The preimplantation embryo - handle with care

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The last decade has seen considerable advances in our understanding of intrinsic developmental mechanisms associated with gametogenesis and embryogenesis, and accompanying applications in the fields of reproductive medicine, embryonic stem cell biology and nuclear reprogramming. However, a new focus has recently emerged concerning the homeostatic regulation of embryonic cells, how this is set and how it may influence the longitudinal progression and optimisation of the developmental programme, and indeed the phenotype of the offspring. Attention has been drawn to the preimplantation stage of development as a sensitive 'window' when in vitro and in vivo manipulations, such as culture conditions or maternal diet, may have critical consequences. In this article, we review how changes in environmental conditions, mediated via a range of epigenetic, cellular and metabolic mechanisms in the preimplantation embryo, may alter the pattern of cell division, gene expression, morphology and potential. We consider how fetal and postnatal phenotype may become susceptible to the plasticity of the preimplantation embryo, and the risks for adult health and physiology.

Key words

Epigenetics, gene expression, in vitro culture, maternal diet, preimplantation embryo.

INTRODUCTION

Mammalian preimplantation development is subject to both genetic determinants and environmental factors. During this time the early embryo undergoes cleavage and blastocyst morphogenesis and the first two cell lineages segregate, the outer trophectoderm (TE) which will form the chorio-allantoic placenta later in pregnancy, and the inner cell mass (ICM) from which will derive the entire fetus. Homeostatic responses by the embryo to environmental cues, both in vivo and in vitro, have been shown to affect the long-term potential for health or predispose to adult disease (1-4; Fig.1). Several studies using rodent and domestic animal models have demonstrated that environmental conditions experienced during early development shape critical aspects of future growth, metabolism, gene expression and physiology. Thus, changes in periconceptual maternal diet can impact upon ovulatory fitness and blastocyst proliferation, leading to altered fetal metabolic and cardiovascular development and adult hypertension (5-11). Similarly, in vitro culture of embryos can associate with changes in fetal growth (4, 12-15), gene expression and regulation (15-17), postnatal behaviour (18, 19), and raised systolic blood pressure (20). These longitudinal associations are pertinent for preventative healthcare concerning nutrition during pregnancy as well as the safety of assisted reproduction technologies (ART) in the treatment of infertility, and underlie the need for elucidation of causal mechanisms and protective strategies.

EMBRYO DEVELOPMENT AND ENVIRONMENTAL SENSITIVITY IN VIVO

To evaluate the causes of environment-induced changes in embryo developmental potential, we first consider the nature of maternal-embryonic interactions within the relatively undisturbed reproductive tract. This may shed light on how the embryo may

respond to sub-optimal conditions and the consequences of this response. The spectrum of maternal-embryonic signalling is broad and a range of metabolites, growth factors, macromolecules and developmental cues are involved in regulating embryo metabolism and growth (21, 23-26). The metabolic requirements of the embryo change as it travels from the oviduct towards the uterus; during early cleavage, the embryo predominantly metabolises pyruvate, but switches to glucose during compaction and throughout blastocyst development (27-30). Coupled with this, non-essential amino acids and glutamine increase the rate of cleavage in mouse (31, 32), human (33), bovine (34) and sheep (22) pre-compaction embryos, whilst post-compaction, essential amino acids are more influential (22, 31, 32). In response to these changing requirements, the oviduct is relatively rich in pyruvate and low in glucose and provides the embryo with the necessary non-essential amino acids. Conversely, the uterine environment is richer in glucose and essential amino acids (35, 36). Recent proteomic research indicates that the interaction between the reproductive tract and gametes and embryos is both dynamic and critical for reproductive progression (37, 38). Interestingly, recent studies have shown the metabolic activity of the early human embryo may be indicative of its potential, in that lower rates of amino acid exchange, perhaps symptomatic of low metabolic stress, correlate with improved viability after transfer (39-41).

