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The SNAT4 isoform of the system A amino acid transporter is functional in human placental microvillous plasma membrane

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Placental system A activity is important for the supply of neutral amino acids needed for fetal growth. There are three system A isoforms: SNAT1, SNAT2 and SNAT4, but the contribution of each to system A-mediated transport is unknown. Here, we have used immunohistochemistry to demonstrate that all three isoforms are present in the syncytiotrophoblast suggesting each plays a role in amino acid transport across the placenta. We next tested the hypothesis that the SNAT4 isoform is functional in microvillous plasma membrane vesicles (MVM) from normal human placenta using a method which exploits the unique property of SNAT4 to transport both cationic amino acids as well as the system A-specific substrate MeAIB. The data show that SNAT4 contribution to system A-specific amino acid transport across MVM is higher in first trimester placenta compared to term (approx. 70% and 33%, respectively, $P < 0.01$). Further experiments performed under more physiological conditions using intact placental villous fragments suggest a contribution of SNAT4 to system A activity in first trimester placenta but minimal contribution at term. In agreement, Western blotting revealed that SNAT4 protein expression is higher in first trimester MVM compared to term ($P < 0.05$). This study provides the first evidence of SNAT4 activity in human placenta and demonstrates the contribution of SNAT4 to system A-mediated transport decreases between first trimester and term: our data lead us to speculate that at later stages of gestation SNAT1 and/or SNAT2 are more important for the supply of amino acids required for normal fetal growth.

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During intrauterine life an adequate supply of amino acids from the maternal circulation is essential for normal fetal growth and development. In humans the maternal and fetal circulations are separated by a polarized epithelium in the placenta known as the syncytiotrophoblast. Amino acid transfer across the syncytiotrophoblast is mediated by a number of transporters found in the maternal-facing microvillous plasma membrane (MVM) and fetal-facing basal plasma membrane (BM) (Moe, 1995; Jansson, 2001). Each class of transporter is specific for a particular group of amino acids and, based on competitive interactions between amino acid substrates, at least 15 transport systems for neutral, basic and acidic amino acids have been identified in the syncytiotrophoblast analogous to those described for other epithelia (Moe, 1995; Jansson, 2001).

System A is a Na^+-dependent transporter that actively transports small, zwitterionic, neutral amino acids with short, unbranched side chains such as alanine, serine, and glutamine (Johnson & Smith, 1988). It can also transport *N*-methylated amino acids such as α-(methylamino)isobutyric acid (MeAIB) (Christensen *et al.* 1965). This non-metabolizable amino acid analogue has been used extensively to study system A activity in the placenta (Mahendran *et al.* 1993; Novak *et al.* 1996; Jozwik *et al.* 1998; Jansson *et al.* 2003). Reduced system A activity has been demonstrated in MVM vesicles isolated from placentas in which the fetus was growth restricted (Dicke & Henderson, 1988; Mahendran *et al.* 1993; Glazier *et al.* 1997; Jansson *et al.* 2002). Whether this represents a primary event in cases of intrauterine growth restriction (IUGR) or is secondary to the growth restriction is unknown. However, evidence from three separate animal studies suggests reduced system A activity may be a cause of, rather than merely be associated with, altered fetal growth. Inhibition of system A in pregnant rats leads to fetal growth restriction (Cramer *et al.* 2002); in a mouse model of IUGR (Constancia *et al.* 2002) and

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also in a dietary model of IUGR in the rat (Jansson *et al.* 2006), fetal growth restriction is preceded by alterations in placental system A activity.

Molecular characterization of system A has revealed that there are three highly homologous protein subtypes of this Na^+-coupled neutral amino acid transporter known as SNAT1, SNAT2, and SNAT4, which are encoded by three members of the SLC38 gene family (*SLC38A1*, *SLC38A2*, and *SLC38A4*, respectively) (Hediger *et al.* 2004; Mackenzie & Erickson, 2004). *SLC38A2* is expressed ubiquitously in mammalian tissues (Hatanaka *et al.* 2000; Sugawara *et al.* 2000*a*) and expression of *SLC38A1* has been shown in various tissues including human placenta (Wang *et al.* 2000; Desforges *et al.* 2006). Functionally, SNAT1 and SNAT2 operate via similar mechanisms (Hatanaka *et al.* 2000; Wang *et al.* 2000; Yao *et al.* 2000). SNAT4 has a lower substrate affinity for neutral amino acids and MeAIB than SNAT1 and SNAT2 (Sugawara *et al.* 2000*b*; Hatanaka *et al.* 2001) and also has a unique ability amongst the other system A isoforms to interact with cationic amino acids in a Na^+-independent manner (Hatanaka *et al.* 2001). It was originally thought that *SLC38A4*/SNAT4 was a liver-specific isoform (Hatanaka *et al.* 2001). However, we have demonstrated expression in human placenta (Desforges *et al.* 2006), and expression in rat (Novak *et al.* 2006) and mouse placenta (Mizuno *et al.* 2002; Smith *et al.* 2003) has also been documented.

The relative contribution of each SNAT isoform to total placental system A activity is not known; indeed it is not certain that all three isoforms are active. We have previously shown that SNAT4 protein is localized to the MVM in human placenta at first trimester and term (Desforges *et al.* 2006), a location consistent with a role in uptake of amino acids into the syncytiotrophoblast. Our laboratory has also demonstrated SNAT2 protein localization to the syncytiotrophoblast of term placenta (Champion *et al.* 2004) but localization in first trimester is unknown and SNAT1 localization has yet to be investigated.

System A activity in human placental MVM increases between first trimester and term (Mahendran *et al.* 1994) coinciding with the increased fetal nutrient demand as gestation advances and fetal growth rate accelerates. Our previous investigations have shown SNAT4 protein expression increases in placental homogenates (containing a mixture of all placental cell types) between first trimester and term (Desforges *et al.* 2006) suggesting amino acid transport by this isoform could be particularly important for maintaining normal fetal growth. Furthermore, in a mouse model of IUGR which displays alterations in placental structure and function similar to those found in human IUGR (Sibley *et al.* 2004), altered placental MeAIB transport is specifically associated with altered *Slc38a4* mRNA expression (Constancia *et al.* 2005).

