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REVIEW

Impact of the periconceptional environment on the programming of adult disease

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The periconceptional period of mammalian development has been identified as an early 'developmental window' during which environmental conditions may influence the pattern of future growth and physiology. Studies in humans and animal models have revealed that factors such as maternal nutritional status or *in vitro* culture and manipulation of developing gametes and preimplantation embryos can impact upon the long-term health and physiology of the offspring. However, the mechanisms involved in the programming of adult disease in response to altered periconceptional development require increased investigation. The role of epigenetic modifications to DNA and chromatin organisation has been identified as a likely mechanism through which environmental perturbations can affect gene expression patterns resulting in phenotypic change. This study will highlight the sensitivity of two critical stages in early mammalian development, gametogenesis and preimplantation development. We will detail how changes to the immediate environment can not only impact upon developmental processes taking place at that time, but can also affect long-term aspects of offspring health and physiology. We will also discuss the emerging role of epigenetics as a mechanistic link between the environment and the later phenotype of the developing organism.

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Introduction

It is now well established that the risk of developing cardiovascular and metabolic disease in adulthood may stem not only from lifestyle factors and genetic predisposition, but can also be attributed to the nature of development *in utero*. Retrospective epidemiological observations made by Barker and colleagues in the late 1980s identified a causal association between sub-optimal foetal growth and an increased propensity for cardiovascular disease in later life.[1,2] Since then, similar observations have been reported from several large independent human cohort studies from around the world,[3–6] identifying a link between sub-optimal foetal growth and increased risk of developing type 2 diabetes, hypertension, elevated cholesterol and cardiovascular disease in adulthood. Interestingly, studies have revealed both short and long term effects on development, dependent on the timing and nature of the altered intrauterine environments. Analysis of women who became pregnant during the Dutch Hunger Winter, a period of 5 months famine in the winter of 1944–1945 in Amsterdam, revealed that maternal undernutrition during embryonic or early foetal development induced an enhanced risk of coronary heart disease, increased body mass index, and glucose intolerance in their offspring.[3,7,8] However, mothers who became pregnant prior to the famine and experienced malnutrition during the later stages of pregnancy gave birth to children of reduced birth weights but who then became obese in adulthood.[8,9] Similarly, protein-derived energy levels during early pregnancy have been positively associated with placental and birth weights,[10] whilst low maternal dietary protein intake during late pregnancy results in babies that are thinner at birth.[11]

The use of animal models provides an invaluable tool with which it is possible to precisely control and target maternal nutrition to specific gestational periods, given the obvious barriers to such studies in humans. Numerous animal studies have demonstrated that the earliest stages of development display particular sensitivity to the effects of altered maternal nutrition, response to which may influence the pattern of future growth and physiology via several developmental pathways. Early gestational nutrient restriction in sheep alters cardiovascular function and circulating cortisol levels in the foetus,[12,13] and results in the hypertrophy of the cardiac muscle in adult offspring.[9,14,15] Our own studies have revealed that whilst maternal low protein diet (LPD) given throughout gestation in the mouse induces hypertension within the adult offspring, targeting the LPD exposure exclusively to the preimplantation period exacerbates the effect, inducing a spectrum of alterations in postnatal cardiovascular and behavioural phenotype.[16] Similarly, in the rat model, maternal protein restriction exclusive to the preimplantation period is sufficient to cause adult hypertension.[17] In response to studies like these, attention is now beginning to focus on understanding the molecular mechanisms by which environmental conditions can affect long-term development, such as epigenetic change within the genome of the developing offspring.

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Epigenetics and developmental programming

Epigenetic modifications result in specific packaging of chromatin to generate either a transcriptionally permissive or repressed state at a given locus, which can vary in a temporal or tissue-specific manner. As such, these modifications can influence the gene expression status of specific regions of DNA, without altering the underlying nucleotide sequence, thus providing a causal mechanism linking a single genotype with multiple phenotypic outcomes.[18] In mammals, these modifications include the methylation of DNA at cytosine residues in CpG dinucleotides, addition of methyl, phospho or acetyl groups to specific residues within histone tails and the recruitment of multiprotein chromatin remodelling complexes.[19] During both gametogenesis and preimplantation development the mammalian genome undergoes an extensive reorganisation of epigenetic marks, in order to establish the appropriate patterns for differentiation and tissue-specific gene expression.[20] Investigations into environmentally induced epigenetic changes during development frequently focus on imprinted genes, wherein parent-specific expression is achieved by epigenetic silencing of one allele and monoallelic expression subsequently occurs from the active allele. However, genes expressed in a tissue-specific manner and regions of non-coding repetitive DNA, both of which are frequently epigenetically modified, are just as likely to be influenced by environmental factors during development.[21] *In vitro* or *in vivo* environmental conditions may feasibly impact on the epigenetic remodelling which takes place during germline and early embryonic development, thus altering the methylation status of gene promoters and potentially resulting in downstream alterations in their expression and implications for offspring long-term health.[22,23]