Maternal diabetes and hyperglycaemia

The link between embryo in vivo metabolic environment and longer-term health is evident in women with maternal diabetes where the hyperglycaemic environment increases the risk of spontaneous abortion and fetal abnormalities (42, 43). Blastocysts recovered from diabetic mice and rats have higher rates of chromatin

degradation, nuclear fragmentation and apoptosis of ICM cells than embryos from controls (44, 45). Similarly, the culture of normal mouse or rat blastocysts in high glucose concentrations increases the expression of pro-apoptotic markers (45). One mechanism through which elevated glucose may induce blastocyst apoptosis is via the down regulation of glucose transporters (GLUTs), resulting in decreased intracellular glucose concentration and increased apoptosis (46-48). Blastocysts from *Glut1* (*Slc2a1*) knock-down mice show increased apoptosis and reduced glucose transport (49), indicating appropriate intracellular glucose availability is critical for embryo survival (50). Moreover, a high embryonic glucose environment also impacts negatively upon fetal development with increased frequency of resorptions and malformations and reduced fetal growth (46, 48, 49). Decreased insulin/IGF-1 signalling via the PI3-K and Akt pathway may be a central mediator of reduced embryo potential (50). Thus, activation of the PI3-K pathway by platelet activating factor has been shown to be essential for preimplantation embryo development and survival (51), whilst inhibition of the PI-3K/Atk pathway in murine blastocysts results in reduced GLUT1 expression and glucose uptake, impaired blastocyst development and increased apoptosis and nuclear fragmentation (50-52).

Mitochondrial function and blastocyst potential

Central to the relationship between glucose metabolism and apoptosis is the mitochondrion. Whilst mitochondrial ATP production is essential for oocyte maturation, Ca^{2+} homeostasis and post-fertilisation development (53-55), mitochondrial dysfunction and subsequent changes in calcium signalling can result in apoptosis in the oocyte and early embryo (53).

There also exists a secondary mechanism through which mitochondria can influence aspects of post-fertilisation development. In the maturing oocyte, mitochondria undergo rapid rates of division followed by replication arrest during cleavage (56). Suboptimal environmental conditions can increase reactive oxygen species (ROS) levels causing increased mitochondrial DNA (mtDNA) mutations (57). Thus, any mtDNA mutations incurred during oocyte maturation will be maintained during early embryonic development. Alongside this, heterogeneity in the distribution of mitochondria in the oocyte could result in asynchronous mitochondrial loads in the resultant blastomeres, altering future developmental and metabolic profile (58, 59).

Maternal diet

An altered uterine environment leading to adverse embryo responses can also derive from changes to maternal diet. Maternal low protein diet (LPD) given to rat dams exclusively during the preimplantation period (0-4.25 days) resulted in mild transient maternal hyperglycaemia, reduced blastocyst cell numbers, gender-specific programming of imprinted gene expression and altered postnatal growth and hypertension (5, 6). This treatment also increased the expression of the 11β-hydroxysteroid dehydrogenase type 1 (*Hsd11b1*) gene responsible for activating glucocorticoid in fetal liver, together with increased expression of phosphoenolpyruvate carboxykinase (*Pepck*, *Pck1*) gene, encoding the rate-limiting enzyme for gluconeogenesis (7). Studies in sheep have further demonstrated that either periconceptional maternal undernutrition or a high protein diet can lead to reduced developmental viability, abnormal fetal and postnatal growth and changes in fetal endocrinology (8, 9, 60, 61). In the sheep, periconceptional undernutrition may act through enhanced stimulation of the HPA axis leading to increased fetal blood

pressure in twins and postnatal cardiovascular dysfunction in singletons (8-11, 62, 63) as well as changes in the fetal IGF axis (64, 65).

Domestic animal studies have also shown follicular growth and oocyte quality to be affected by a range of dietary manipulations (66-69). Dietary energy levels influence both morphology and developmental competence of bovine oocytes (70) although increased protein intake may also elevate ammonia content in follicular fluid resulting in reduced blastocyst development (71-74). High levels of maternal nitrogen metabolism prior to embryo collection and in vitro culture has been shown to increase fetal development and alter gene expression (73).