In the current study we have used immunohistochemistry to compare localization of SNAT1, SNAT2 and SNAT4 in first trimester and term human placenta and then examined any changes in the specific localization of SNAT4 to the MVM between these two gestations using Western blotting. We then performed experiments to test the hypothesis that the SNAT4 isoform is functional in normal human placenta and to estimate its contribution to total system A activity both in the first trimester and at term. Definitively identifying the contribution of SNAT4 to total system A transport in placenta, which expresses all three SNAT isoforms, is complicated by their overlapping substrate specificities. Therefore our initial experiments were performed using isolated MVM vesicles which allowed optimization of experimental conditions, outside the normal physiological range, to measure SNAT4 activity. Our experimental approach was to exploit the unique property of SNAT4 to transport cationic amino acids as well as MeAIB by measuring the arginine-inhibitable component of MeAIB uptake by MVM vesicles. Subsequently, experiments were performed using intact placental villous tissue fragments to examine whether SNAT4 activity could be measured under more physiological conditions in tissue from first trimester and term pregnancy.

Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Co. Ltd (Poole, UK) or VWR International (Lutterworth, UK) unless otherwise stated.

Tissue acquisition and ethical approval

All tissue was obtained with written informed consent as approved by the Central Manchester Research Ethics Committee (REC Ref: 03/CM/031). Gestational age was estimated from the date of last menstrual period and confirmed by ultrasound dating. First trimester placentas (6–13 weeks gestation) were obtained following elective medical or surgical termination of pregnancy. Term placentas (38–40 weeks gestation) were collected following caesarean section or vaginal delivery from uncomplicated singleton pregnancies.

Immunohistochemistry

Placental tissue samples from first trimester and term were fixed in 10% neutral buffered formalin overnight at 4°C before thorough washing in Tris buffered saline (TBS) and paraffin embedding. Sections of 5 μm were de-waxed and rehydrated prior to antigen retrieval by microwaving for 2 × 5 min at 800 W in 0.01 M citrate buffer (pH 6.0). Non-specific staining was prevented by quenching

endogenous perxoidase activity with 3% aqueous H_2O_2, followed by a 30 min incubation with non-immune block (10% normal swine serum, 2% normal human serum ('in-house'), 0.1% Tween-20 in TBS). Primary antibodies (affinity-purified rabbit IgG) were custom-made (Eurogentec: Seraing, Belgium) using the following amino acid sequences of the human system A isoforms as antigen: SNAT1 – VPEDDNISNDSNDFT (residues 18–32); SNAT2 – SNLGKKKYETEFHPG (residues 55–69); SNAT4 – YGEVEDELLHAYSKV (residues 391–405). Antibodies were diluted to 1 μg ml^{-1} in non-immune block and applied to sections overnight at 4°C. For negative controls, and to ensure antibody specificity, primary antibodies were preadsorbed with the appropriate purified antigenic peptide (10× excess) overnight at 4°C before applying to serial sections at 1 μg ml^{-1}. Following repeated washes with TBS containing 0.6% Tween-20, antibody binding was detected by sequential application of biotinylated swine anti-rabbit IgG (Dako: Ely, UK; 1 : 200 dilution) and avidin-peroxidase (20 ng ml^{-1}), with the chromogen diaminobenezidine, to produce a brown precipitate. Sections were counterstained with Harris' haematoxylin, dehydrated and mounted with DPX.

Preparation of MVM vesicles

MVM vesicles were prepared using the method of Mg^{2+} precipitation and differential centrifugation as previously described (Glazier *et al.* 1988; Mahendran *et al.* 1994) with the modification that Mg^{2+} concentration was increased to 12 mM for first trimester MVM. Once isolated, MVM vesicles were suspended in intravesicular buffer (IVB: 30 mM KCl, 110 mM choline chloride, 20 mM mannitol, 6.5 mM Tris, and 13.5 mM Hepes, pH 7.4). MVM protein concentration was determined by the method of Lowry *et al.* (1951). Vesicle purity was assessed by measuring enrichment of alkaline phosphatase activity as previously described (Glazier *et al.* 1988; Mahendran *et al.* 1994). Alkaline phosphatase enrichment factors (mean ± S.E.M.) for first trimester and term MVM vesicles ($n = 6$ for both) were 18 ± 2 and 19 ± 2, respectively. Statistical analysis revealed no significant difference between the two groups ($P = 0.82$, Mann–Whitney U-test). All vesicles intended for activity experiments were stored at 4°C prior to measurement of amino acid uptake, performed within 48 h of isolation. MVM vesicles intended for Western Blot analysis were stored at −80°C.

Western blot analysis

In order to compare SNAT4 protein expression in MVM from first trimester and term placenta, 30 μg protein was mixed at a ratio of 1 : 1 with loading buffer (22% glycerol, pH 6.8, 154 mM SDS, 4.4 M urea, 0.002% bromophenol blue, and 10% v/v 2-mercaptoethanol, 139 mM Tris-HCl) and heat reduced for 5 min at 95°C. BeWo choriocarcinoma cell homogenate was included as a negative control for SNAT4 expression as shown previously (Desforges *et al.* 2006) in order to confirm primary antibody specificity. Polyacrylamide gel electrophoresis was performed using 3% stacking gel and 10% resolving gel. Proteins were then electrotransferred onto nitrocellulose membrane. The membranes were blocked for 1 h at room temperature with 5% dried skimmed milk powder in phosphate-buffered saline (PBS; 172 mM NaCl, 3.35 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and then washed for 3 × 5 min in PBS–0.1% Tween-20 (PBS-T). A rabbit polyclonal to SNAT4 (Abcam: Cambridge, UK) was used at a 1 : 300 dilution in PBS-T. Primary and HRP-conjugated goat anti-rabbit secondary antibody (Dako, Ely, UK; 1 : 2000 dilution in PBS-T) incubations were carried out for 1 h at room temperature. Immunoreactive signals were detected by enhanced chemiluminescence (ECL). Following ECL, the nitrocellulose membrane was reprobed for β-actin (mouse anti-β-actin monoclonal, 1 : 1000 dilution) to confirm equal protein loading.

