RESEARCH ARTICLE

Mouse early extra-embryonic lineages activate compensatory endocytosis in response to poor maternal nutrition

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ABSTRACT

Mammalian extra-embryonic lineages perform the crucial role of nutrient provision during gestation to support embryonic and fetal growth. These lineages derive from outer trophectoderm (TE) and internal primitive endoderm (PE) in the blastocyst and subsequently give rise to chorio-allantoic and visceral yolk sac placentae, respectively. We have shown maternal low protein diet exclusively during mouse preimplantation development (Emb-LPD) is sufficient to cause a compensatory increase in fetal and perinatal growth that correlates positively with increased adult-onset cardiovascular, metabolic and behavioural disease. Here, to investigate early mechanisms of compensatory nutrient provision, we assessed the influence of maternal Emb-LPD on endocytosis within extra-embryonic lineages using quantitative imaging and expression of markers and proteins involved. Blastocysts collected from Emb-LPD mothers within standard culture medium displayed enhanced TE endocytosis compared with embryos from control mothers with respect to the number and collective volume per cell of vesicles with endocytosed ligand and fluid and lysosomes, plus protein expression of megalin (Lrp2) LDL-family receptor. Endocytosis was also stimulated using similar criteria in the outer PE-like lineage of embryoid bodies formed from embryonic stem cell lines generated from Emb-LPD blastocysts. Using an in vitro model replicating the depleted amino acid (AA) composition found within the Emb-LPD uterine luminal fluid, we show TE endocytosis response is activated through reduced branched-chain AAs (leucine, isoleucine, valine). Moreover, activation appears mediated through RhoA GTPase signalling. Our data indicate early embryos regulate and stabilise endocytosis as a mechanism to compensate for poor maternal nutrient provision.

KEY WORDS: Mouse embryo, Trophectoderm, Primitive endoderm, Embryoid body, Endocytosis, Maternal diet, RhoA

INTRODUCTION

Environmental factors around the time of conception including maternal nutrition, health and body condition together with in vitro assisted reproduction treatments (ART) have all been shown to influence the course of mammalian development. This has been demonstrated in different animal and human models and leads to altered physiology and health of offspring (Fleming et al., 2004; Duranthon et al., 2008; Fleming et al., 2012; Laguna-Barraza et al., 2013). In the context of maternal nutrition, we have used rat (Kwong et al., 2000) and mouse (Watkins et al., 2008; Watkins et al., 2010; Watkins et al., 2011) models to show that low protein diet fed during the ‘periconceptional’ (PC) period alone (Emb-LPD) before return to control diet for the remainder of gestation and throughout postnatal life is sufficient to change the rate of fetal and postnatal growth and lead to adult-onset cardiovascular, metabolic and behavioural disease. Poor maternal nutrition in large domestic animals during the PC window also results in increased adult disease risk (Gardner et al., 2004; Sinclair et al., 2007; Torrens et al., 2009). Likewise, in animal ART models, embryo culture and PC treatments cause altered growth rates and increased adult hypertension, metabolic dysfunction and behavioural deficits (Ecker et al., 2004; Fernández-Gonzalez et al., 2004; Watkins et al., 2007; Banrezes et al., 2011). Moreover, in human ART, the selection of commercial embryo culture medium has been shown to change birth weight (Dumoulin et al., 2010) and children born have an increased risk of cardiometabolic disease (Ceelen et al., 2008).

These diverse examples of PC environmental sensitivity with long-term consequences indicate that the extensive chromatin epigenetic reprogramming occurring at this time (Cantone and Fisher, 2013) may be affected by in vivo and in vitro conditions and lead to heritability of an epigenetic profile that can change gene expression pattern across emerging cell lineages and into postnatal life (Young et al., 2001; Fernández-Gonzalez et al., 2007; Morgan et al., 2008; Calle et al., 2012). Additionally, the manner in which early embryos interact with their environment suggests a range of cellular and physiological mechanisms are at work to modulate the inherent developmental programme and confer plasticity to support survival in challenging conditions. For example, we find in our mouse model that maternal Emb-LPD changes the allocation and ratio of cells within blastocyst lineages with a higher proportion within the outer trophectoderm (TE; progenitor of chorio-allantoic placenta) and a lower complement within the inner cell mass (ICM; progenitor of fetus and primitive endoderm lineage) (Eckert et al., 2012). This early consequence of poor maternal diet has also been identified by other laboratories using related mouse and large mammal models (Kakar et al., 2005; Mitchell et al., 2009). We also find increased TE proliferation coincides with increased motility and spreading activity of these cells as they outgrow as trophoblasts, probably to increase their invasiveness into the endometrium during implantation (Eckert et al., 2012).

