Do little embryos make big decisions? How maternal dietary protein restriction can permanently change an embryo's potential, affecting adult health

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Abstract

Periconceptional environment may influence embryo development, ultimately affecting adult health. Here, we review the rodent model of maternal low protein diet specifically during the preimplantation period (Emb-LPD) with normal nutrition during subsequent gestation and postnatally. This model, studied mainly in mouse, leads to cardiovascular, metabolic and behavioural disease in adult offspring with females more susceptible. We evaluate the sequence of events from diet administration that may lead to adult disease. Emb-LPD changes maternal serum and/or uterine fluid metabolite composition notably with reduced insulin and branched-chain amino acids (BCAAs). This is sensed by blastocysts through reduced mTORC1 signalling. Embryos respond by permanently changing the pattern of development of their extra-embryonic lineages, trophectoderm (TE) and primitive endoderm (PE), to enhance maternal nutrient retrieval during subsequent gestation. These compensatory changes include stimulation in proliferation, endocytosis and cellular motility and epigenetic mechanisms underlying them are being identified. Collectively, these responses act to protect fetal growth and likely contribute to offspring reproductive competitive fitness. However, the resulting growth adversely affects long-term health since perinatal weight positively correlates with adult disease risk. We argue that periconceptional environmental responses reflect developmental plasticity and 'decisions' made by embryos to optimise their own development - but with lasting consequences.

Introduction

The discovery that adult health could be significantly affected by conditions in utero and early infancy first emerged with robust datasets from epidemiological research some 25 years ago, driven in particularly by Professor David Barker at Southampton UK (Barker, Osmond *et al.* 2009; Barker and Thornburg 2013). The Hertfordshire and Helsinki cohort studies demonstrated that risk factors for adult-onset cardiovascular and metabolic disease, including coronary heart disease and type-2 diabetes, positively correlated with low birth weight and 'catch-up' growth in infancy. Such associations, also including neurological disease risk, are evident in males and females and have been replicated in different cohorts across the world (Barker and Thornburg 2013). The concept of fetal programming of non-communicable disease emerged from such studies, now known as the Developmental Origins of Health and Disease (DOHaD). Another critical human dataset that has been central in establishing the DOHaD hypothesis is from the Dutch Hunger Winter, a 5 month famine experienced during WW2 by the citizens of Amsterdam. Here, the effects of the famine experienced during gestation have revealed a similar elevated risk of cardiometabolic and neurological disease in later life than for pregnancies occurring outside the famine period (Roseboom, Painter *et al.* 2011). Human health is therefore critically dependent upon maternal provision of nutrients during pregnancy that influence fetal growth with lasting consequences. Research studies by any groups using animal models have searched for mechanisms of developmental programming to understand why the consequences of in utero environment might be so profound. There are some excellent reviews embracing rodent and larger animal models and considering the programming effects of both maternal undernutrition and overnutrition (Ford and Long 2011; Langley-Evans 2013; Langley-Evans 2014; Williams, Seki *et al.* 2014; Zambrano, Guzman *et al.* 2014).

A major theme to emerge from across these studies is that nutrition during pregnancy is indeed an environmental factor 70 that can activate physiological interactions between mother and conceptus, mediated through diverse mechanisms including hormonal signalling that may cause to epigenetic changes in regulatory genes mechanisms within target tissues. Such interactions may modify placental efficiency, fetal growth and metabolic character, setting the foundation for disease when gestational and postnatal nutrient availability are discordant. Commonly, the physiological 'markers' of adult cardiometabolic dysfunction from across over- or under-nutritional models can be quite similar, with typically high blood pressure, poor glucose handling, increased adiposity and renal insufficiency all likely (Langley-Evans 2013). A second theme to emerge from animal models is the relevance of how different developmental stages may respond to a specific nutritional challenge in differing ways with the accumulative effects contributing to postnatal health (Langley-Evans, Welham *et al.* 1996). The periconceptional period has become recognised as a particularly vulnerable period for nutritional programming since maturing gametes and early embryos may show enduring responses to environmental conditions due to the unique metabolic, epigenetic and developmental events associated

with this reproductive stage (Fleming, Velazquez *et al.* 2012; MacLaughlin and McMillen 2007; Turner and Robker 2014; Zhang, Rattanatray *et al.* 2011) (see also later). In this context, it is apparent that paternal nutrition and physiology also contribute to developmental programming, either directly or indirectly through modulation of the maternal tract via the composition of the seminal plasma (Braun and Champagne 2014; Bromfield, Schjenken *et al.* 2014; Watkins and Sinclair 2014).