Measurement of total system A activity in MVM vesicles

MVM vesicles from first trimester and term placenta were used at 5 μg ml^{-1} and 10 μg ml^{-1}, respectively (diluted in IVB). MVM vesicles were then incubated in 4 μm valinomycin, a potassium ionophore, for 1 h at room temperature as previously described (Ayuk *et al.* 2000) before transport assays were performed. [^{14}C]MeAIB (0.4 mM, Perkin Elmer) uptake was measured at room temperature (21–24°C) following addition of 20 μl MVM vesicles to 100 μl extravesicular buffer (EVB: 30 mM KCl, 110 mM NaCl, 20 mM mannitol, 14 mM Tris, 6 mM Hepes, pH 8.5) by the method of rapid filtration (Mahendran *et al.* 1993, 1994; Ayuk *et al.* 2000). Experimental conditions were modified to promote system A activity; EVB composition achieved an inwardly directed Na^+ gradient of ∼90 mM across MVM plasma membranes creating an optimal driving force for MeAIB transport and an alkali pH of 8.5 was used to stimulate SNAT activity (Hatanaka *et al.* 2001). [^{14}C]MeAIB uptake in the presence of 30 mM MeAIB was also measured and considered to be non-system A mediated because at this high concentration of substrate it is predicted that all three SNAT isoforms would be saturated. The difference between total [^{14}C]MeAIB uptake and uptake in the presence of 30 mM MeAIB was calculated to give a measure of system A-specific uptake. Previous studies have shown that [^{14}C]MeAIB uptake by MVM after 30 s provides a reasonable estimate of initial rate (Mahendran *et al.*

1993; Kuruvilla *et al.* 1994; Glazier *et al.* 1997). Due to the different experimental conditions used in the current study, [^{14}C]MeAIB uptakes over a 1 min time course were performed to ensure measurement at initial rate had been achieved (see Results).

Measurement of SNAT4-specific activity in MVM vesicles

In conjunction with the system A activity experiments described above, [^{14}C]MeAIB uptake by MVM vesicles was also performed in the presence of 10–30 mM arginine. The arginine-inhibitable component of system A-specific [^{14}C]MeAIB uptake by MVM vesicles was taken as a measure of SNAT4-mediated activity as this is the only system A isoform which supports this cationic amino acid as a substrate (Hatanaka *et al.* 2001). SNAT4 has a relatively low affinity for MeAIB compared to the other SNAT isoforms and therefore [^{14}C]MeAIB was used at a higher concentration (0.4 mM) than in previous publications (0.165 mM; Mahendran *et al.* 1994; Glazier *et al.* 1997) to ensure contribution to uptake by SNAT4. These experiments, performed under non-physiological conditions (i.e. pH 8.5, manipulated transmembrane Na^+ gradient, and the use of supraphysiological concentrations of arginine), were designed to confirm SNAT4 activity in MVM and provide an estimate of its contribution to system A transport.

Measurement of total system A activity in placental villous fragments

Villous fragments represent a more physiologically relevant model than isolated MVM vesicles because tissue architecture, cell-to-cell interactions, and intracellular signalling pathways remain intact, important for the maintenance of the normal driving forces for amino acid transport. Therefore, system A activity measurements were also performed using this model. Placental villous fragments were dissected from first trimester and term placenta and rinsed in 1 : 3 mix of Dulbecco modified Eagle medium (DMEM)/control Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 5.6 mM glucose, pH 7.4). Uptake of [^{14}C]MeAIB (10 μm) into villous fragments over 20 min was carried out in control and Na^+-free Tyrode's buffer (135 mM choline chloride replaced NaCl, pH 7.4) as previously described (Jansson *et al.* 2003; Ericsson *et al.* 2005; Greenwood & Sibley, 2006). The Na^+-dependent component of [^{14}C]MeAIB uptake, representing system A-specific uptake, was calculated by subtracting [^{14}C]MeAIB uptake in the absence of Na^+ from uptake in the presence of Na^+. Fragment protein content (μg) was determined using the method of Bradford (1976) using a commercial kit (Bio-Rad Laboratories Ltd, Hemel Hampstead, UK). Na^+-dependent [^{14}C]MeAIB uptake by first trimester and term fragments is expressed per mg fragment protein over 20 min, a time previously confirmed to be at initial rate (Greenwood & Sibley, 2006).

Measurement of SNAT4-specific activity in placental villous fragments

[^{14}C]MeAIB uptake by first trimester and term fragments, in both control and Na^+-free conditions, was also measured in the presence of 5 mM MeAIB. The Na^+-dependent component of uptake in each condition was calculated and expressed per mg protein over 20 min. The transport function of SNAT1 and SNAT2 in human retinal pigment endothelial (HRPE) cells is saturable with increasing concentrations of MeAIB over the concentration range of 0.05–5 mM (Hatanaka *et al.* 2000; Wang *et al.* 2000) whereas the K_m of SNAT4 for MeAIB is 6.7 mM. Therefore, any measurable Na^+-dependent [^{14}C]MeAIB uptake by placental villous fragments in the presence of 5 mM MeAIB was taken to be that specifically mediated by SNAT4. These experiments, performed at physiological pH in intact placental tissue, were designed to provide an estimate of placental SNAT4 activity *in vivo*.

RT-PCR

In order to examine *SLC38A4* mRNA expression in other human tissues besides placenta we used RT-PCR. RNA (100 ng) from various human tissues including brain, stomach, lung, small intestine, heart, kidney, liver (Ambion, Cambridge, UK) and placenta (Stratagene), were reverse transcribed simultaneously to generate cDNA, ensuring comparable reverse transcription efficiency, and PCR was performed using *SLC38A4*-specific primers (Desforges *et al.* 2006). The thermal cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation, annealing and amplification (95°C for 30 s, 58°C for 1 min, 72°C for 30 s). β-Actin mRNA expression was also examined by PCR in the same samples as previously described (Lacey *et al.* 2005) to confirm cDNA integrity. *SLC38A4* and β-actin amplification products were visualized by ethidium bromide staining following agarose gel electrophoresis.

Statistical analysis

Following Western blotting, the mean intensity of signal for immunoreactive species was assessed using Image J software. These data are presented as median and interquartile range and any difference between expression

in first trimester and term MVM was determined by a Mann–Whitney *U*-test. Raw data of [^{14}C]MeAIB uptake by MVM vesicles over the 1 min time course are presented as mean ± S.E.M. Two-way ANOVA was used to statistically analyse [^{14}C]MeAIB uptake by MVM vesicles in the presence of arginine over the 1 min time course. The calculated data of system A-specific and SNAT4-mediated [^{14}C]MeAIB uptake by MVM vesicles and villous fragments are presented as median and interquartile range. Any difference in system A-specific or SNAT4-mediated [^{14}C]MeAIB uptake between first trimester and term placenta was determined by a Mann–Whitney *U*-test. All statistical analyses were made using GraphPad Prism software and in all cases, $P < 0.05$ was considered significant.