EMBRYONIC DEVELOPMENT AND ENVIRONMENTAL SENSITIVITY IN VITRO

Despite advances in the composition and properties of embryo culture media for ART and domestic animal biotechnology purposes, in vitro culture generally remains inferior to the in vivo environment for the rate of embryo development (1, 4, 12, 20, 75). An early, and perhaps the most dramatic, example of embryo culture having an adverse effect on the developmental programme is the phenomenon of ‘large offspring syndrome’ (LOS). LOS was observed after culture of sheep and cattle embryos in the presence of serum resulting in increased weight at birth, increased muscle mass, cerebellar dysplasia, skeletal and facial malformations, changes in the normal size and weight of internal organs and sudden perinatal death (76-80). A LOS phenotype has also been observed following the process of cloning by nuclear transfer, the co-culture of embryos on granulosa cells, the transfer of embryos to an asynchronous uterine environment and the exposure of embryos to a high progesterone uterine environment (76-79, 81). It is of interest to note that sheep

embryos cultured in the presence of serum display increased glycolysis when compared to embryos derived in vivo or cultured in the absence of serum (22). These changes may arise from increased mitochondrial degeneration observed for embryos cultured in serum compared with serum-free conditions (82, 83). As outlined above, mitochondrial function and morphology in response to environmental perturbations may be a useful indicator of embryo developmental potential. Serum-free culture conditions have been successfully employed for the human (84), mouse (32) and ruminant species (85) to protect embryo proliferation and fetal growth. Recently, it has been shown that the pre-compact ovine embryo is more sensitive to serum in culture than the post-compact embryo, although culture in the absence of serum was most beneficial for maintaining normal fetal growth (86).

Other long-term effects mediated by embryo culture indicate vulnerability to a wide range of postnatal conditions. In vitro embryo culture and transfer of mouse preimplantation embryos has been shown to adversely alter postnatal behaviour and memory and growth (18, 19) but not longevity (87). Also in the mouse, in vitro culture for either a prolonged (2-cell to blastocyst) or brief period (1-2 hours as blastocysts) prior to transfer resulted in elevation of adult blood pressure and activity of the cardiovascular regulator, serum angiotensin converting enzyme (ACE) and the gluconeogenesis controller, hepatic phosphoenolpyruvate carboxykinase (PEPCK) in female offspring (20).

Short-term effects of embryo culture alter the global pattern of gene expression within blastocysts, indicating the sensitivity of several gene pathways. (88). In vitro derived bovine blastocysts exposed to serum display altered expression

profiles of metabolic and growth regulator genes including *BAX*, leukemia inhibitory factor (*LIF*), LIF-Rβ, basic fibroblast growth factor (*bFGF*), insulin-like growth factor-I receptor (*IGF1R*) and superoxide dismutase (*SOD*) compared to embryos cultured in serum-free medium or developing in vivo (89-91). In vitro produced bovine embryos also alter their expression profiles of *GLUT1* in response to changes in oxygen concentrations (92) while in the mouse, expression of *Glut1* (*Slc2a1*), *Glut3* (*Slc2a3*) and *Vegf* (*Vegfa*) are all increased by 2% versus 20% oxygen (93). These changes in metabolic regulators indicate conservation in mechanisms with in vivo environmental sensitivity, particularly diabetes and hyperglycaemia.

Despite this apparent negative effect of in vitro culture, a range of modifications to the culture environment have been shown to significantly improve embryo development such as the addition of amino acids and/or growth factors, reduction in the level of ammonium ions, and culture in a low O_2 atmosphere (4). It has been proposed that the composition of culture medium is of more influence upon developmental outcome than the procedure of in vitro culture itself (4). The addition of physiological concentrations of insulin and IGF-1 increase the number of cells within mouse, bovine and human preimplantation embryos (94-99), predominantly via the stimulation of ICM proliferation (94, 95, 98-102) and the inhibition of apoptosis (96-99). Insulin and IGF-1 also stimulate embryo metabolism, increasing protein endocytosis whilst reducing the catabolism of the incorporated protein (103, 104). Mouse embryos cultured in the presence of insulin and albumin result in increased fetal weight after transfer (105). More recently, the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) to the culture medium has been shown to alleviate some of the detrimental influences of mouse embryo culture on

fetal viability and growth, predominantly mediated through the alteration of placental morphogenesis (106). Leptin has also been shown to enhance in vitro mouse embryo development and stimulate trophectoderm proliferation (107), whilst ghrelin, know to modulate feeding behaviour and energy metabolism, negatively influences mouse preimplantation embryo development in vitro (108).