The focus of this short review is to evaluate in mechanistic terms the effect of maternal low protein diet specifically during the periconceptional period on long-term health of offspring using our rodent models. We consider the adult phenotypic consequences but 95 go back in time to assess in a stepwise manner the likely sequence of events through changes in maternal body composition caused by diet, how the embryo may interface with these changes, and what the responses may be that lead to adverse developmental programming. Lastly, we consider broader consequences of periconceptional environment outside of nutritional influences that may affect adult health.

Rodent maternal low protein diet and embryo programming

The low protein diet (LPD) used in our lab comprises 9% casein as a protein source versus the control normal protein diet (NPD) which has 18% casein; other macro- and micronutrients have the same concentration except corn starch and sucrose which are elevated by ~14% in the LPD to ensure the two diets are isocaloric. The diet was formulated by Professor Alan Jackson's laboratory in Southampton (Langley and Jackson 1994) and has been used extensively in rodent models of DOHaD by many laboratories worldwide (Langley-Evans 2013). It should be recognised that LPD at this protein concentration is a relatively mild restriction and would be a suitable nutrient base for a non-pregnant rodent; the effects of the diet can therefore be viewed as representative of potential variation across the normal range rather than an attempt to model starvation. Within our laboratory, we have used two maternal LPD treatments fed *ad libitum*, either from the time of detection of the copulatory plug in the morning after natural mating and for the duration of pregnancy (or late gestation prior to sacrifice for fetal/placental analyses; LPD) or exclusively from the time of detection of the copulatory plug until blastocyst formation (E4.25 rat; E3.5 mouse; Emb-LPD) before switching to NPD for the remainder of gestation (**Figure 1**). Control dams are fed NPD throughout gestation or until termination. During and after weaning, standard chow is provided for both experimental and control animals. The Emb-LPD diet offers the opportunity to assess the importance of the preimplantation period in nutritional programming.

Long-term effects of maternal LPD and Emb-LPD:

In both rat and mouse models, LPD and Emb-LPD had no effect on gestation length, litter size or the gender ratio of pups, reflecting the mild nature of the dietary challenge. In our original rat model (Kwong, Wild *et al.* 2000), Emb-LPD caused a slight decrease in birthweight in female offspring but

was followed by more substantial and sustained increased growth in both male and female Emb-LPD offspring from weaning until 7 weeks of age. The stimulation in postnatal growth following Emb-LPD was coupled with increased mean systolic blood pressure in both male and female offspring but only significant in males at 4 and 11 weeks. Male Emb-LPD offspring also exhibited altered increased kidney and reduced liver/body weight ratios (Kwong, Wild *et al.* 2000). More detailed studies on rat Emb-LPD livers during fetal development demonstrated abnormality in expression of key genes in hepatic function comprising phosphoenolpyruvate carboxykinase (PEPCK, rate limiting enzyme for gluconeogenesis) in males and 11beta-hydroxysteroid dehydrogenase type 1 (a reductase for production of active glucocorticoid) in females (Kwong, Miller *et al.* 2007). Moreover, Emb-LPD fetal liver revealed abnormal imprinted gene expression in a gender specific manner (Kwong, Miller *et al.* 2006).

Our studies in mice of long-term effects of Emb-LPD have been more detailed (**Figure 2**) but in general support and extend findings from the rat (**Figure 2**). Thus, a gender-influenced comorbidity of cardiovascular, metabolic and behavioural dysfunction is coupled with abnormal growth leading to late adult increased adiposity, with female offspring moreseverely affected (Watkins, Lucas *et al.* 2010; Watkins, Lucas *et al.* 2011; Watkins, Ursell *et al.* 2008). The cardiovascular disorder comprises a combination of physiological conditions evident for at least one year of postnatal life including relative hypertension present in both males and females and coupled with reduced capacity for arterial dilatation in response to isoprenaline, increased lung angiotensin-converting enzyme activity and, in females, reduced size of the heart, all factors that may contribute to the raised blood pressure (Watkins, Lucas *et al.* 2010; Watkins, Ursell *et al.* 2008). The Emb-LPD metabolic status in of Emb-LPD offspring, whilst not affecting serum glucose and insulin levels, reflected a change in adipose homeostasis with an increase (non-significant) in mean collective fat pad weight of over 20% in females coupled with increased gene expression of the insulin and IGF-1 receptors in retroperitoneal fat, suggestive of an energy-storage phenotype (Watkins, Lucas *et al.* 2011). The behavioural profile of Emb-LPD offspring was also compromised preferentially within females, evident within repeat open-field tests for locomotory activity and showing sustained hyperactivity but which may also be contributed by poorer memory (Watkins, Ursell *et al.* 2008). It should be further noted that the cardiovascular, metabolic and behavioural comorbidity defined from the Emb-LPD model is similar but not identical to a related periconceptional model where LPD is restricted exclusively to the period of oocyte maturation before fertilisation (Watkins, Wilkins *et al.* 2008).