Results

SNAT1, SNAT2 and SNAT4 localization in human placenta

Figure 1 shows representative images from immunohistochemical analysis of SNAT1 (Fig. 1*A–F*), SNAT2 (Fig. 1*G–L*), and SNAT4 (Fig. 1*M–R*) localization in first trimester ($n = 7$) and term ($n = 6$) placental tissue. Immunostaining was predominantly localized to the syncytiotrophoblast for all SNAT proteins. Cytoplasmic staining was observed in all samples with heterogeneous but intense MVM staining apparent for all three isoforms (Fig. 1*A*, *G* and *M*). In first trimester placenta, the intensity of staining for SNAT1 was less in cytotrophoblasts than syncytiotrophoblast (Fig. 1*A* and *B*) but the two cell types had similar levels of staining for SNAT2 (Fig. 1*G* and *H*) and SNAT4 (Fig. 1*O*). SNAT1 staining was also detected in stromal Hofbauer cells and the vascular endothelium of first trimester placenta (Fig. 1*B* and *C*), whilst at term, this isoform of system A was exclusively localized to syncytiotrophoblast (Fig. 1*D* and *E*). SNAT 2 and 4 were also present in endothelial cells in first trimester placenta (Fig. 1*H* and *N*), with faint staining persisting in larger intermediate and stem villous vessels at term (Fig. 1*L* and *Q*). Subpopulations of stromal cells were positive for SNAT2 and 4 at both stages of gestation (Fig. 1*H*, *O* and *Q*). Spatial immunostaining patterns were consistent in all samples examined, but considerable variation was detected in staining intensity. Negative control sections lacked detectable stain (Fig. 1*F*, *I* and *R*).

SNAT4 protein expression in first trimester and term MVM

SNAT4 protein expression was compared in first trimester and term MVM samples ($n = 4$ for each). Figure 2*A* shows that a band representing protein with a molecular weight corresponding to that of SNAT4 (∼70 kDa) was present in all MVM samples. There was no detectable immunoreactive signal in the BeWo sample used as a negative control, confirming antibody specificity. Densitometric analysis of the blots shown in Fig. 2*A* revealed that SNAT4 protein expression was higher in first trimester MVM samples compared to term MVM (Fig. 2*C*), which is also visually apparent. To ensure this was not attributable to unequal protein loading, following ECL the nitrocellulose membrane was reprobed for β-actin (Fig. 2*B*). Densitometric analysis revealed there was no significant difference in β-actin protein expression in first trimester compared to term MVM samples (Fig. 2*D*), confirming equal protein loading.

System A activity in first trimester and term MVM vesicles

Total [^{14}C]MeAIB uptake by first trimester and term MVM vesicles was linear ($P < 0.0001$ for both, linear regression) over 1 min (see control uptake in Fig. 3*A* and *B*) indicating this measurement approximated to initial rate. Figure 3*C* shows system A-specific [^{14}C]MeAIB uptake (calculated by subtracting uptake in the presence of 30 mM MeAIB from control uptake) by term MVM vesicles was significantly higher than uptake by first trimester vesicles at 30 s.

Arginine-inhibitable MeAIB uptake by MVM vesicles

[^{14}C]MeAIB uptake by term MVM was inhibited by 20 mM and 30 mM arginine (Fig. 3*B*). Thirty millimolar arginine had the most pronounced effect and, at this concentration, also significantly inhibited [^{14}C]MeAIB uptake by first trimester MVM (Fig. 3*A*). In experiments using term MVM vesicles, the effect of 40 mM and 50 mM arginine were also investigated but these did not cause any further inhibition of [^{14}C]MeAIB uptake (data not shown). Inhibition of [^{14}C]MeAIB uptake by 30 mM arginine was therefore used to calculate SNAT4-specific activity in MVM vesicles. In order to ensure that the inhibitory effect of 30 mM arginine was specific for MeAIB uptake, and not due to a general effect on amino acid uptake by MVM vesicles, we performed additional experiments to investigate the effect of 30 mM arginine on [^{3}H]taurine uptake. Taurine is a specific substrate for the Na^+-dependent amino acid transporter system β which is present in human placental MVM (Roos *et al.* 2004). Thirty millimolar arginine did not inhibit [^{3}H]taurine uptake by MVM vesicles (data not shown) confirming the inhibitory effect of 30 mM arginine was specific to [^{14}C]MeAIB uptake.