These early changes in TE behaviour in response to poor maternal diet have significance, we believe, for subsequent development into adulthood. The Emb-LPD treatment leads to stimulation in fetal growth, increased birth weight and, in females, to enhanced postnatal growth into adulthood (Watkins et al., 2008). The increase in Emb-LPD fetal growth appears ‘programmed’ by the blastocyst...
stage, as transfer of Emb-LPD blastocysts into control-fed (NPD) recipients results in enhanced fetal growth despite a normal maternal dietary background (Watkins et al., 2008). Maternal dietary protein restriction has also been shown to increase capacity for maternal-fetal nutrient transfer across both the chorio-allantoic (Coan et al., 2011) and yolk sac (Watkins et al., 2008) placenta during later gestation. Lastly, we find Emb-LPD perinatal weight is significantly positively correlated with adult weight and cardiovascular and behavioural disease risk in later life (Watkins et al., 2008).

Collectively, these findings indicate that early embryos can ‘sense’ the nutrient quality of their immediate environment and activate responses to aid survival and protect fetal growth crucial for competitive fitness but which may have a “trade-off” of increased disease risk in later life. Our studies with the mouse Emb-LPD model indicate that insulin and branched-chain amino acid (AA) signalling through the mTORC1 pathway within the uterine environment may be the sensing mechanism for TE responsiveness (Eckert et al., 2012).

Here, we explore alternate mechanisms that may contribute to early embryo responsiveness to poor maternal nutrient provision to aid developmental survival but lead to long-term disease risk; we focus on the potential for endocytosis to fulfil this role (Luzio et al., 2009; Lamb et al., 2013). Preimplantation embryos differentiate a polarised endocytic and lysosomal system within the outer TE lineage during cleavage that preferentially internalises fluid from the outer apical membrane domain (Fleming and Pickering, 1985), comprises megalin (Lrp2 – Mouse Genome Informatics) and cubilin LDL-family receptors (Gueth-Hallonet et al., 1994; Moestrup and Verrout, 2001; Assémat et al., 2005) and is sensitive to insulin (Dunglison et al., 1995). Endocytic, histiotrophic characterises the maternal-fetal nutrient pathway of the visceral yolk sac (VYS) placenta (Beckman et al., 1997; Zohn and Sarkar, 2010), and we have shown that megalin-mediated VYS endocytosis is enhanced in late gestation in response to maternal protein restriction (Watkins et al., 2008). This second extra-embryonic lineage derives from the primitive endoderm (PE) formed in the late blastocyst at the blastocoelic face of the ICM (Rossant and Tam, 2009). Our data indicate stimulated endocytosis is used by both TE and PE extra-embryonic lineages to compensate for poor maternal nutrition, evident in ligand internalisation, receptor expression and lysosome production. We find dietary induction of enhanced TE endocytosis is activated and stabilised through branched-chain amino acid signalling and involves RhoA GTPase and actin remodelling.

RESULTS
Maternal Emb-LPD stimulates trophoderm endocytosis
We first assessed endocytosis and lysosome presence in blastocysts immediately following collection from Emb-LPD and NPD mothers in KSOM medium containing BSA-BODIPY (to detect digested ligand) and Lyso-Tracker (to detect lysosomes) followed by washing, fixation, confocal microscopy and image analysis. Using both single TE cell complete scan (Fig. 1) and whole embryo scan methods (supplementary material Fig. S1), Emb-LPD blastocysts displayed increased numbers and/or collective volume of labelled vesicles per cell (Fig. 1A-C; supplementary material Fig. S1A-C) in the outer TE layer. Labelled vesicles were mainly localised close to the nucleus rather than in peripheral cytoplasm. In Emb-LPD blastocysts, a higher proportion of the total Lyso-Tracker vesicular pool in TE cells was co-labelled with BSA-BODIPY (Fig. 1D), but the distribution of vesicles with respect to distance to the nucleus was not changed by diet (Fig. 1E; supplementary material Fig. S1D).