Gametogenesis

Mammalian gametogenesis is a highly orchestrated series of events, resulting in the differentiation and production of highly specialized, developmentally competent haploid gametes. However, despite the critical function of gametogenesis in reproduction and development, the mechanisms regulating this process are at present poorly defined. Human and animal gametes are routinely subjected to non-physiological conditions both *in vitro* and *in vivo*, despite our limited knowledge about their sensitivity to such manipulations and the potential long-term impacts on the resultant offspring.

Mammalian oogenesis has been shown to display sensitivity to its immediate environment, such that gene expression patterns within the ovary,[24] oocyte[25] and resultant blastocysts[26] can be altered. Both *in vitro* culture and suboptimal maternal diet during oocyte development and maturation have been shown to impact on embryo development[27] and the future health of offspring in several species.[28–30] In domestic animals, follicular growth and oocyte quality have been shown to be altered by a range of dietary manipulations.[31] Follicle morphology, number and the composition of follicular fluid in ewes and heifers are all affected by maternal dietary energy levels.[32,33] Increased intake of dietary protein and urea elevate ammonia levels in follicular fluid and lead to reduced blastocyst development.[34,35] In mice, significantly decreased cell numbers were observed in the blastocyst inner cell mass (ICM) after feeding the females with diets of high or low in protein for several weeks prior to fertilization.[36] These morphological changes occurred in unison with elevated markers of metabolic stress in high protein diet embryos, whilst an apparent reduction of metabolic activity was observed in LPD embryos.[36] Such changes may have far-reaching consequences for the developing offspring. Maternal periconceptional undernutrition in the sheep increases foetal blood pressure, alters growth dynamics of the fetus and placenta and increases the activation of the foetal hypothalamic-pituitary-adrenal axis.[37–40] Feeding sheep diets deficient in B vitamins and methionine, which are essential factors for the methyl cycle, during the periconceptional period resulted in offspring who became heavier and fatter than controls, displayed altered immune responses, became insulin-resistant and had elevated blood pressure.[29] These authors also reported wide spread changes in methylation status of the foetal liver genome, demonstrating the interaction between maternal environment and epigenetic modification.[29] While the findings of such studies have resulted from dietary intervention for several weeks preceding conception, the window of oocyte sensitivity has been refined even further to encompass just the final stages of maturation with the demonstration that maternal LPD provided for just 3.5 days prior to fertilisation resulted in offspring displaying elevated blood pressure, impaired vascular responsiveness, altered anxiety-related behaviour and changes in kidney glomerular numbers.[30]

One manipulation routinely used in human assisted reproduction and in domestic animal production and conservation, as well as in experimental settings, is the process of superovulation. Large doses of hormones are used to induce the maturation and ovulation of multiple oocytes at the same time. However, this practice has been reported to affect the developmental competence of the embryo in mice,[41] as well as leading to the appearance of abnormal DNA methylation patterns within embryonic and foetal DNA,[42] and altered DNA methylation patterns in the sperm of male offspring, with subsequent transgenerational inheritance.[43] The establishment of appropriate methylation patterns at imprinted gene loci takes place during meiosis I in the growing oocyte,[44] thus the premature recruitment of oocytes by superovulation may result in incomplete establishment of genomic imprints and subsequent alterations in allelic expression of these genes. Temporal differences have been reported in the timing of DNA methylation establishment both at maternally imprinted genes during oocyte maturation,[45] as well as at paternally imprinted genes during different stages of spermatogenesis,[46] thus the specific timing of adverse environmental stimuli may affect different genes and result in varied phenotypic outcomes. *In vitro* maturation of mouse, sheep and human

oocytes has also been shown to reduce viability and developmental competence,[47–50] and has been shown to alter foetal growth,[48] again likely due to altering the temporal dynamics of the maturation process and the exposure to non-physiological culture conditions.