The most revealing postnatal Emb-LPD phenotype is an altered body weight profile since this has allowed an understanding both of the origins of adult diseases and the derivation of the altered developmental programming from the preimplantation period onwards. As in the rat model, postnatal excess growth, synonymous to the ‘catch-up’ growth recorded across the DOHaD literature (Barker, Osmond *et al.* 2009; Langley-Evans 2014), is evident in response to maternal Emb-LPD but in the

mouse this occurs earlier than in the rat resulting in increased birthweight for male and female offspring combined and increased body weight gain for females alone (Watkins, Ursell *et al.* 2008). Thus, fetal growth is stimulated in late gestation in mouse Emb-LPD pregnancies, leading to increased birthweight and likely representing a compensatory response to poor maternal nutrition. Critically, the increased weight evident at birth following Emb-LPD appeared an effective biomarker of future disease, correlating positively with weight, systolic blood pressure and behavioural dysfunction in adulthood (Watkins, Ursell *et al.* 2008). In female Emb-LPD offspring, the weight advantage at birth is maintained through to adulthood compared to NPD controls but in males, perhaps through appetite regulation, postnatal weight becomes equivalent to controls (Watkins, Ursell *et al.* 2008).

Emb-LPD adult disease: the link to preimplantation environment

The comorbidity following maternal Emb-LPD in mice broadly matches the combination of non-communicable disease conditions found in the wider literature of rodent maternal LPD sustained throughout the gestation period (Langley-Evans 2001; Langley-Evans 2013; Watkins, Ursell *et al.* 2008). This clearly suggests that periconceptional development may be the major period of susceptibility to maternal dietary programming affecting lifelong health. The human epidemiological datasets associated with the Dutch Hunger Winter substantiate this and show that persons *conceived* during the famine (rather than 'experienced' it during later gestation) exhibit in adulthood a doubled rate of coronary heart disease, a more atherogenic plasma lipid profile, increased risk of schizophrenia and depression, were more responsive to stress and performed worse in cognitive tasks, collectively a sign of accelerated ageing (Roseboom, Painter *et al.* 2011). The simple conclusion is that the earlier maternal nutritional deprivation occurs during development the more widespread and critical the disease condition in adult life.

This periconceptional sensitivity may reflect the small number of totipotent and becoming pluripotent cells in the embryo, the co-occurrence of embryonic and extra-embryonic cell lineage allocation, and the extensive epigenetic restructuring that characterises this period. Such a combination of circumstances provides an *opportunity* for the embryo to optimise its future developmental programme according to environmental conditions, a form of plasticity consistent with the predictive adaptive responses concept in DOHaD (Gluckman and Hanson 2004). It also identifies a *vulnerability* for the embryo in that the entirety of subsequent conceptus lineages will be subject to this plasticity (Eckert and Fleming 2011). To understand why periconceptional maternal undernutrition has such a profound effect on adult health requires knowledge of the sequence of events from the time of maternal dietary intervention, whether and how the embryo may perceive dietary quality, and how the embryo may express plasticity and change its pattern of development as a consequence (**Figure 2**). In this review, we argue that early embryos make 'decisions' based upon their environment that can ultimately control their metabolic homeostasis and adult disease risk.

Embryo sensing of maternal dietary quality

The rat and mouse Emb-LPD models have been shown to lead to depleted levels of insulin and amino acids, particularly the branched chain amino acids (BCAAs), leucine, isoleucine and valine, within maternal serum during the period of blastocyst morphogenesis (Kwong, Wild *et al.* 2000; Watkins, Ursell *et al.* 2008). We consider this dietary effect as significant since insulin and BCAAs are key activators of cellular growth through the mTORC1 signalling pathway (Dowling, Topisirovic *et al.* 2010; Proud 2007; Wang and Proud 2009). Moreover, insulin and BCAAs have been shown to have stimulatory effects on preimplantation embryo biosynthetic activity, cell proliferation and endocytosis, with enduring effects on fetal growth (Dunglison, Jane *et al.* 1995; Harris, Gopichandran 220 *et al.* 2005; Heyner 1997; Kaye and Gardner 1999; Kaye and Harvey 1995; Lane and Gardner 1997; Martin, Sutherland *et al.* 2003) and blastocysts are known to utilise mTORC1 signalling to regulate trophectoderm motility (Gonzalez, Martin *et al.* 2012; Martin and Sutherland 2001). To substantiate the potential significance of Emb-LPD changes on in maternal serum, we investigated BCAA concentrations in uterine fluid during the time of blastocyst formation and expansion. This study demonstrated a ~25-30% depletion of all three BCAAs in Emb- LPD versus NPD uterine fluid (Eckert, Porter *et al.* 2012).