NPD and Emb-LPD blastocysts were also immunolabelled for localisation of clathrin and megalin followed by confocal microscopy and image analysis. Both clathrin (Fig. 2A) and megalin (Fig. 2C) were concentrated along the apical surface of TE cells and within vesicular structures in the apical cytoplasm. Using standardised settings and the whole embryo scan method, clathrin (Fig. 2B) and megalin (Fig. 2D) intensity of staining was increased in Emb-LPD blastocysts. Moreover, the increased megalin vesicular staining in Emb-LPD TE cells was located more within the central domain of the cells and closer to the nucleus than in NPD TE cells (Fig. 2E). Blastocyst immunoblots confirmed increased megalin protein in Emb-LPD embryos but not increased clathrin (Fig. 3A,B). However, megalin mRNA was not increased in Emb-LPD blastocysts (supplementary material Fig. S2A).

Changes in ICM endocytosis upon Emb-LPD are unknown, as the tight junction seal of the TE layer prevents diffusion of the BSA-BODIPY probe towards the ICM (Fig. 2F).

Maternal Emb-LPD stimulates primitive endoderm endocytosis
We have previously shown VYS endoderm endocytosis and megalin protein expression at late gestation to be increased by maternal LPD (i.e. low protein diet provided throughout pregnancy) and Emb-LPD (Watkins et al., 2008). To determine whether this change is mediated from the blastocyst stage and evident within the PE lineage, we conducted similar endocytosis assays to those on blastocyst TE. However, to improve accessibility and to evaluate the heritability of changed phenotype over many cell cycles from the time of maternal dietary treatment, we used embryoid bodies (EBs) formed from embryonic stem cell (ESC) lines derived from NPD and Emb-LPD blastocysts. EBs were generated from passage six ESCs in ESC culture medium without leukemia inhibitory factor (Li) over 5.5 days during which time an outer layer, two to three cells thick, of PE-like cells form, expressing the PE markers Gata6 and Dab2, also detected by western blotting (Fig. 4A) (Koike et al., 2007). Analysis of endocytosis using BSA-BODIPY revealed EBs derived from Emb-LPD blastocysts had increased vesicle number and collective volume per outer PE-like cell when compared with NPD EBs (Fig. 4B-D). Although lysosome number and volume per cell detected by Lyso-Tracker were also higher in Emb-LPD EBs, the increase was not significant (Fig. 4B-D). Immunoblot analysis of Emb-LPD EBs, however, showed increased expression of megalin and Lamp1 (lysosome marker) but not clathrin compared with NPD EBs (Fig. 3C,D).

Collectively, these data indicate that both TE and PE lineages exhibit a stimulation of endocytosis in response to maternal Emb-LPD that becomes stabilised and maintained beyond the period of diet treatment within a standard culture environment. Next, we assessed the mechanistic basis for enhanced endocytosis using the blastocyst TE model.

Induction and regulation of blastocyst TE response to maternal diet
To understand the mechanistic basis of enhanced endocytosis, we assessed the effects of altered environmental protein upon endocytosis in the blastocyst TE model. Embryos from Chow-fed mothers were cultured from the two-cell stage [embryonic day (E) 1.5] to blastocyst stage (E3.5) in KSOM medium containing either 1 or 4 mg/ml bovine serum albumin (BSA) concentration before evaluating TE endocytosis activity using the fluid marker fluorescein isothiocyanate (FITC)-dextran in the same medium. BSA was chosen because albumin is the major protein found within
uterine fluid (Velazquez et al., 2010; Faulkner et al., 2012). We found both the number and collective volume of FITC-dextran labelled vesicles within blastocyst TE to be increased after culture in 1 mg/ml BSA (Fig. 5A-C) using the whole embryo scan method. Our previous work has shown that Emb-LPD blastocysts transferred to NPD recipients are already ‘programmed’ to initiate the compensatory growth response in late gestation induced by the PC maternal protein restriction and do not require continuance of an LPD maternal environment (Watkins et al., 2008). This induced state is also indicated in our analysis of TE and PE-like cell endocytosis in diet-derived samples maintained in culture medium with standard composition (see above). To assess whether the stimulation in endocytosis in medium with reduced protein concentration was similarly stabilised, we compared blastocyst endocytosis when BSA concentration was changed for 1 hour before FITC-dextran culture commenced. Here, blastocysts cultured in 1 mg/ml BSA from the two-cell stage but switched to 4 mg/ml BSA for the terminal 1 hour period retained a high level of endocytosis (Fig. 5B,C). However, blastocysts cultured in 4 mg/ml from the two-cell stage but switched to a terminal 1 mg/ml culture changed their pattern of endocytosis to a high level (Fig. 5B,C). These data indicate that low environmental protein during preimplantation development can activate compensatory endocytosis in a sustained way, mimicking the effect observed both in our in vivo model and after culture of diet-derived embryos.