Less attention has been focused on the environmental sensitivity of spermatogenesis, however, several studies have shown adverse effects of *in vitro* and *in vivo* environments on sperm motility,[51,52] DNA breakage[53] and apoptosis.[54] These changes have been further associated with alterations in DNA methyltransferase expression and transgenerational inheritance of altered phenotype.[54,55] The impact of maternal gestational nutritional status has also been highlighted as being fundamental for successful spermatogenesis. In rats, maternal dietary vitamin B_{12} deficiency results in defects of the testis development and spermatogenesis in male offspring.[56] The subsequent feeding of a diet sufficient in B_{12} to male offspring, following exposure to a maternal vitamin B_{12} deficient diet, did lead to some recovery in seminiferous tubule development and spermatogenesis, although still not comparable to control animals.[56] *In vitro* organ culture experiments have also demonstrated specific windows of oestrogen-sensitivity in rat testicular development,[57] wherein cell proliferation is reduced specifically in testicular samples taken during foetal but not neonatal development, whilst inhibition of testosterone production is suppressed continually by oestrogen exposure.[57]

The apparent sensitivity of the maturing gametes to environmental factors clearly has implications for the success of embryonic and foetal development, as well as for the health of the resulting adult. However, this sensitivity is certainly not limited to the gametes and post-fertilisation environment may be equally as important to the future phenotype and disease propensity.

Preimplantation embryo development

The relatively short developmental time from fertilisation to implantation encompasses several critical events. These include the first cleavage division, the timing of which can be an indicator of developmental competence;[58] a switch in the regulation of development from maternally stored transcripts and proteins to the activation of the embryonic genome; and embryo compaction at the morula stage which permits the formation of the blastocyst. At the blastocyst stage, the embryo is composed of two distinct cell populations; the outer polarised epithelial trophectoderm (TE) cells and the inner pluripotent non-polar ICM cells. From these two lineages the chorioallantoic placenta and foetal tissues are derived respectively. The establishment of differential DNA methylation and histone tail modifications of the ICM and TE lineages marks a major differentiation landmark in the developing embryo, and the allocation of cells to these lineages can be predicted by the asymmetry of histone modifications within the chromatin of blastomeres during the cleavage stages.[59,60] Manipulation of the extent of DNA methylation in mouse embryonic stem (ES) cells, which are derived from the ICM, has been shown to be capable of influencing cell fate decisions. ES cells deficient in the maintenance DNA methyltransferase, *Dnmt1*, and thus hypomethylated, have been reported to differentiate readily into trophoblast derivatives in association with a loss of methylation at the *Elf5* promoter region.[61] Since *Dnmt1* null embryos fail to develop past E8.5, in association with a failure to differentiate during gastrulation,[62] the importance of establishing appropriate methylation patterns during early embryonic development cannot be underestimated.

During preimplantation development, changing metabolic requirements and sensitivity to the stimulatory effects of essential and non essential amino acids, as well as the expression of different growth factors and their receptors, occur in a spatial and temporal fashion.[63–65] The maternal reproductive tract responds to these changing requirements by providing oviductal and uterine environments that match precisely the needs of the developing embryo,[66,67] with oviductal culture resulting in a embryonic gene expression profile almost identical to that of *in vivo* derived blastocysts[68] and culture in oviductal fluid improving cleavage and blastocysts rates.[69] It is therefore imperative that a better understanding of this intimate maternal-embryo interaction and the implications of manipulating are gained in order to minimise the potential for negative consequences during pregnancy and post-gestational life.

Environmental manipulation in vitro *and* in vivo

In vitro culture and embryo manipulation are widely used for treatment of human infertility, derivation of ES cell lines and the preservation of endangered or important domestic and wild animal species. However, despite advances in these techniques and the development of enhanced culture media, *in vitro* culture conditions still remain relatively sub-optimal in relation to the *in vivo* environment when examining blastocyst developmental rates, ICM and TE cell numbers, gene expression and post-transfer viability.[70–73]

Variations in culture media components have been shown to impact on the developmental potential of mouse embryos *in vitro* in several studies in a strain specific manner.[74] The addition of insulin, insulin-like growth factor-1 and albumin to the media stimulates mouse embryo metabolism, increases the number of cells within the ICM and stimulates foetal growth post transfer.[75–77] Embryo culture in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), when in the absence of albumin, can enhance blastocyst cell numbers and ICM development.[78] The addition of GM-CSF to the culture medium has also been shown to prevent the negative impacts on adverse foetal, placental and postnatal development associated with embryo culture and transfer and to increase the surface area of placental trophoblast available for nutrient exchange.[79] Changes in metabolic and signalling activity in response to *in vivo* or *in vitro* environments are more than likely to be accompanied by