Whilst this provides good circumstantial evidence that maternal insulin and BCAA concentrations may act as metabolic factors important in Emb-LPD programming, it is important to establish whether blastocyst mTORC1 signalling is sensitive to this change and altered in response to the dietary challenge. Critically, we have found that the mTORC1 downstream target S6 ribosomal protein, a translational activator of terminal oligopyrimidine-dependent transcripts encoding ribosomal proteins active in protein translation and cell cycling (Kim 2009; Peterson and Schreiber 1998), was significantly deactivated in blastocysts in response to Emb-LPD, exhibiting a reduced phosphorylation:total protein ratio in quantitative immunoblots (Eckert, Porter *et al.* 2012). This direct evidence of altered mTORC1 signalling in Emb-LPD blastocysts coincident with depleted maternal insulin and BCAA availability leads us to conclude that this pathway is utilised for nutrient sensing in vivo. This conclusion is further substantiated by the fact that blastocyst amino acid composition is quantifiably altered by the maternal Emb-LPD diet with five being significantly altered and 17 out of 19 amino acids analysed showing a reduced mean concentration following Emb-LPD treatment (Eckert, Porter *et al.* 2012).

From embryo sensing to altered developmental programming

How might the maternal-embryonic interaction occurring through metabolite concentration downstream of dietary composition lead to altered developmental programming? Is the metabolic change detected within the Emb-LPD blastocyst sufficient for programming to occur? We have transferred Emb-LPD

and NPD blastocysts into opposite horns of NPD recipients to test this possibility. We have found that transferred Emb-LPD blastocysts develop into significantly heavier conceptuses in late gestation compared with transferred NPD blastocysts within an NPD maternal environment, providing compelling evidence that the blastocyst stage becomes programmed for an altered growth trajectory independent of the subsequent maternal dietary environment (Watkins, Ursell *et al.* 2008). Moreover, culturing embryos throughout cleavage in medium with reduced insulin and BCAAs as in Emb-LPD mothers can lead to altered postnatal growth and increased blood pressure similar to Emb- LPD offspring (Velazquez, Sheth *et al*. 2014). We consider this as critical evidence that the blastocyst mTORC1 sensing of deficient insulin/BCAA composition is the mechanism activating adverse long-term programming.

From embryo programming to compensatory fetal growth

If the reduction in maternal nutrient availability sensed by the Emb-LPD blastocyst via mTORC1 signalling programmes an increased fetal growth trajectory, can we identify how this compensatory response is mediated? We have studied the extra-embryonic lineages in our mouse Emb-LPD model to find such evidence (**Figures 2 and 3**). Could plasticity in the maturation of these lineages, both trophectoderm as progenitor of the chorio-allantoic placenta, and primitive endoderm as progenitor of the visceral yolk sac placenta, provide a physiological mechanism to link poor maternal diet with increased efficiency in maternal- embryonic/fetal nutrient delivery to drive fetal growth? Indeed, our recent 269 studies provide several lines of evidence for compensatory responses within the extra-embryonic lineages.

Compensation within trophectoderm lineage:

Cellular studies on the mouse Emb-LPD blastocyst have shown that the trophectoderm (TE) lineage is stimulated through increased proliferation relative to the inner cell mass (ICM). Increased proliferation within the Emb-LPD TE leads to increased total cells within the blastocyst at E3.75 (Eckert, Porter *et al.* 2012). In our rat Emb-LPD model, blastocyst TE numbers were unchanged in the early blastocyst but declined in the late blastocyst, an inconsistency with the mouse that may associate with the delay in compensatory growth in the rat until after birth (Kwong, Wild *et al.* 2000). The mouse Emb-LPD blastocyst TE further shows evidence of increased endocytosis as a compensatory response, reflecting an increase in nutrient quantity internalised in conditions of reduced nutrient quality (Sun, Velazquez *et al.* 2014). The stimulation in endocytosis is comprehensive and comprises increased number and/or volume of endocytosed vesicles and lysosomes, together with increased expression and vesicular distribution of the major endocytic receptor in TE, the megalin LDL-family receptor (Assemat, Vinot *et al.* 2005; Gueth-Hallonet, Santa-Maria *et al.* 1994; Moestrup and Verroust 2001; Sun, Velazquez *et al.* 2014). In vitro studies further demonstrated compensatory endocytosis was stimulated through reduced environmental protein concentration, as shown previously (Dunglison and Kaye 1995) and was induced

rapidly upon protein deprivation yet was sustained even if environmental protein was restored. Moreover, stimulated endocytosis could be activated specifically by mimicking in vitro the reduced insulin and BCAA levels found in Emb-LPD mothers (Sun, Velazquez *et al.* 2014), thereby showing mechanistic continuity between the dietary challenge and embryo response. This cell biological response to poor 294 maternal diet is regulated downstream from insulin/BCAA signalling via the actin cytoskeleton, specifically mediated through activation of the RhoA small GTPase which can modulate actin polymerisation (Bohdanowicz and Grinstein 2013; Garred, Rodal *et al.* 2001). Thus, specific inhibition of RhoA GTPase in Emb-LPD blastocysts was able to inhibit stimulation of endocytosis (Sun, Velazquez *et al.* 2014). The stimulation of TE endocytosis by maternal Emb-LPD had no effect on cellular autophagy suggesting the response was focused on retrieving more extracellular nutrients present in the uterine fluid rather than increasing cellular catabolism (Sun, Velazquez *et al.* 2014).