We next considered the possible signal pathway and specific nutrient sensor that may activate compensatory endocytosis in our model. We have shown that the uterine luminal fluid environment at the time of blastocyst formation in Emb-LPD mothers becomes depleted in branched-chain AAs (BCAAs; leucine, isoleucine and valine) (Eckert et al., 2012). These BCAAs are involved with insulin in the mTORC1 signal transduction pathway regulating cellular growth (Wang and Proud, 2009) and Emb-LPD blastocysts show a reduction in mTORC1 signalling (Eckert et al., 2012). Therefore, we investigated whether this same BCAA signal may also be responsible for the activation of compensatory endocytosis in TE cells.

Embryos from chow-fed mothers were collected at the two-cell stage and cultured until blastocysts in KSOM medium without BSA but with insulin (1 ng/ml) and AAs (including the BCAAs) at a
concentration found in NPD uterine fluid (Eckert et al., 2012). In addition, we cultured embryos in the same medium but with low (50%) or absent (0%) BCAAs. All three groups developed normally to the blastocyst stage, at which time embryos were examined for endocytosis using the FITC-dextran assay using the whole embryo scan method. We found treatment with low BCAAs stimulated endocytosis with both FITC-dextran vesicle number and collective volume per cell increased over the control normal concentration (Fig. 5A,D,E). Embryos cultured in the absence of BCAAs also exhibited an increase in endocytosis but not to a significant level. Collectively, these data indicate that embryo endocytosis is sensitive to environmental protein concentration and reduced uterine fluid BCAA levels, as found in maternal Emb-LPD mothers. The latter may act specifically to induce increased blastocyst TE endocytosis, but this mechanism functions within the physiological range rather than in complete absence of BCAAs.

We next evaluated the response mechanism within blastocyst TE following collection from diet-fed mothers. As blastocysts exhibit
reduced mTORC1 signalling in response to Emb-LPD (Eckert et al., 2012) and reduced mTORC1 activity is known to positively regulate autophagy and catabolism within cells to supplement nutrient availability (Martina et al., 2012; Jewell et al., 2013), we assessed whether the increased nutrient supply provided by endocytosis was also supplemented by increased autophagy (Lamb et al., 2013). We examined diet blastocysts by immunolabelling for LC3 protein, a marker for cellular autophagy (Florey and Overholtzer, 2012). LC3 was present as cytoplasmic puncta within both TE and ICM cells (supplementary material Fig. S3A). Comparison between NPD and Emb-LPD blastocysts showed no difference in LC3 puncta number or intensity (supplementary material Fig. S3B,C), indicating that embryo autophagy was not responsive to diet treatment.

Endocytosis and vesicular transport is known to be regulated from external signals via Rho-GTPases and downstream effector Rho-associated kinase (Rock) modulation of the actin cytoskeleton (Foerg et al., 2007; Nelson, 2009; Chi et al., 2013). We examined the localisation of RhoA and actin in NPD and Emb-LPD blastocysts following FITC-dextran incubation to quantify endocytosis. We found FITC-dextran endocytosis was increased to trend level (P<0.1) in Emb-LPD blastocysts (collective volume per TE cell; Fig. 6A,B). RhoA localised at the apical surface of TE cells commonly as a ring of punctate staining around the apicobasal column of endocytic vesicles within the central domain of the cells; this was more pronounced in Emb-LPD than NPD blastocysts (Fig. 6A). Actin was predominant in the cytoskeletal regions associated with the cell membrane (Fig. 6A). Quantification of RhoA fluorescence intensity revealed increased levels in Emb-LPD than NPD blastocysts (Fig. 6C) and also to trend level (P<0.1) for actin fluorescence (Fig. 6C).

These data implicate a role for RhoA in stimulation of endocytosis in Emb-LPD blastocysts. To evaluate this directly, we assessed FITC-dextran endocytosis in the presence or absence of the Rho GTPase family inhibitor, C3 transferase (Krijnen et al., 2010) together with immunolabelling for RhoA and actin (Fig. 6A, right column). Here, FITC-dextran endocytosis was again enhanced in Emb-LPD versus NPD blastocysts in the absence of C3 transferase (Fig. 6D) but in the presence of C3 transferase, RhoA staining was diminished and FITC-dextran endocytosis was reduced and to equivalent levels in both NPD and Emb-LPD blastocysts (Fig. 6D). C3 transferase incubation reduced FITC-dextran endocytosis by ~40% in NPD blastocysts and by ~70% in Emb-LPD blastocysts (Fig. 6D). These data implicate roles for RhoA both in blastocyst endocytosis and upregulation of endocytosis following Emb-LPD treatment.