The findings that in vitro culture conditions can have dramatic consequences on postnatal health of mice and domestic livestock pose questions as to the long term health of ART children. Despite the increased ability to culture and manipulate gametes and embryos, the pregnancy rate from ART remains low with only approximately 23% of women undergoing treatment becoming pregnant (109). Incidences of multiple pregnancies are increased in patients receiving ART and as a consequence, children conceived through ART are more likely to be born premature and with a low birth weight (<2500g) (110, 111). A recent comprehensive review of the data currently available on postnatal development of children conceived via ART concluded that the combination of the low birth weight and the inherent defects in parental gametogenesis were the most likely cause of any altered postnatal development, and that children born after ART were healthy and developmentally similar to children naturally conceived (110). However, as the oldest person born from ART is still under the age of 30, it is difficult to gauge how long-term health and development may be affected. Indeed, the data from numerous animal studies has shown that the physiological consequences arising from altered embryo development may not manifest themselves till adulthood (20, 112). It will therefore be of particular interest to follow the long term health and development of children conceived via ART. As well as this, we feel that certain aspects of postnatal physiology are being

under-investigated. Data from our laboratory has demonstrated consistently that cardiovascular function is perturbed in mice and rats derived from embryos which have experienced altered rates of preimplantation embryo development (5, 20, 113). However, in studies examining the postnatal development of ART children, the main emphasis has been upon neurological development, rates of surgical interventions and growth rather than cardiovascular health (112).

THE SEARCH FOR MECHANISMS

From the evidence presented above, environmentally-induced changes in embryos leading to altered developmental potential associate with a broad range of outcomes affecting gene expression, cell proliferation, embryo/fetal growth, metabolic and cardiovascular physiology, and neurological criteria, especially behaviour. This breadth is indicative of multiple and interacting mechanisms at molecular, cellular and systems levels (1). An enduring requirement is the cellular heritability and longevity of the response activated at the onset of development yet persisting into adult life. The fundamental mediator of cellular heritability in this context appears to reside in the epigenetic status of embryos and how this may be vulnerable to environmental conditions. The term epigenetics in the modern sense refers to heritable changes in gene expression without alterations of DNA sequence, mediated by altered methylation of the DNA and remodeling of chromatin (114). DNA modifications in mammals occur as cytosine methylation at CpG dinucleotides. While most CpGs are methylated throughout the genome, CpGs in gene regulatory elements can be differentially methylated as an epigenetic modification. In addition, the core histones forming the nucleosome can be subject to a vast number of posttranslational

modifications such as methylation, acetylation, ubiquitylation, phosphorylation, sumoylation and deimination of specific amino acid residues, affecting accessibility and transcriptional activity of chromatin (recently reviewed in (115). DNA methylation marks as well as histone modification marks are thought to interact and confer heritability while allowing for plasticity and reversibility if necessary. One of the best known examples for an epigenetic mechanism is the regulation of mammalian imprinted genes with roles in growth regulation during pregnancy and beyond. These genes exhibit parent-of-origin-specific monoallelic expression, such that growth-promoting genes are expressed exclusively from the paternal allele, whereas for growth-inhibiting genes only the maternal allele is active.

Together with gametogenesis, preimplantation development is one of two periods of dynamic epigenetic reprogramming during the mammalian life cycle, as recently reviewed by Morgan et al. (116). This period starts with fertilisation where the paternal chromatin sees the sperm protamines replaced by acetylated histones (117, 118), followed by active DNA demethylation, which however spares paternally methylated imprinted loci (119), intracisternal A particle (IAP) transposons (120) and heterochromatic centromere regions (118, 121). The new histones recruited to the male pronuclear DNA carry a high level of acetylation marks compared to the maternal chromatin, whereas other histone modifications are present in the female pronucleus early on, but are only gradually acquired during male pronuclear development. Following syngamy, through the early cleavage divisions, there is a loss of genome methylation, apparently by a passive mechanism. However this excludes imprinted alleles which retain their status. In the ICM this is followed by extensive remethylation from the morula stage onward, likely mediated by the *de novo* DNA methylase Dnmt3b which is detectable in the ICM but not in the trophectoderm (122).

In marked contrast, the placental DNA remains close to the undermethylated ground state created by the combination of active and passive demethylation. We will next consider the evidence why epigenetics may be at the heart of environment-induced embryo programming.