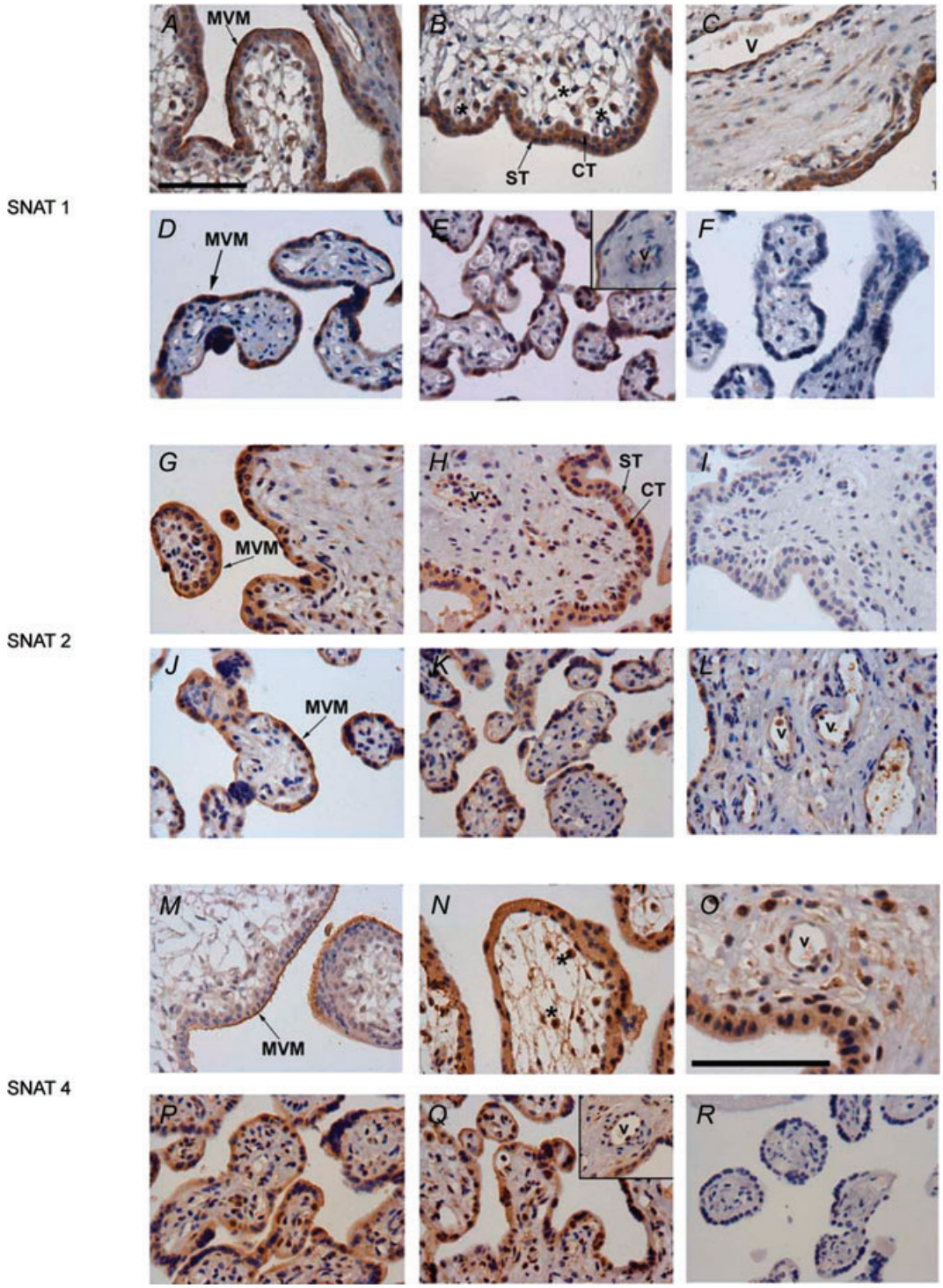

Figure 1. Immunolocalization of SNAT1, SNAT2 and SNAT4 in first trimester and term placentas
Representative photomicrographs for SNAT1 in first trimester (*A*–*C*) and term placenta (*D*–*E*). Immunostaining is predominantly in trophoblast cells (cytotrophoblast cells: CT and the syncytiotrophoblast: ST), with heterogeneous intense staining on the microvillous membrane (MVM) (*A* and *D*). Additional staining was present in stromal Hofbauer cells (asterisks) and vascular endothelium (V) in first trimester (*B* and *C*), but not term (*E* inset), placenta. SNAT2 exhibited a similar immunostaining pattern in first trimester (*G*–*I*) and term (*J*–*L*) placenta, but with endothelial staining present in vessels throughout pregnancy (*H* and *L*). Representative images for SNAT4 immunohistochemistry in first trimester (*M*–*O*) and term (*P*–*R*) placenta, illustrate a similar localization. Immunostaining is predominantly syncytial, with MVM staining more pronounced in first trimester (*M*) than term (*P*). Negative controls are shown in *F*, *I* and *R*. Scale bars represent 50 μm; scale bar on image *A* refers to all images, except image *O*.

SNAT4-specific MeAIB uptake by MVM vesicles

The arginine-inhibitable component of system A-specific [^{14}C]MeAIB uptake, representing SNAT4-mediated transport (mean ± S.E.M.), in first trimester and term vesicles was 0.083 ± 0.014 and 0.06 ± 0.01 pmol (mg protein^{-1} (30 s)$^{-1}$, respectively ($P = 0.24$, unpaired t test). Figure 3*D* shows that when these data are expressed as a percentage of system A-specific [^{14}C]MeAIB uptake, the SNAT4 contribution to total system A activity was significantly higher in first trimester MVM vesicles compared to term vesicles under these experimental conditions.

MeAIB uptake by human placental villous fragments

Figure 4*A* shows that Na^+-dependent [^{14}C]MeAIB uptake, representing system A-mediated transport, by term placental villous fragments is significantly higher than uptake by first trimester fragments. This increase could not be attributed to differences in fragment size (Greenwood & Sibley, 2006) as the protein content of first trimester and term placental villous fragments was comparable (mean ± S.E.M. = 501.8 ± 46.3 and 625.7 ± 45.6 μg, respectively, unpaired t test, $P = 0.07$).

SNAT4-specific MeAIB uptake by villous fragments

In the presence of 5 mM MeAIB, Na^+-dependent [^{14}C]MeAIB uptake by term villous fragments was not significantly different from zero (−1.1 ± 2.5 pmol (mg protein)$^{-1}$ (20 min)$^{-1}$, $P = 0.38$, Wilcoxon's signed rank test, $n = 3$) whereas Na^+-dependent [^{14}C]MeAIB uptake by first trimester villous fragments in the presence of 5 mM MeAIB was 43.9 ± 12.7 pmol (mg protein)$^{-1}$ (20 min)$^{-1}$ (Fig. 4*B*). These data suggest that, under physiological conditions, there is a contribution of SNAT4 to system A-mediated transport in first trimester placenta, whereas in term placenta, SNAT4 activity is minimal.

SLC38A4 mRNA expression in human tissue

The amplification product generated following *SLC38A4* PCR was visualized as a single band of appropriate size (152 bp) following agarose gel electrophoresis (Fig. 5*A*). The data reveal *SLC38A4* mRNA is expressed in all human tissues examined, including human placenta and liver as positive controls (Desforges *et al.* 2006). The amplification product generated following β-actin PCR was also visualized on agarose gel (Fig. 5*B*) and confirmed cDNA integrity in all samples.

Discussion

We have used immunohistochemical staining to confirm all three SNAT isoforms are present in the syncytiotrophoblast MVM of human placenta during first trimester and at term suggesting each has the potential to mediate amino acid transport from maternal blood in the intervillous space, into the placenta, and towards the fetus. We proceeded to design experiments to explore whether