A final compensatory response mediated by Emb-LPD blastocyst TE was increased motility and invasiveness of the primary trophoblast cells that emerge at the time of implantation after hatching from the zona pellucida. In an in vitro outgrowth model of Emb-LPD blastocysts, the area of trophoblast migration per unit time was significantly increased versus NPD blastocysts (Eckert, Porter *et al.* 2012). Collectively, we consider these three responses detected within mouse Emb-LPD blastocyst TE, of increased proliferation, increased endocytosis of extracellular fluid and stimulated outgrowth motility and invasiveness phenotype, represent developmental plasticity and the capacity to enhance nutrient uptake during gestation despite maternal protein restriction. Indeed, recent studies show the placenta of LPD mice during later gestation exhibit morphological and functional adaptations to optimise nutrient transfer to the fetus, consistent with the early extra-embryonic mechanisms described above (Coan, Vaughan *et al.* 2011).

Compensation within primitive endoderm lineage

The primitive endoderm (PE) forms on the blastocoel face of the ICM in 318 the expanding late blastocyst and is the progenitor of the visceral endoderm epithelial layer of the yolk sac. The yolk sac regulates histiotrophic nutrition through endocytosis and lysosomal breakdown of maternal uterine lumen proteins and the delivery of liberated amino acids for post implantation embryo growth, particularly before the chorio-allantoic placenta is functional (Beckman, Lloyd *et al.* 1997; Bloomfield, Jaquiery *et al.* 2013; Zohn and Sarkar 2010). Our analyses of Emb-LPD extra-embryonic PE lineage compensatory responses have shown some similarities with TE responses. Thus, both PE and its visceral endoderm derivative in later gestation exhibit stimulated endocytosis. We have used mouse embryonic stem cell (mESC) lines derived from Emb-LPD and NPD blastocysts cultured to form embryoid bodies in which a PE forms of the surface to assess endocytic activity. Like TE within earlier blastocyst stages, the PE in embryoid bodies show increased ligand uptake, cellular lysosomes and megalin receptor expression compared with NPD embryoid bodies (Sun, Velazquez *et al.* 2014). During later gestation,

ex vivo LPD or Emb-LPD yolk sac visceral endoderm exhibited increased endocytosis of exogenous radiolabelled tracer, increased numbers of endocytic vesicles at the ultrastructural level, and increased megalin protein expression (Watkins, Ursell *et al.* 2008). Emb-LPD embryoid bodies grew to a larger size per unit time than NPD embryoid bodies, suggesting that Emb-LPD PE cells may also undergo increased proliferation (Sun, Denisenko *et al.* 2015) (Sun, Denisenko *et al*. 2014).

Epigenetic regulation of the Emb-LPD PE phenotype

The extra-embryonic lineage compensatory responses indicate true programming characteristics in that they are maintained irrespective of whether the initiating stimulus (Emb-LPD) is preserved. For example TE or PE stimulated endocytosis following Emb-LPD is retained in standardised culture medium and, in the case of PE, even after mESC derivation and culture over several passages in standard culture medium. The persistence of these responses indicates epigenetic regulation to retain altered levels of gene expression. The Emb-LPD embryoid body model has allowed us to investigate epigenetic regulation of Emb- LPD developmental programming. We found that Emb-LPD embryoid bodies, despite growing to a larger size than NPD embryoid bodies, had reduced expression at gene and protein levels of the transcription factor Gata6 (Sun, Denisenko *et al*. 2014) (Sun, Denisenko *et al.* 2015), known to be the key regulator of PE differentiation (Artus, Piliszek *et al.* 2011; Rossant, Chazaud *et al.* 2003; Schrode, Saiz *et al.* 2014). Previously, suppression of Gata6 expression has been shown to coincide with increased proliferation and reduced differentiation of several ovarian carcinoma cell lines (Cai, Caslini *et al.* 2009; Caslini, Capo chichi *et al.* 2006) and reduced Gata6 expression occurs in ex vivo Emb-LPD yolk sacs, all indicating a role in stimulating proliferation and suppressing differentiation in our Emb-LPD model. We have compared histone modifications of the Gata6 promoter within Emb-LPD and NPD embryoid bodies using chromatin immunoprecipitation assay and found significant reduction in histone H3 and H4 acetylation and RNA polymerase II binding in the Emb-LPD Gata6 promoter, all markers of reduced transcription. In addition, the histone deacetylase Hdac-1gene expression was upregulated in Emb-LPD embryoid bodies which may drive the deacetylation of the Gata6 promoter and contribute to reduced expression of the gene (Sun, Denisenko *et al.* 2015)(Sun, Denisenko *et al*. 2014).