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changes in gene expression patterns. Comparison of bovine blastocysts derived from *in vitro* or *in vivo* environments have revealed that culture induces significant changes in transcript levels of genes involved in a wide array of key developmental pathways such as transcription and translation, cellular metabolism,[69] nutrient transport, apoptosis and gap junction formation.[80] Interestingly, these changes occur in a temporal fashion,[81,82] indicating that specific pathways may vary in the timing of their sensitivity to the environment. Survival rates following embryo transfer have also been shown to be compromised following the culture or manipulation of bovine embryos.[74,83] The bovine endometrium has been shown to display a plasticity in its gene expression patterns in response to embryos derived from somatic-cell-nuclear-transfer (SCNT) or *in vitro* fertilisation (IVF),[83] suggesting that the influence of the *in vitro* culture environment may persist even after the culture period. Furthermore, the inclusion of non-physiological levels of some nutrients, such as methyl-folate cycle cofactors, may affect the establishment and maintenance of epigenetic modifications such as DNA methylation during embryo culture[84] with consequences likely to extend into foetal and adult life.

There is now increasing evidence that the effects of *in vitro* culture and embryo manipulation do in fact extend far beyond preimplantation and foetal development, altering perinatal and adult development. In cattle and sheep, the phenomenon of large offspring syndrome has been recorded following a range of embryo environment manipulations, including *in vitro* culture in the presence of serum, cloning by SCNT, exposure to a maternal uterine environment high in progesterone, and granulosa cell co-culture.[85–87] As a result a range of foetal and postnatal consequences including enhanced foetal growth and weight at birth, alterations in organ sizing and elevated rates of sudden perinatal death have been reported.[85,88,89] Studies from our own laboratory have demonstrated that both prolonged (two-cell to blastocyst) or relatively short-term (approximately 2 h immediately prior to blastocyst transfer) periods of *in vitro* culture significantly elevated offspring postnatal systolic blood pressure.[72] Significant changes in the activity of key homoeostatic enzymes regulating both cardiovascular (serum angiotensin converting enzyme) and metabolic (hepatic phosphoenolpyruvate carboxykinase; PEPCK) pathways were also observed in female offspring.[72] Significant changes in postnatal memory and growth profiles have also been demonstrated following the *in vitro* culture and transfer of mouse embryos.[70,90] Recently, it has been demonstrated that the effects of embryo culture can even perpetuate into a second generation. Significant changes in postnatal growth and organ sizes following mouse embryo culture and transfer were transmitted to the F2 generation, despite no further manipulations being performed.[91] This suggests that a 'memory' of the environmental change experienced by the F0 dam is retained in the genome of the offspring, likely by an epigenetic change within the germline.

With approximately 1%–3% of births in developed countries involving some form of assisted reproduction technology (ART),[92] an increasing number of long-term follow-up studies into the health and development of ART children are being conducted. Increased incidences of prematurity and low birth weight have been widely associated with ART, but often have been attributed to a higher frequency of multiple births following the transfer of several embryos.[93,94] However, there is evidence to suggest that even singleton ART children are at risk from preterm birth and low birth weight.[95] Some studies have reported slight but non-significant specific neuro-developmental conditions in ART children[96–98] and several studies have shown an association between IVF and an increased incidence of cerebral palsy.[99,100] Additionally, elevated systolic and diastolic blood pressure, and elevated fasting glucose levels have been reported in pre-pubertal IVF children, independent of early life factors or parental characteristics.[101]

In humans, the use of ART has also been associated with increased incidence of several imprinting disorders, including Beckwith-Wiedemann syndrome, Angelman syndrome and Silver-Russel syndrome.[95] Abnormal DNA methylation profiles at a cluster of imprinted genes on human chromosome 11p15.5, in association with loss of imprinted mono-allelic expression, have been reported to associate with the incidence of these syndromes.[95] However, a recent study has shown altered methylation status of the KvDMR imprinting control region (ICR) at this locus even in clinically normal children after assisted conception.[102] Investigation into the impact of ICR hypomethylation on gene expression in these children would be needed to identify a functional outcome of the altered methylation status, and to tease out the possible role of specific *in vitro* manipulations. Importantly, imprinted genes are not the only genomic loci that demonstrate epigenetic sensitivity during early development.[103] The *Agouti viable yellow* (A^{vy}) allele in mice is one example whereby a repetitive element situated within a gene (in this case controlling coat colour) can be regulated by DNA methylation. Environmental conditions, such as diet or embryo culture, have been used to experimentally alter the methylation status of this locus,[104,105] providing a visual read-out of methylation change via the coat colour of the mouse. *In vitro* mouse embryo manipulations have been shown to reduce methylation at the A^{vy} locus, shifting coat colours from pseudo-agouti (brown) to mottled or yellow coats, with a significant increase in proportion of animals with yellow or mottled coats observed with increasing duration/extent of manipulation.[104] Maternal dietary manipulation can also affect the methylation status at the A^{vy} locus. Supplementation of maternal diet with methyl cycle donors and cofactors, including choline, folic acid and vitamin B_{12}, increases methylation levels resulting in a coat colour shift in offspring towards the pseudo-agouti brown associated with CpG methylation, and thus silencing, of the IAP repetitive element within the A^{vy} gene.[105]