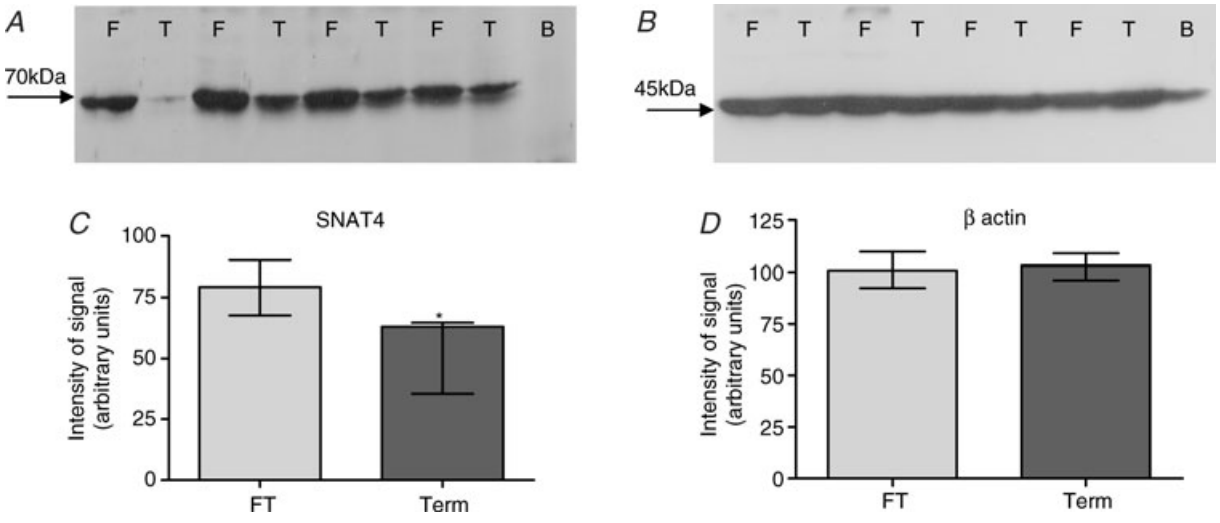

Figure 2. SNAT4 expression in first trimester and term MVM
Western blot of 4 first trimester (F) and 4 term (T) MVM samples probed for *A*, SNAT4 and *B*, β-actin using BeWo (*B*) as a negative control for SNAT4 expression. After 2 s exposure a single immunoreactive signal was observed in all lanes at the expected size of ∼45 kDa for β-actin. After 5 min exposure a single immunoreactive signal representing SNAT4 protein was observed in all MVM samples at ∼70 kDa. *C* and *D*, bar chart to show densitometric analysis of SNAT4 (*C*) and β-actin (*D*) protein expression in first trimester and term MVM samples (median and interquartile range, $n = 4$ for each group). *$P < 0.05$, Mann–Whitney *U*-test.

the SNAT4 isoform of system A is functional in human placental MVM during first trimester and at term. This study is the first to investigate the activity of a single SNAT isoform in the placenta, or in any tissue, which expresses all three system A isoforms.

Western blotting revealed higher SNAT4 protein expression in first trimester MVM compared to term. SNAT4 protein expression was also considerably variable between term MVM samples. The emerging pattern of SNAT4 expression and activity in human placenta across gestation appears to be quite complex. We have previously shown SNAT4 protein expression is lower in first trimester human placental homogenates, comprising many cell types, compared to term (Desforges *et al.* 2006). However, our current Western blotting data suggest that during first trimester a greater proportion of the SNAT4 protein pool is localized to the MVM. Consistent with this observation, immunohistochemical detection of SNAT4 shows strong staining in the cytoplasm of the syncytiotrophoblast of term placental tissue whereas in first trimester syncytiotrophoblast there is relatively more MVM staining of the syncytiotrophoblast layer. This accords with evidence from other cell types that shows preformed SNAT proteins are stored in cytoplasmic compartments before translocation to the plasma membrane takes place (Hundal *et al.* 1994; Ling *et al.* 2001; Hyde *et al.* 2002). As SNAT4 protein was also localized to fetal blood vessels, which are widespread at term but poorly developed during the first trimester, this may contribute to the higher SNAT4 protein expression previously observed in term placental homogenates (Desforges *et al.* 2006).