**DISCUSSION**

Our study investigated whether cellular endocytosis was utilised by early extra-embryonic lineages as a compensatory mechanism to supplement nutrient uptake in response to poor maternal nutrition during the preimplantation period. We found both blastocyst TE and EB PE-like lineages activate enhanced endocytosis in response to maternal Emb-LPD with increased uptake of endocytosed fluid and ligand and increased numbers of lysosomes. These responses are accompanied by an increase in expression of the major LDL-receptor family member, megalin (Moestrup and Verroust, 2001; Marzolo and Farfán, 2011) in both extra-embryonic lineages. Moreover, diet-induced enhanced endocytosis is maintained upon *in vitro* culture in medium with standard composition; this stability is most striking in our EB model of PE-like cells, maintained over many passages from original derivation of ESCs from Emb-LPD blastocysts. Our studies also implicate a role for protein concentration generally and reduced BCAA concentration specifically in activation of enhanced TE endocytosis, mimicking the altered uterine fluid environment induced by maternal Emb-LPD treatment. Lastly, we show a role for RhoA signalling in regulating the diet-induced change in endocytosis rate.

The effect of poor maternal diet on stimulation of extra-embryonic lineage endocytosis provides a coherent mechanism for
compensatory nutrient provision to support conceptus survival in challenging conditions. Histiotrophic nutrition, whereby exogenous macromolecules are internalised and degraded and the products made available for conceptus biosynthesis is a characteristic function of the rodent VYS, particularly during organogenesis and before the chorio-allantoic placenta becomes fully active, thereby regulating fetal growth (Bloomfield et al., 2013). Similarly, yolk sac-mediated histiotrophic nutrition is crucial during the first trimester of human development (Burton et al., 2002). Our earlier findings that maternal LPD stimulates VYS endocytosis in late gestation (Watkins et al., 2008) is now extended to show evidence of induction and propagation of this mechanism within the progenitor PE lineage from the PC period. As the TE layer completely encloses the ICM, and we show our endocytosis probe (BSA-BODIPY) does not have access to the ICM (Fig. 2F), we anticipate that the TE performs a similar histiotrophic role to the PE in provision of lysosome-digested nutrients acquired by endocytosis to support the development of the ICM.

Both TE and PE epithelial lineages engage in polarised endocytosis, previously characterised for maturation steps during differentiation (Fleming and Pickering, 1985), actin cytoskeletal regulation (Fleming et al., 1986), and receptor-mediated uptake via megalin and cubulin (Gueth-Hallon et al., 1994; Assémat et al., 2005). The complexity of different endocytic pathways regulating cellular internalisation of varied constituents including fluid, nutrients, ligands and pathogens is increasingly recognised. Clathrin-mediated endocytosis is primarily but not exclusively actin-independent, whereas clathrin-independent macro- and micropinocytosis, including apical epithelial surface internalisation of fluid, ligands and macromolecules, is more clearly actin dependent and can be regulated through small GTPases including RhoA (Garred et al., 2001; Sandvig et al., 2008; Bohdanowicz and Grinstein, 2013). Our data indicate that maternal Emb-LPD stimulates TE and PE fluid and ligand endocytosis, increases expression of megalin but not clathrin, and is dependent upon active RhoA coinciding with an increased presence of actin. Collectively, this suggests that clathrin-independent endocytosis is the primary mechanism involved. RhoA GTPase is activated in the GTP-bound state mediated by external stimuli and regulates the downstream effector Rho kinase 1 (Rock1) to modulate actin polymerisation, organisation and myosin contraction and thereby membrane transport and endocytosis (Hall, 2005). The increase in TE apical
surface RhoA coincident with increased actin staining in Emb-LPD blastocysts implicates nutrient sensing to stimulate endocytosis by this pathway. The exoenzyme C3 transferase derived from Clostridium botulinum is a potent inhibitor of RhoA, B and C proteins by ADP-ribosylation in the effector-binding domain (Benink and Bement, 2005; Krijnen et al., 2010). C3 transferase treatment preferentially inhibited FITC-dextran endocytosis by Emb-LPD TE cells by ~70% and to a lesser extent in NPD TE cells by ~40% resulting in an equivalent level of reduced internalisation across treatment groups. This indicates that RhoA-mediated endocytosis is specifically enhanced in response to Emb-LPD but that other regulators of endocytosis exist. Endocytosis in blastocysts is known to be insulin sensitive (Dunglison et al., 1995), but because Emb-LPD reduces maternal insulin level (Eckert et al., 2012) it is unlikely to be a contributory factor. The stimulation in megalin expression by Emb-LPD in both TE- and PE-like cells indicates that receptor-mediated endocytosis is enhanced either through actin-mediated or independent mechanisms. Moreover, clathrin immunostaining was increased although expression is unchanged in response to Emb-LPD, indicating a possible minor involvement in the upregulation of endocytosis, probably mediated through a combination of several pathways.

