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Regulation of amino acid transporters in pluripotent cell populations in the embryo and in culture; novel roles for sodium-coupled neutral amino acid transporters☆

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ABSTRACT

The developmental outcomes of preimplantation mammalian embryos are regulated directly by the surrounding microenvironment, and inappropriate concentrations of amino acids, or the loss of amino acid-sensing mechanisms, can be detrimental and impact further development. A specific role for L-proline in the differentiation of embryonic stem (ES) cells, a cell population derived from the blastocyst, has been shown in culture. L-proline acts as a signalling molecule, exerting its effects through cell uptake and subsequent metabolism. Uptake in ES cells occurs predominantly through the sodium-coupled neutral amino acid transporter 2, Slc38a2 (SNAT2). Dynamic expression of amino acid transporters has been shown in the early mammalian embryo, reflecting functional roles for amino acids in embryogenesis. The expression of SNAT2 and family member Slc38a1 (SNAT1) was determined in mouse embryos from the 2-cell stage through to the early post-implantation pre-gastrulation embryo. Key changes in expression were validated in cell culture models of development. Both transporters showed temporal dynamic expression patterns and changes in intracellular localisation as differentiation progressed. Changes in transporter expression likely reflect different amino acid requirements during development. Findings include the differential expression of SNAT1 in the inner and outer cells of the compacted morula and nuclear localisation of SNAT2 in the trophectoderm and placental lineages. Furthermore, SNAT2 expression was up-regulated in the epiblast prior to primitive ectoderm formation, an expression pattern consistent with a role for the transporter in later developmental decisions within the pluripotent lineage. We propose that the differential expression of SNAT2 in the epiblast provides evidence for an L-proline-mediated mechanism contributing to the regulation of embryonic development.
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1. Introduction

Inadequate amino acids supplementation in the culture environment of early mammalian embryos leads to poor embryonic outcomes, suggesting a requirement for these key metabolites (Gardner and Lane, 1993; Lane and Gardner, 1994; McKiernan et al., 1995). Functionally, amino acids protect the early embryo by acting as osmolytes (Van Winkle et al., 1990c), buffers of intracellular pH (Edwards et al., 1998), antioxidants (Liu and Foote, 1995) and chelators (Lindenbaum, 1973), as well as playing canonical roles as biosynthetic precursors, energy sources (Crosby et al., 1988; Rieger et al., 1992) and regulators of carbohydrate metabolism (Gardner, 1998; Lane and Gardner, 2005). Additional roles in cell signalling and determining cell allocation and differentiation within preimplantation embryos have been demonstrated (Lane and Gardner, 1997; Martin and Sutherland, 2001; Martin et al., 2003; Wu and Morris, 1998). Not surprisingly, therefore, developmental outcomes of cultured early mammalian embryos are sensitive to amino acid concentrations within the medium, and successful culture requires manipulation and optimisation of the medium amino acid composition (Gardner, 1998; Gardner and Lane, 2014a; Lane and Gardner, 1997). Conversely, death occurs around the time of implantation in embryos deficient in the amino acid sensor mTOR (Gangloff et al., 2004; Murakami et al., 2004), implying a requirement for nutrient sensing in late preimplantation development and differentiation. Despite these observations, little is known about the role of amino acid signalling in embryogenesis.

Amino acid transporters can be highly regulated during early embryonic development (Pelland et al., 2009; Van Winkle, 2001, 2013; Van Winkle et al., 1990b). The glycine transporter Slc6a9 (GLYT1) is essential for regulating oocyte size. GLYT1 is activated as the oocyte undergoes meiotic maturation, and activity gradually decreases with

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development, returning to baseline levels by the 8-cell stage (Tartia et al., 2009). Betaine uptake by the betaine/proline transporter, Slc6a20 (SIT1), can be detected in 1- and 2-cell embryos, with activity on either side of this window at baseline (Anas et al., 2007, 2008). GLYT1 and SIT1 transport osmolyte regulators, glycine and betaine, which protect the embryo from osmotic stress during the early cleavage stages (Baltz, 2013; Lane, 2001; Van Winkle et al., 1990c). Slc7a9 ($B^{0,+}$), increases in activity 30-fold between the 1-cell embryo and the preimplantation blastocyst, after which the transporter is inactive (Van Winkle et al., 1990b). Leucine uptake through $B^{0,+}$ activates the mTOR pathway and induces the formation of motile trophoblast cells, thereby regulating the onset of embryo penetration into the uterine wall (Van Winkle et al., 2006). These observations suggest that the expression and activity of amino acid transporters in the embryo are dynamic, and are consistent with the known roles of, and the requirement for, amino acids in mammalian embryogenesis.

The sodium-coupled neutral amino acid transporter 2, Slc38a2 (SNAT2), is expressed by pluripotent cells in culture and transporter activity is required for the formation of early primitive ectoderm-like (EPL) cells from embryonic stem (ES) cells (Tan et al., 2011). EPL cell formation is induced from primed ES cells by L-proline (Casalino et al., 2011; Rathjen et al., 1999; Tan et al., 2011; Washington et al., 2010). The activity associated with L-proline is specific; other amino acids (bar ornithine) and analogues of proline do not exhibit bioactivity (Casalino et al., 2011; Tan et al., 2011; Washington et al., 2010). Transport of L-proline into the cell by SNAT2 is required for L-proline activity (Tan et al., 2011). SNAT2 is the primary transporter of L-proline in ES cells. Competitive inhibition of the transporter prevents over 60% of total L-proline transport and stops the formation of EPL cells from ES cells (Tan et al., 2011).