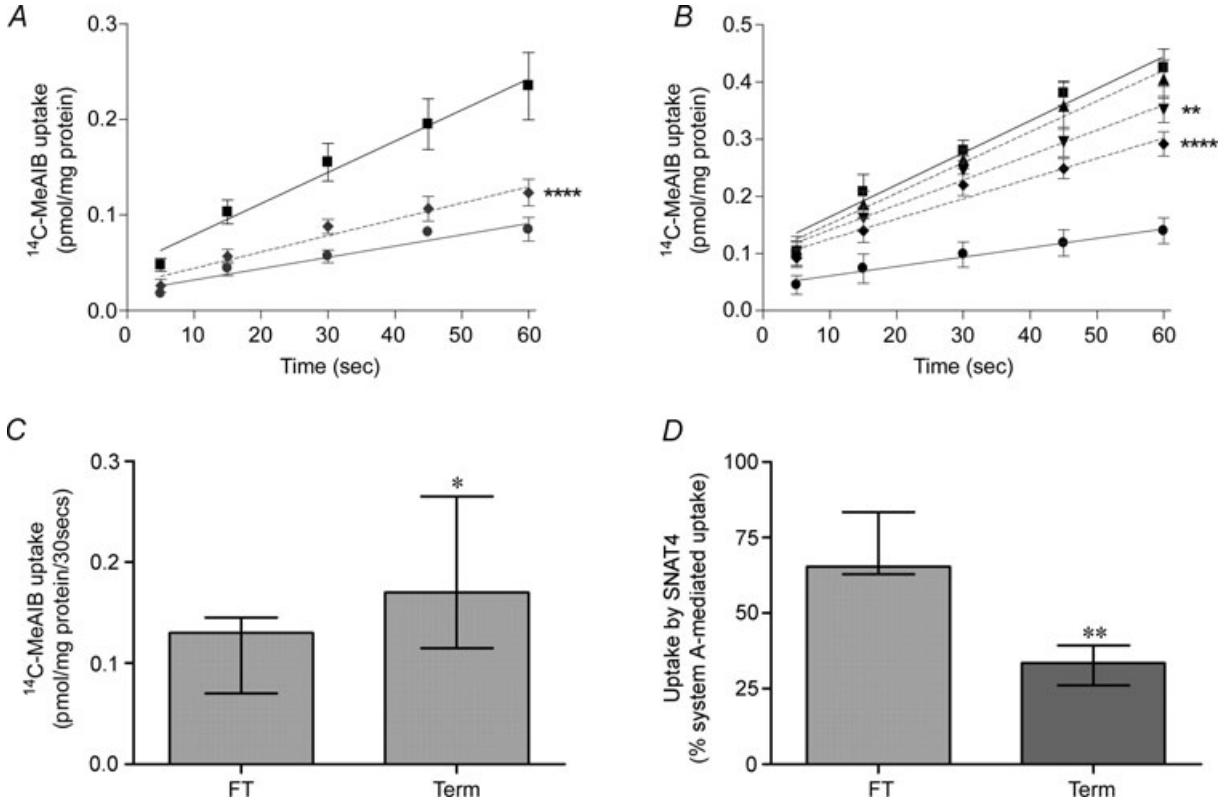

Figure 3. [^{14}C]MeAIB uptake by MVM vesicles
A, the effect of arginine on [^{14}C]MeAIB uptake by first trimester (FT) MVM and *B*, term MVM vesicles ($n = 6$ in each group). Data presented are mean ± S.E.M. and statistical analysis by 2-way ANOVA. Symbols shown are; ■, control; ▲, + 10 mM arginine; ▼, + 20 mM arginine; ♦, + 30 mM arginine; ●, + 30 mM MeAIB. $^{**}P < 0.01$, $^{****}P < 0.0001$ *versus* control. [^{14}C]MeAIB uptake in the presence of 30 mM MeAIB was significantly lower than [^{14}C]MeAIB uptake in all other conditions ($P < 0.0001$) and was considered non-system A-mediated due to saturation of all isoforms by this high concentration of substrate. In all conditions [^{14}C]MeAIB uptake significantly increased with time ($P < 0.0001$). *C*, comparison of system A-mediated [^{14}C]MeAIB uptake (calculated by subtracting [^{14}C]MeAIB uptake in the presence of 30 mM MeAIB from total [^{14}C]MeAIB uptake) by FT and term MVM after 30 s. Data presented are median and interquartile range, $n = 6$ in each group, $^{*}P < 0.05$ (Mann–Whitney *U*-test). *D*, arginine-inhibitable component of system A activity (expressed as a percentage of system A-mediated [^{14}C]MeAIB uptake) by FT and term MVM. Data presented are median and interquartile range, $n = 6$ in each group, ** $P < 0.01$ (Mann–Whitney *U*-test).

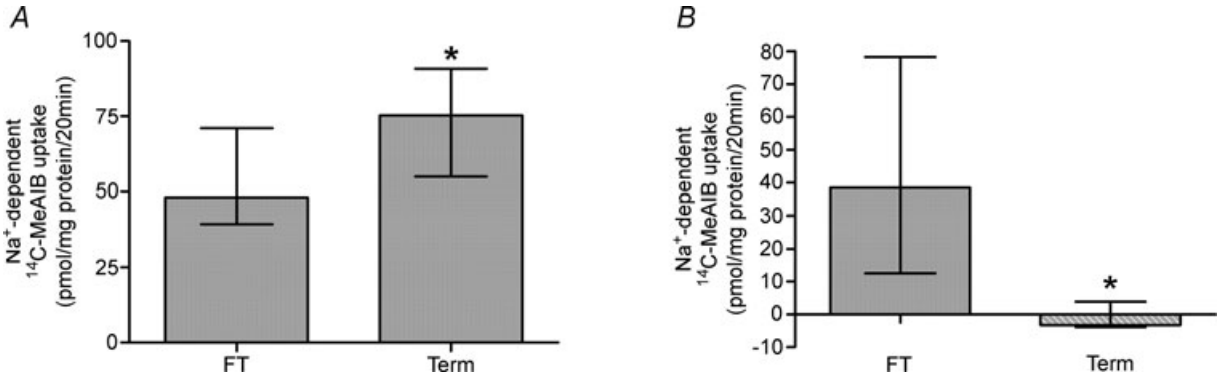

Figure 4. [^{14}C] MeAIB uptake by placental villous fragments
A, Na^+-dependent [^{14}C]MeAIB uptake by first trimester (FT) and term placental villous fragments ($n = 16$ in each group). These data represent system A-mediated transport of [^{14}C]MeAIB and are presented as median and interquartile range. System A activity is significantly higher at term compared to during first trimester (*$P < 0.05$, Mann–Whitney *U*-test). *B*, Na^+-dependent [^{14}C]MeAIB uptake by first trimester ($n = 6$) and term ($n = 3$) villous fragments in the presence of 5 mM MeAIB, representing SNAT4-mediated activity. SNAT4-mediated activity is significantly lower at term compared to during first trimester (*$P < 0.05$, Mann–Whitney *U*-test).

Following consideration of the substrate specificity and kinetic characteristics of SNAT4 we defined SNAT4-mediated transport by MVM vesicles as arginine-inhibitable [^{14}C]MeAIB uptake. The strategy of using the cationic amino acid arginine as a SNAT4 substrate is confounded by its specificity also for the amino acid transporter systems y^+ and y^+L which are active in MVM of first trimester and term human placenta (Ayuk *et al.* 2000). However these two cationic amino acid transporter systems do not support MeAIB as a substrate (Fei *et al.* 1995; Pfeiffer *et al.* 1999; Broer *et al.* 2000) strengthening the rationale for using this competitive inhibition approach. In the current study, experimental conditions using MVM vesicles were designed to promote MeAIB transport by SNAT4: the pH and the Na^+ gradient set across MVM vesicles were optimized to promote neutral amino acid transport by SNAT4 (Hatanaka *et al.* 2001). Furthermore, we used a relatively high concentration of [^{14}C]MeAIB (0.4 mM) compared to previous studies of system A activity in placenta (0.165 mM) in order to achieve contribution to transport across MVM by SNAT4. This was based on functional expression studies of the cloned SNAT transporters in HRPE cells, which demonstrate SNAT4 has a relatively low affinity for MeAIB ($K_m = 6.7$ mM) compared to the other SNAT isoforms ($K_m = 0.89$ and 0.39 mM for SNAT1 and SNAT2, respectively) (Hatanaka *et al.* 2000, 2001; Wang *et al.* 2000).

In agreement with previous observations (Mahendran *et al.* 1994), and using a modified assay system, we have shown that total system A activity in placental MVM is higher at term than during first trimester. Consistent with these observations, we have also demonstrated increased placental system A activity between first trimester and term using freshly isolated villous fragments. In contrast, Ericsson *et al.* (2005) reported no significant difference in Na^+-dependent transport of MeAIB into villous fragments between first trimester and term. The reason for these differing observations could be related to the size of fragments used (Greenwood & Sibley, 2006) or due to the concurrency of our experiments in contrast to the Ericsson *et al.* (2005) study, which compared first trimester data to term data arising from an earlier study by Jansson *et al.* (2003).