The upstream inductive mechanism leading to stimulated endocytosis in blastocyst TE by Emb-LPD probably derives from nutrient levels within the immediate maternal tract environment or through indirect changes in factors regulating cellular behaviour mediated through the diet. We show that reduction in protein (BSA) concentration in vitro is sufficient to activate an increase in endocytosis as has been reported previously (Dunglison and Kaye, 1995). Interestingly, activation of endocytosis by reduced protein concentration is induced rapidly yet is sustained upon increase of in vitro protein level. These characteristics imply a compensatory process designed for longer-term protection in response to nutrient restriction even if conditions are fluctuating and further identify the PC period as key in setting the future growth trajectory. We do not know whether protein concentration is reduced within the maternal
tract in response to Emb-LPD, but we have found BCAAs to be depleted by ~30% concentration in Emb-LPD uterine fluid (Eckert et al., 2012). Reduced BCAAs was also sufficient to activate enhanced endocytosis in blastocyst TE.

Our data therefore show continuity from maternal Emb-LPD reducing both serum and uterine fluid BCAAs exactly at the time of blastocyst formation, which in turn coincides with reduced mTORC1 signalling (Eckert et al., 2012) and stimulated endocytosis. Surprisingly, as reduced mTORC1 is commonly associated with enhanced autophagy to supplement nutrient supply for growth (Martina et al., 2012; Jewell et al., 2013), this would seem unlikely, although the relationship between mTORC1 activity and endocytosis rate and processing is remarkably underexplored. Rather, we expect the link between mTORC1 signalling of reduced nutrient levels via BCAA availability in the Emb-LPD condition to enhanced endocytosis to be mediated indirectly through the associated and interacting mTORC2 complex. Unlike mTORC1, mTORC2 is insensitive to rapamycin and functions primarily in cytoskeletal reorganisation and cell survival mechanisms in response to external stimuli (Pépulo et al., 2012). Moreover, mTORC2 modulates cytoskeletal organisation via Pkca (Pkca – Mouse Genome Informatics), Akt (Akt1 – Mouse Genome Informatics) and the activation state of RhoA GTPase (Liu et al., 2010). Lastly, whereas mTORC1 signalling was clearly reduced via the S6 downstream effector in Emb-LPD blastocysts, the increased motility and invasiveness phenotype subsequently occurring in Emb-LPD
trophoblast outgrowths was rapamycin-insensitive, therefore likely to be mediated through mTORC2 cytoskeletal reorganisation to support implantation potential (Eckert et al., 2012).

In summary, our data indicate that maternal Emb-LPD acts to stimulate endocytosis within TE and PE lineages as a compensatory mechanism to protect nutrient provision and enhance survival. We find that this mechanism once activated becomes stable and is induced through reduced nutrients, specifically BCAAs, and mediated through RhoA activation of actin cytoskeletal organisation. In combination with our early work, we propose that this early response to adverse nutrition is mediated through both mTORC1 and mTORC2 complexes. Lastly, we show the feasibility of an ESC derived model for developmental programming of cellular and physiological criteria, providing an opportunity to reduce animal use and evaluate underlying mechanisms.

MATERIALS AND METHODS
Animals, diet treatment and embryo collection
MF1 mice, under UK Home Office license and local ethics approval, were bred in-house (University of Southampton Biomedical Research Facility) on a 07:00-19:00 light cycle with standard chow. Virgin females (7-8.5 weeks) were mated naturally overnight with MF1 males and plug positive females were housed individually the following morning and assigned randomly to either normal protein diet (18% casein, NPD) or isocaloric low protein diet (9% casein, Emb-LPD) until E3.5. Diet composition has been described previously (Kwong et al., 2000; Watkins et al., 2008). Alternatively, chow-fed females were used for in vitro culture experiments. Embryos were collected at different time points during preimplantation development after cervical dislocation. Following dissection of the reproductive tract, two-cell embryos (E1.5) and blastocysts (E3.5) were flushed from the oviducts and uterus, respectively, with H6 medium with 4 mg/ml BSA (H6+BSA) (Watkins et al., 2007).