ROLE FOR EPIGENETICS IN EMBRYO LINEAGE ALLOCATION

The first lineage allocations in mouse take place early in development before implantation and are under genetic control, as reviewed elsewhere (123). It is thought that the first blastomeres represent totipotent precursor cells. These will undergo lineage decisions based on antagonisms between transcription factor genes. In the first decision, morula ICM (inner cell mass) cells are characterised by continued *Oct4* expression, whereas the outer cells express *Cdx2* and become trophectoderm. A second lineage separation takes place in the blastocyst ICM between epiblast cells expressing *Nanog*, and the cells facing the blastocoel which express *Gata6* and become primitive endoderm. This might be taken as evidence that perturbations of gene expression patterns during the preimplantation period, possibly acting through epigenetic mechanisms, could lead to changes in lineage allocation and altered developmental trajectories for the embryo and extraembryonic tissues. A recent report reinforced this notion and focused attention to the very first days of mammalian development (124). As early as the four cell stage, the authors demonstrated differential histone modification between blastomeres, which was predictive of their respective lineage contributions. Overexpression of the implicated histone methylase in individual blastomeres induced expression of specific transcription factors concomitant with certain lineage allocations. These data implicate the precompaction

stage of mammalian development in lineage allocation by epigenetic mechanisms, and thus as a window of development potentially sensitive to environmental cues or noxious influences.

SUBOPTIMAL IN VITRO CULTURE INDUCES EPIGENETIC CHANGES AT IMPRINTED GENES.

There is growing evidence that embryo culture conditions induce changes in epigenetic status. As outlined above, LOS in sheep and cattle has been linked to fetal calf serum exposure during embryo culture. The timing of this exposure and the similarities between LOS and human imprinting disorders (such as Beckwith-Wiedemann syndrome) implicated epigenetic modifications of genes with functions in growth and development (125). Indeed, LOS sheep fetuses after embryo culture had reduced DNA methylation and expression of the normally active maternal allele of the IGF2 receptor gene (126). This is consistent with the overgrowth of *Igf2r* knockout mice (127, 128) and repressed *Igf2r* expression upon loss of maternal methylation in *Dnmt1* knockout mice (129). When different time windows were tested for the impact of serum exposure on later fetal growth, the highest effect of culture with serum was seen before embryo compaction, implicating the precompaction phase of development as particularly sensitive to changes in embryo environment (86). Imprinted gene loci normally escape the genome-wide demethylation during early cleavage divisions, retaining their germline imprints, but it is not clear what DNA/chromatin marks are required for this or which Dnmt sustains methylation of these loci. Whatever the mechanism by which imprinted loci retain their correct status, it appears to be disturbed by serum exposure during the first cleavage divisions in ruminants.

Several studies were conducted to determine the effects of serum on rodent embryos. Preimplantation exposure to FCS caused mouse fetuses derived by uterine transfer to express decreased levels of the imprinted *Igf2* and *H19* gene, concomitant with increased DNA methylation at the imprinting control region (130). There were also effects on imprinted growth factor binding genes: *Grb10* expression was increased, while *Grb7* expression was lowered. In another mouse study (19), embryos harvested from superovulated females were cultured from the one cell stage in KSOM containing either 10% serum or 1 g/l BSA (control). The serum-cultured group showed abnormalities in imprinted gene expression by the blastocyst stage: maternally expressed *Grb10/Meg1* and paternally expressed *Igf2* and *Mest/Peg1* mRNA were all reduced with no significant difference in *H19*. Notably, and similar to the sheep LOS syndrome data, the period of exposure to serum included cleavage divisions of the early blastomeres, and when such blastocysts were allowed to develop following uterine transfer, a pattern of long-term abnormalities was evident including increased body weight, changed organ allometry, and anxiety-, memory- and locomotor-related abnormalities (19).

Several studies have found altered imprinted gene expression in response to embryo culture parameters other than serum exposure. Mouse blastocysts cultured in Whitten's medium showed aberrant *H19* expression from the normally silent paternal allele concomitant with a loss of DNA methylation at the upstream imprinting control region, whereas *H19* expression and methylation were more in-vivo-like upon culture in KSOM with amino acids (131). Further, this loss of *H19* imprinting induced by culture in Whitten's medium was shown to persist in the fetus, whereas placenta expression from the normally silent allele was demonstrated for a number of genes, namely *H19*, *Ascl2*, *Snrpn*, *Peg3*, *Xist* (132).