Despite there being increased total system A activity in human placenta between first trimester and term, our data demonstrate that SNAT4 activity in first trimester MVM is comparable to in term MVM; however, the contribution of SNAT4 to total system A activity is lower in term MVM compared to first trimester. Collectively these data indicate, firstly, that this broader specificity

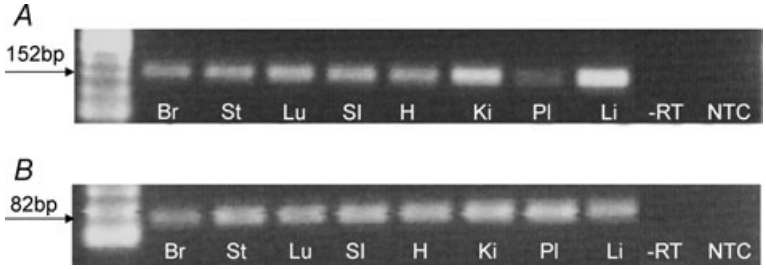

Figure 5. Amplification products for *SLC38A4* (*A*) and *β*-actin (*B*) PCR visualized on agarose gel using a 50-bp ladder
The human tissue samples include: Br, brain; St, stomach; Lu, lung; SI, small intestine; H, heart; Ki, kidney; Pl, term placenta; Li, liver; –RT, negative control (no reverse transcriptase enzyme); NTC, no template control. *SLC38A4* mRNA was expressed in all tissues.

isoform has a particularly important role in delivering amino acids to the fetus in early pregnancy, and secondly, that the gestational increase in system A transporter activity between first trimester and term is likely to be mediated by up-regulation of SNAT1 and/or SNAT2. It will therefore be important to determine the contribution of these particular SNAT isoforms to system A transport across MVM during normal pregnancy in order to further understand the complex gestational changes associated with this transporter.

MVM vesicles lack the intracellular regulatory components present in intact placental tissue which influence amino acid transporter activity. Therefore, *in vivo*, the absolute contribution of SNAT4 to total system A activity may be somewhat different from that suggested by the vesicle data (i.e. approximately 70% in first trimester compared to 33% at term). To address this issue we performed experiments designed to measure SNAT4 activity under more physiological conditions in villous fragments from human placenta. Any measurable Na^+-dependent uptake of [^{14}C]MeAIB (0.01 mM) in the presence of 5 mM MeAIB was considered to be SNAT4-mediated system A activity based on kinetic data showing that SNAT1 and SNAT2 activity is saturable at this concentration of MeAIB (Hatanaka *et al.* 2000; Wang *et al.* 2000). It is therefore interesting that there was measurable Na^+-dependent uptake of [^{14}C]MeAIB by first trimester villous fragments but not by term villous fragments in the presence of 5 mM MeAIB. These data suggest there is little or no SNAT4-mediated transport of neutral amino acids in term placenta and higher SNAT4-mediated transport in first trimester placenta, a trend which agrees well with the MVM vesicle data. Expression of SNAT4 protein in term MVM does suggest a functional role in amino acid transport and therefore it is reasonable to speculate that SNAT4 could mediate the transport of cationic, rather than neutral, amino acids at term. Although this is a possibility, our laboratory has previously demonstrated evidence for only two transport systems for cationic amino acids in the MVM of term human placenta, identified to be systems y^+ and y^+L (Ayuk *et al.* 2000).

Numerous studies have shown there is reduced MVM system A activity in pregnancies associated with IUGR compared to normal pregnancy (Dicke & Henderson, 1988; Mahendran *et al.* 1993; Glazier *et al.* 1997; Jansson *et al.* 2002). In a mouse model of IUGR which displays alterations in placental structure and function that are similar to those found in human IUGR (Sibley *et al.* 2004), reduced placental system A activity is specifically associated with altered *Slc38a4* expression (Constancia *et al.* 2005). Furthermore, ablation of the major placental promoter for *Slc38a4* in mice is associated with fetal growth restriction (G. Kelsey and M. Constância, personal communication). These data suggest the SNAT4 isoform of system A plays an important role in the provision of amino acids for normal fetal growth in mice. In mouse placenta *Slc38a4* is imprinted, being paternally expressed (Mizuno *et al.* 2002; Smith *et al.* 2003), which further suggests a role in promoting fetal growth (Coan *et al.* 2005; Fowden *et al.* 2006). It will therefore be interesting to investigate placental *SLC38A4*/SNAT4 expression in human IUGR and measure SNAT4 activity across MVM using the methods described in this study, in conjunction with the fragment experiments also described, to determine any differences from normal pregnancy. One study has shown placental *SLC38A1* and *SLC38A2* mRNA expression in human IUGR is comparable to that in normal pregnancies but the authors did not investigate *SLC38A4* expression (Malina *et al.* 2005). As shown in the current study, there is broad distribution of SNAT isoforms in human placenta highlighting the importance of performing functional assays in parallel to expression studies. Development of isoform-specific knockdown protocols for each of the three SNAT isoforms in cytotrophoblast cells and intact placental tissue will greatly assist in defining the relative contribution of each to placental system A activity over gestation and in relation to fetal growth anomalies.

SLC38A4 mRNA is expressed in various tissues from mouse (Mizuno *et al.* 2002) and also cattle (Zaitoun & Khatib, 2006). In the current study we demonstrated expression of *SLC38A4* in various human tissues previously shown to be devoid of *SLC38A4* expression (Hatanaka *et al.* 2001). These observations confirm this isoform of system A should no longer be considered liver-specific, and suggest it may be important for the transport of amino acids required for metabolism and protein synthesis in a variety of tissues. The experimental approaches described in this study for measuring SNAT4 activity could be applied to other human tissues besides placenta. This would be useful for identifying the involvement of SNAT4 in altered system A activity associated with certain diseases. For example in diabetes there is increased system A activity in the liver (Handlogten & Kilberg, 1984; Rosenthal *et al.* 1985; Schenerman & Kilberg, 1986), a key gluconeogenic organ. As gluconeogenesis primarily involves the conversion of amino acids and the recycling of glucose-derived carbon skeletons to glucose (Felig, 1975), with alanine an important glucose precursor, an altered system A activity in the liver could impact on the rate of alanine-supported gluconeogenesis and influence circulating glucose levels. *SLC38A2* and *SLC38A4* mRNA expression is increased in experimentally induced diabetic liver (Varoqui & Erickson, 2002). It would therefore be interesting to investigate whether the increased hepatic system A activity in diabetes can be attributed to increased SNAT4 activity using similar approaches to those described in this study.

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