ESC culture and EB formation
Mouse ESC lines were prepared using standard procedures from blastocysts derived from mothers fed NPD or Emb-LPD. This comprised culture of hatched blastocysts in ES culture medium containing knockout-Dulbecco’s modified Eagle medium [high glucose] (DMEM [high glucose], Gibco) supplemented with 20% knock out serum replacement (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine, penicillin [50 U/ml] and streptomycin [50 μg/ml] (Gibco) and 1000 U/ml Lif on 0.1% gelatin-coated dish with feeder layer cells at 37°C in humidified air with 5% CO2 for 5.5 days to form EBs. Mouse ESC lines were prepared using standard procedures from blastocysts derived from mothers fed NPD or Emb-LPD. This comprised culture of hatched blastocysts in ES culture medium containing knockout-Dulbecco’s modified Eagle medium [high glucose] (DMEM [high glucose], Gibco) supplemented with 20% knock out serum replacement (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine, penicillin [50 U/ml] and streptomycin [50 μg/ml] (Gibco) and 1000 U/ml Lif on 0.1% gelatin-coated dish with feeder layer cells at 37°C in humidified air with 5% CO2. Mouse embryonic fibroblasts treated with 10 μg/ml mitomycin C (Sigma) were used as feeder layer. After substantial outgrowth of the ICM (2.5 days) ICM-Embryos at the two-cell stage were cultured in defined KSOM medium comprising either BSA-BODIPY (0.5 mg/ml; Invitrogen) and Lyso-Tracker (100 nM; Invitrogen) for 1 hour or FITC-dextran as above plus 0.5 mg/ml BSA for 1 hour. Lyso-Tracker and FITC-dextran were combined in some experiments using KSOM plus 0.5 mg/ml BSA. The FITC-dextran assay was also conducted after culture of blastocysts in KSOM plus 0.5 mg/ml BSA and 8 μg/ml C3 transferase (Cytoskeleton, CT04) for 2 hours to inhibit Rho-GTPases. Endocytosis was also assayed on uniform-sized EBs after differentiation from ESCs but using ESC culture medium without Lif.

In vitro embryo culture
Embryos at the two-cell stage were cultured in defined KSOM medium (Sigma) under mineral oil at 37°C in 5% CO2 until the blastocyst stage either with variable protein or BCAC concentration. Protein levels were 1, 2 or 4 mg/ml BSA (Sigma) or switches between these concentrations. For AA experiments, control medium consisted of KSOM supplemented with insulin (1 ng/ml) and the ureteric luminal fluid AA concentration [including the BCAAs valine (0.46 mM), isoleucine (0.21 mM) and leucine (0.32 mM)] found in mice fed NPD at E3.5 (Eckert et al., 2012). In treatment groups BCAA concentration was either decreased (50%, LAAs) or omitted (0%, 0AA) compared with control (100%, NAA). After culture, embryos were incubated for 1 hour in the same medium but containing 5 mg/ml FITC-dextran (40 kD; Sigma) to label fluid phase endocytosis.

Endocytosis assays
Blastocysts (E3.5) from NPD or Emb-LPD fed mothers were collected in H6+BSA and immediately cultured for analysis of endocytosis in KSOM medium comprising either BSA-BODIPY (0.5 mg/ml; Invitrogen) and Lyso-Tracker (100 nM; Invitrogen) for 1 hour or FITC-dextran as above plus 0.5 mg/ml BSA for 1 hour. Lyso-Tracker and FITC-dextran were combined in some experiments using KSOM plus 0.5 mg/ml BSA. The FITC-dextran assay was also conducted after culture of blastocysts in KSOM plus 0.5 mg/ml BSA and 8 μg/ml C3 transferase (Cytoskeleton, CT04) for 2 hours to inhibit Rho-GTPases. Endocytosis was also assayed on uniform-sized EBs after differentiation from ESCs but using ESC culture medium without Lif.