Sodium-coupled neutral amino acid transporter activity has been demonstrated in the embryo with the identification of a 2-methyl(amino)isobutyric acid (MeAIB) sensitive transporter active in the inner cell mass (ICM) (Jamshidi and Kaye, 1995). This transporter could be SNAT2, consistent with the activity of SNAT2 in pluripotent cells in culture, or the related transporter Slc38a1 (SNAT1) (Tan et al., 2011), as both these transporters can be identified by their ability to transport MeAIB. SNAT1 transcripts have been detected in ES and EPL cells in culture (Tan et al., 2011). Here, the expression patterns of SNAT1 and SNAT2 in the cell lineages of the early mouse embryo have been examined here. For each transporter highly dynamic expression patterns were detected, and evidence of temporal and spatial regulation in multiple lineages of the early embryo, and key expression patterns were recapitulated using cultured cell models. We speculate that the regulation of SNAT2 in the pluripotent lineage of the embryo mirrors the role of this transporter in in vitro differentiation.

2. Experimental procedures

2.1. Mice

Naturally mated six-week-old female Swiss mice were used for embryo generation. Embryos were termed E0.5 on midday of the day a vaginal plug was detected. Mice were killed by cervical dislocation on E1.5, E2, E2.25, E3.5, E4.5 and E5.5. Embryos between E1.5 and E2.25 were flushed from the oviduct with G-MOPS with 5 mg/ml HSA (GMOPS+) (Gardner and Lane, 2014b) using a 10G gauge needle. Blastocysts were flushed from of the uterine horns with G-MOPS+ with a 27G gauge needle. E5.5 embryos were dissected from the decidua.

2.2. Cell culture

D3 ES cells (Doetschman et al., 1985), were as described previously (Rathjen and Rathjen, 2003; Tan et al., 2011). EPL cells were formed in response to MEDII supplementation as described (Rathjen and Rathjen, 2003). WA30 ES cells were sourced from Dr. Marnie Blewitt, Walter and Eliza Hall, Australia and maintained in 2i medium with LIF as described (Ying et al., 2008). To form primed WA30 ES cells, cells were cultured in serum- and LIF-containing medium (Rathjen and Rathjen, 2003). The mouse TS cell line (Tanaka et al., 1998), BeWo choriocarcinoma cell line (ATCC CCL-98) and JAR choriocarcinoma cell line (ATCC HTB-122) were a gift from Professor Paul Robson (The Jackson Laboratory for Genomic Medicine, Farmington, CT, USA). BeWo cells were cultured in DMEM:F12 with 10% FCS. JAR cells were cultured in RPMI with 10% FCS. TS cells were maintained as described previously (Tanaka et al., 1998).

Human ES cells (MEL2 cells, Australian Stem Cell Centre) were maintained in mTeSR™1 medium (Stemcell™ Technologies) at 37 °C and 5% CO_2 in air on tissue culture treated plates (BD Falcon™) coated with Matrigel (BD Biosciences #354277) according to the manufacturer's instructions.

2.3. Immunofluorescence and confocal microscopy

Cells were cultured to 80% confluency on gelatin-treated coverslips in 6-well tissue culture cluster dishes. Cells were fixed in 4% PFA/PBS for 30 min at RT. Embryos were fixed in 4% PFA/PBS for 24 h at 4 °C. Cells/embryos were permeabilized with PBST (30 min/RT) before blocking in PBS with 1% serum from the animal source of the secondary antibody) (30 min/RT). Cells/embryos were incubated overnight at 4 °C with the primary antibody, washed 3× with PBS and incubated in secondary antibody solution at RT for 90 min. Primary and secondary antibodies were diluted according to Table S1. Cells/embryos were stained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) in PBS and mounted in Vectorshield mounting medium (Vectorlabs). Imaging was performed on a Zeiss LSM 510 Meta confocal microscope or an Olympus Fluoview FV10i-LIV confocal microscope system. Images were prepared using the ImageJ software package (Schneider et al., 2012).

2.4. Real-time quantitative PCR

RNA was isolated, DNaseI treated and cDNA was synthesized as previously described (Tan et al., 2011). The reaction mix comprised 5× HOT FIREPol® EvaGreen® mix (Solis BioDyne), 200 nM forward and reverse primers and 1 μl of cDNA. Samples were amplified using a Roche Lightcycler® 480 Instrument II (Roche) or ViiA™ 7 Real-Time PCR System (Life Technologies™). The samples were initially heated to 95 °C for 15 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. The cycle threshold (Ct) values for *β-actin* was normalized across samples and the raw Ct values were analysed using Q-Gene software package (Simon, 2003).

2.5. Nuclear extractions and Western blotting

Nuclear extractions were performed using a NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Protein Biology Products; Thermo Scientific) according to manufacturer's instructions. Western blotting was performed as described previously (Tan et al., 2011). Antibodies were diluted in TBST according to Table S1. Detection was performed using the Immun-Star™ WesternC™ Chemiluminescence Kit (Bio-rad #170-5070) and imaged using the Bio-rad ChemiDoc™ XRS^{+} System, with images manipulated using the Image Lab™ software (Bio-rad #170-8265).

2.6. Statistics

Statistical analysis was performed using GraphPad Prism software. Gene expression data were analysed using the Student's *t*-test. Differences were considered statistically significant at $p = 0.05$. Data are presented as mean ± SEM.

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