Conclusions

The maternal Emb-LPD model has demonstrated a clear association and sequence of events linking maternal dietary consumption during the periconceptional period and adult onset non communicable disease. We find diet changes the composition of maternal fluids including within the uterine lumen and that embryos residing there can sense the quality of these nutrients, in this case via BCAA and insulin levels and the mTOR signalling pathway. This information, we propose, allows little embryos (<100 μm diameter) to make some big and apparently permanent decisions leading to adverse programming of adult health. This occurs even if the embryo is transferred to a mother having normal nutrition

throughout. We find some key decisions are to activate a series of compensatory responses within both the trophectoderm and primitive endoderm pathways of the extra-embryonic lineages. The compensations are designed to improve nutrient delivery from the mother to overcome the deficiency of low nutrient quality. Such a response is effective in protecting fetal growth and likely reflects an evolutionary mechanism to retain reproductive fitness of offspring. The trade-off, however, is an increased risk of adult onset disease with less evolutionary significance. We need to develop maternal nutrient supplementation strategies to alleviate the risk of reduced protein availability during periconceptional life.

One interesting aspect of the response to maternal undernutrition within our model is the gender specificity evident in particular postnatally with female offspring showing more severe growth and behavioural consequences (Watkins, Ursell *et al.* 2008). In other animal based, maternal dietary models focussed on the periconceptional period, gender-specific cardiometabolic or growth phenotype differences have been observed in offspring (Bermejo- Alvarez, Rizos *et al.* 2011; Sinclair, Allegrucci *et al.* 2007). Similarly, in ART-related embryo environment models, sexual dimorphism in embryo survival and adult offspring phenotype has been recorded (Fernandez-Gonzalez, Moreira *et al.* 2004; Feuer, Donjacour *et al.* 2014; Sjoblom, Roberts *et al.* 2005; Tarin, Garcia-Perez *et al.* 2014). Why might this occur following dietary challenge at the preimplantation stage? Differing environmental sensitivities by male and female embryos before gonadal development and direct sex hormone effects can be influential most likely derive from sex chromosome dosage effects on transcriptional activity (Bermejo-Alvarez, Rizos *et al.* 2011). Downstream from transcriptional differences, gender-specific variance in metabolism and growth rates may emerge in response to environmental challenge (Bermejo-Alvarez, Rizos *et al.* 2011; Erickson 1997), and this may be substantiated by X-inactivation in females (Tarin, Garcia- Perez *et al.* 2014) and by sex hormone effects during subsequent fetal development. The emergence and propagation of sexual dimorphic responses to dietary challenge from the preimplantation period onwards will be one important direction for future research.

Do little embryos make big decisions about other forms of their early environment? We know from another mouse model we have developed that maternal bacterial infection around the time of conception, mimicked by a single intraperitoneal injection of bacterial endotoxin, activated changes in blastocyst cellular organisation and alters offspring phenotype irreversibly (Williams, Teeling *et al.* 2011). However, here, the periconceptional endotoxin treatment does not alter the cardiometabolic phenotype of the adult offspring but rather suppresses the innate immune system after challenge, perhaps a programming mechanism to protect itself from a perceived pathogen-rich environment and the risk of auto-immune disease (Williams, Teeling *et al.* 2011). This argues that the early embryo does not activate a single stress-related response to diverse environmental challenges but rather is more selective,

using the cues provided to optimise its future development through epigenetic and physiological mechanisms of plasticity – the embryo has its own agenda distinct from its mother and father. There is growing evidence that assisted reproductive treatments (ART) can lead to small but increased risk of disease in children (Hart and Norman 2013a; Hart and Norman 2013b). We are still uncertain how the embryo 'views' its culture medium and the nature of potential compensatory responses that might unfold. However, the lessons from the Emb-LPD model suggest that the sequence of events should be further explored to protect against unwelcome disease effects.

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Figure legends

Figure 1. Schematic representation of mouse maternal Emb-LPD dietary model treatment. Control and treated females are fed with 18% (NPD) or 9% casein (LPD) respectively from the time of detection of the copulatory plug in the morning following natural mating (embryonic day 0.5 [E0.5]) and for the duration of pregnancy. A second treatment group is fed 9% casein (Emb-LPD) exclusively from the time of detection of the copulatory plug until blastocyst formation (E3.5) before switching to NPD for the remainder of the pregnancy. This latter group allows addressing the importance of the preimplantation period in nutritional programming. Before weaning, dams and offspring are fed with standard chow. At weaning, offspring continues on chow diet until the end of the experiment.