Immunocytochemistry, microscopy and antibodies
Antibodies used for immunolabelling were: mouse monoclonal to megalin (Protein G purified, 1:500) (Meads and Wild, 1993), rabbit polyclonals to E-cadherin (generated in house to mouse E-cadherin GST fusion protein; 1:250), clathrin (Cell Signaling P1663, 1:400), LC3 autophagosome marker (Cell Signaling D11 X, 1:200), RhoA (Santa Cruz sc-418, 1:100), Gata6 (R&D Systems AF1700, 1:50) and Dab2 (BD 610465, 1:1000).

Blastocysts from NPD and Emb-LPD mothers, either after or without endocytosis assay, were treated with acic Tyrode’s medium (Sigma) for 15-30 seconds to remove the zona pellucida, washed in H6+BSA and fixed in 4% paraformaldehyde in PBS for 20 minutes. For megalin, RhoA and E-cadherin immunolabelling, blastocysts were permeabilised with 0.25% Triton X-100 (Sigma) in PBS for 15 minutes, washed in PBS and neutralised with 2.5 mg/ml NH4Cl in PBS for 10 minutes before primary antibody incubation in PBS containing 0.1% Tween 20 (Sigma; PBS-Tween) overnight at 4°C. For clathrin and LC3 immunolabelling, blastocysts were blocked and permeabilised with 5% fetal bovine serum (Sigma) in 0.3% Triton X-100 in PBS before antibody incubation overnight in PBS containing 1% BSA and 0.3% Tween-20. Blastocysts were subsequently washed and incubated in anti-mouse Alexa-546 or anti-rabbit Alexa-488 (Invitrogen, 1:300) in PBS-Tween for 1 hour at room temperature. Blastocysts were washed and stained for actin with Texas Red-X Phalloidin (Cell Signaling D11 X, 1:200), RhoA (Santa Cruz sc-418, 1:100), Gata6 (R&D Systems AF1700, 1:50) and Dab2 (BD 610465, 1:1000).

Endocytosis assays
Blastocysts from NPD and Emb-LPD mothers, either after or without endocytosis assay, were treated with acic Tyrode’s medium (Sigma) for 15-30 seconds to remove the zona pellucida, washed in H6+BSA and fixed in 4% paraformaldehyde in PBS for 20 minutes. For megalin, RhoA and E-cadherin immunolabelling, blastocysts were permeabilised with 0.25% Triton X-100 (Sigma) in PBS for 15 minutes, washed in PBS and neutralised with 2.5 mg/ml NH4Cl in PBS for 10 minutes before primary antibody incubation in PBS containing 0.1% Tween 20 (Sigma; PBS-Tween) overnight at 4°C. For clathrin and LC3 immunolabelling, blastocysts were blocked and permeabilised with 5% fetal bovine serum (Sigma) in 0.3% Triton X-100 in PBS before antibody incubation overnight in PBS containing 1% BSA and 0.3% Tween-20. Blastocysts were subsequently washed and incubated in anti-mouse Alexa-546 or anti-rabbit Alexa-488 (Invitrogen, 1:300) in PBS-Tween for 1 hour at room temperature. Blastocysts were washed and stained for actin with Texas Red-X Phalloidin (Invitrogen, 1:100) and nuclei with DAPI (0.2 μg/ml) for 30 minutes in PBS-Tween as required. Embryos were mounted onto slides with Citifluor or Vector-shield (H-10) and viewed with a Leica SP5 confocal microscope. Images were acquired by accumulation of z-series of whole embryos (~70×1 μm spaced xy-sections) or of single trophoderm cells (~60×0.1 μm continuous xy-sections without spaces) or of EB surface layer (~90×0.2 μm continuous xy-sections without spaces). E-cadherin staining was used to define the borders of TE cells in the blastocyst endocytosis assays.

EBs were fixed and processed for immunofluorescence analysis and confocal microscopy as for blastocysts. Additionally, Gata6 and Dab2 localisation in the EB outer layer was analysed in cryosections and examined using ABC staining to confirm formation of primitive endoderm-like cells as described previously (Gomes et al., 2010).

RNA isolation and real-time PCR
Isolation of RNA and real-time quantitative polymerase chain reaction (qRT-PCR) in embryos and EBs was performed as described previously (Lucas et al., 2011). For EBs, Gapdh and Pdhb were selected from six housekeeping genes with Genorm software. Primers used were: Lrp2 (megalin), sense CAATGGAGGATGCAGCCATATCT and antisense GTGTTGGAC-ACTGCGACTCAG; Gapdh, sense AGCTTGTCATCAACCGGAAG and