In a recent study to investigate the effects of embryo culture conditions frequently used in human IVF, IVF-derived Mus musculus/Mus spretus F1 hybrid embryos, when cultured in human tubal fluid, exhibited abnormal *H19* imprinting concomitant with abormal DNA methylation at a CTCF binding site in the imprinting control region, indicative of the acquisition of a paternal methylation pattern by the normally unmethylated maternal allele (133). This was accompanied by altered histone methylation marks on both alleles at the same CTCF binding site, evidence for a link of abnormal DNA methylation and histone methylation in the aberrant imprinting pattern on the maternal allele caused by the culture conditions. Another example of potentially altered epigenetic status of embryos in response to culture conditions concerns changes in H19 gene expression in blastocysts and longer term fetal abnormalities associated with ammonium build up from amino acid break down (74, 134).

ART AND IMPRINTING DISORDERS

As recently reviewed elsewhere (135), there are a number of studies pertinent to the relationship between ART procedures and the incidence of imprinting disorders. There have been reports on individual ICSI-conceived children with Angelmann Syndrome (AS) where an imprinting defect was demonstrated (136, 137). Likewise, there have been reports of IVF and ICSI children with Beckwith-Wiedemann Syndrome (BWS), albeit without analysis of the underlying defect (reviewed in (138). A number of patient-based studies of the prevalence of BWS, as well as a large case control study have found a three to nine fold increase in ART children (139-142). It should be noted that the underlying defect in the AS as well as BWS cases is hypomethylation of the maternal alleles' imprinting control region. Thus, even though

the risk of an imprinting disorder after ART is very low and not cause for alarm, its increase compared to conventionally conceived children is compatible with an adverse effect of preimplantation manipulation and culture on proper establishment of epigenetic marks.

EVIDENCE FOR EPIGENETIC EFFECTS OF PROTEIN RESTRICTED DIET

Persistent and gene-specific epigenetic changes in response to maternal diet were found in a study on rats (143). When female pregnant rats were fed a protein restricted diet throughout pregnancy (including preimplantation stages) this caused reduced DNA methylation of the peroxisomal proliferator activated receptor (PPAR) alpha and Glucocorticoid receptor (GR) genes and increased their expression in the offspring post-weaning; these effects could be abolished by folic acid supplementation of the protein-restricted diet. Further, GR hepatic promoter showed changed histone marks consistent with transcriptional activation in addition to DNA hypomethylation. Intriguingly, DNA methyltransferase 1 (*Dnmt1*) was implicated in these epigenetic changes because its expression was significantly reduced and its variation could to a large degree explain variation in GR promoter methylation (144). As the maternal protein restriction used extended throughout the entire pregnancy, it is tempting to speculate whether protein restriction during the preimplantation period would be sufficient to induce permanent alterations to Dnmt expression.

In this context, maternal low protein diet treatment to rats exclusively during preimplantation development leading to postnatal phenotypic changes (5) may include an epigenetic mechanism. Thus, this treatment resulted in male blastocysts showing reduced *H19* expression levels, which was mirrored by reduced *H19* and *Igf2*

expression in male fetal livers (6). Importantly, extension of the maternal protein restriction beyond the preimplantation stage abolished the downregulation of *H19* and *Igf2* in fetal liver, indicating that the blastocyst's early response is an appropriate one when protein restriction continues.

In order to understand the mechanisms of preimplantation maladaptive programming during the preimplantation window of sensitivity, it is imperative to investigate its molecular basis, for instance at the level of signalling, gene expression and altered epigenetic modifications in the embryo. Furthermore, we must unravel how the pertinent subsequent phenotypic effects become manifest in the fetus itself and its supply systems (including cross talk between fetus, yolk sac and placenta) from a physiological, endocrine and metabolic perspective.

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Figure captions

Figure 1. Diagram representing potential interactions of the preimplantation embryo with the environment, either in vivo or in vitro, the short term responses, and the long term consequences induced.