Figure 2. Proposed model depicting the short- and long-term effects of low protein exposure exclusively during in vivo preimplantation embryo development in the mouse (an olyovulatory species). Protein undernutrition can decrease concentrations of insulin in blood and amino acids (AA) in both blood and in uterine luminal fluid (ULF) respectively, which in turn induces cellular and molecular changes in both the blastocyst 442 and the conceptus. These changes are associated with an enhanced nutrient intake uptake during in utero, with long-lasting consequences in adult life. The altered phenotype observed during postnatal development can be sex-specific. Model based on data from Watkins et al . (2008), Eckert *et al*. (2012) and Sun *et al*. (2014).

Figure 3. In vitro model to study the effects of protein undernutrition during the preimplantation period on trophoblast and primitive endoderm activity. Blastocysts are collected from females fed with 9% casein (Emb-LPD) exclusively during preimplantation embryo development and used for blastocyst outgrowth and embryoid body production. These studies have revealed an increased motility of the trophoblast and enhanced endocytic activity in embryoid bodies associated with decreased expression of important transcription factors (i.e. Gata6) critical for primitive endoderm formation.

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Figure 1

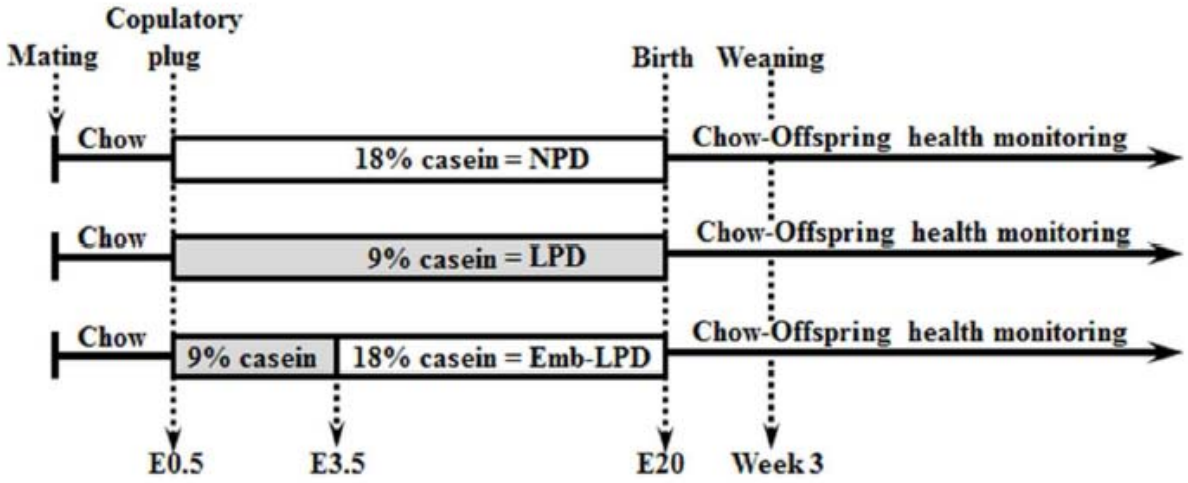

Copulatory
Mating
plug
Birth
Weaning
Chow
18% casein = NPD
Chow-Offspring health monitoring
Chow
9% casein = LPD
Chow-Offspring health monitoring
Chow
9% casein
18% casein = Emb-LPD
Chow-Offspring health monitoring
E0.5
E3.5
E20
Week 3

