38a4*) was determined by SYBR Green qRT-PCR using the relative standard curve method and values were normalized relative to RNA 28S. RNA 28S expression was comparable between NSS and LAT1 OX group. Primer sequences are listed in Table S2.

Western blotting

mTOR signaling pathway activity in placental homogenates was determined by western blot for total and phosphorylated forms of S6 (Ser235/236) for mTORC1 and Akt (Ser473), SGK (Ser-422) and PKCα (Tyr-657) for mTORC2 (70). In addition, AMPK signaling activity was determined using the phosphorylation of AMPK (Thr-172) as the functional readout. Placental homogenates were processed for immunoblotting analyses (Total and phosphorylated forms of Akt (Ser473), Akt (Thr308), SGK (Ser-422), PKCα (Tyr-657), and AMPK (Thr-172) as described) (22, 28). Protein expression of LAT1 was measured in isolated TPM. An antibody targeting the LAT1 was produced in rabbits as described previously (71). LAT1 antibody specificity was validated using gene-silencing approaches (15). List of primary antibodies, their source and working dilutions are listed in Table S3. Target protein expression was normalized to total protein expression.

Total and phosphorylation of S6 (Ser235/236) were measured in placental homogenates using the ProteinSimple Jess capillary immunoblotting system (SM-PN01-1, Protein Simple, San Jose, CA, USA) as previously reported (72). Protein abundance was normalized to total capillary protein measured using the total protein detection module (AM-PN01, Protein Simple, San Jose, CA, USA).

Immunoprecipitation

Placental homogenates were incubated with LAT1 antibody overnight and antibodies were precipitated with protein G-Sepharose. Immunoprecipitates and aliquots of placental homogenates were denatured in sample buffer at 95°C, resolved by electrophoresis, and probed with 4F2hc antibody.

Data presentation and statistics

Data are presented as mean ± SEM or individual value. Statistical significance between NSS and *Slc7a5* overexpression groups was determined by paired Student's t test. A P value <0.05 was considered significant. Frequency distribution of individual fetal weights in NSS and Slc7a5 OX groups was determined by Gaussian curves fitted by least-squares nonlinear regression and compared by extra sum-of-squares F-test.

Supplementary Material

Supplementary material is available at *PNAS Nexus* online.

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Data Availability

All supporting data for this manuscript are included in the figures and the accompanying Supplementary Files.

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