Figure 2

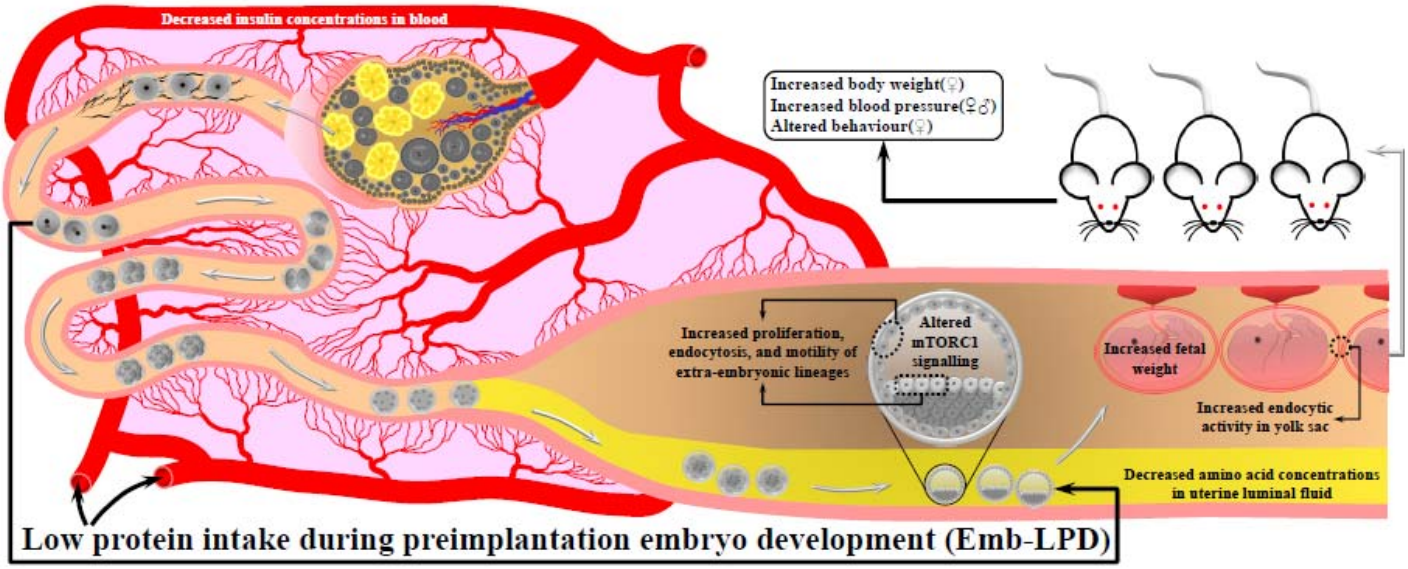
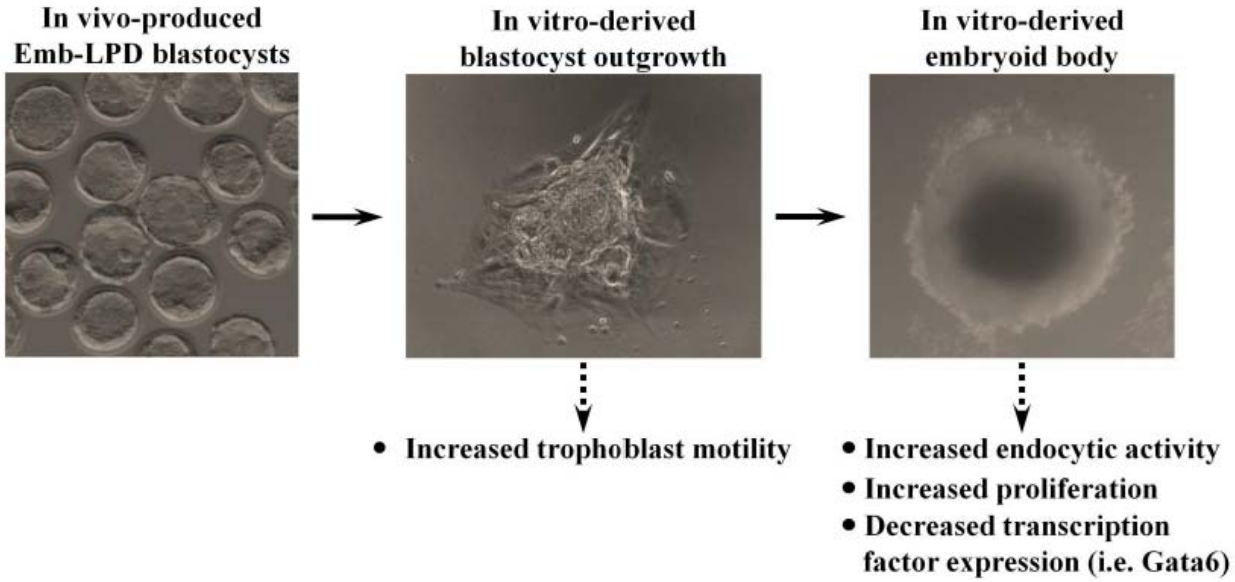

Decreased insulin concentrations in blood
Increased body weight(♀)
Increased blood pressure(♀♂)
Altered behaviour(♀)
Increased proliferation, endocytosis, and motility of extra-embryonic lineages
Altered mTORC1 signalling
Increased fetal weight
Increased endocytic activity in yolk sac
Decreased amino acid concentrations in uterine luminal fluid
Low protein intake during preimplantation embryo development (Emb-LPD)

Figure 3


In vivo-produced
Emb-LPD blastocysts
In vitro-derived
blastocyst outgrowth
In vitro-derived
embryoid body
• Increased trophoblast motility
• Increased endocytic activity
• Increased proliferation
• Decreased transcription
factor expression (i.e. Gata6)