The long-term impact of maternal nutrition during preimplantation development on the programming of offspring development has been extensively covered in recent studies.[55,106,107] The large majority of studies examining the role

Developmental Stage	Environmental Conditions	Associated Adverse Outcomes
Spermatogenesis 	• Smoking • Pollution / environmental Oestrogens • Paternal diet • Grand-maternal diet during Primordial germ cell (PGC) specification	• Impaired semen quality (e.g. motility, morphology, DNA fragmentation) - may lead to reduced fertilisation competence • Altered expression of imprinted genes • Altered sperm DNA methylation patterns. • Defects in testicular development and spermatogenesis
Oogenesis 	• Maternal diet • Superovulation • *In vitro* maturation (IVM) • Grand-maternal diet during primordial germ cell (PGC) specification	• Altered sperm DNA methylation in male offspring. • Increased activation of the fetal hypothalamic-pituitary-adrenal axis • Elevated postnatal blood pressure and cardiovascular risk factors • Postnatal behavioural changes
Pre-Implantation Development 	• *In vitro* culture / embryo transfer Blastomere biopsy for preimplantation genetic diagnosis • Maternal diet • Maternal physiology (e.g. obesity / diabetes)	• Reduced blastocyst cell number (influencing lineage allocation?) • Altered gene expression patterns • Altered metabolism • Reduced viability • Altered fetal / postnatal growth and organ sizing • Adult obesity • Elevated postnatal blood pressure and Cardiovascular risk factors • Behavioural changes • Genome-wide changes in epigenetic status
Post-Implantation and Fetal Development 	• Maternal diet • Maternal physiology (e.g. obesity / diabetes) • Administration of synthetic glucocorticoids • Placental physiology • Maternal infection	• Altered transport of nutrients, gases and waste products • Altered gene expression patterns • Altered fetal and postnatal growth and organ sizing • Adult Obesity • Elevated blood pressure and Cardiovascular risk factors • Genome-wide changes in epigenetic status • Postnatal behavioural changes

Fig. 1. (Colour online) A summary diagram detailing the impact of environmental conditions on gametogenesis and development, and the short- and long term consequences which may be observed.

of maternal diet and its effects on subsequent foetal and postnatal health, have centred on the use of diets altered for protein content, and have typically been coupled with the use of rodent models. In studies from our laboratory, maternal LPD diet targeted exclusively to the rodent preimplantation period results in a range of embryonic, foetal and postnatal consequences, including altered blastocyst cell numbers, altered foetal liver gene expression of 11β-hydroxysteroid dehydrogenase type 1 (Hsd11b1) and phosphoenolpyruvate carboxykinase (Pepck, Pck1), postnatal hypertension increased patterns of anxiety-related behaviour in an open field test, and alter the relative sizes of organs.[16,108,109] Interestingly, we further identified a compensatory response mediated by the visceral yolk sac in response to maternal gestational LPD. We observed that endocytosis of maternal proteins mainly via the multi-ligand megalin low-density lipoprotein receptor-related protein 2 receptor was increased in response to LPD.[16] This elevation of yolk sac histiotrophic function appeared subject to developmental plasticity, providing a mechanism by which embryos could respond to maternal undernutrition in order to protect foetal growth and postnatal competive fitness. In the sheep, maternal undernutrition from days 0–30/31 of pregnancy increases circulating plasma cortisol levels in female lambs and altered cardiovascular function of lambs at 1 year of age.[12,13]

Conclusions

There is now a growing body of evidence that factors such as maternal nutrition, hormonal imbalance, *in vitro* culture

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conditions and other environmental factors can have a profound effect on gamete quality and subsequent embryo development (Fig. 1). This has clear implications and relevance for the *Developmental Origins of Health and Disease* hypothesis. It is also becoming increasingly apparent that changes to the normal pattern of early development can result in long-term changes in offspring health and physiology. It is therefore imperative that a better understanding of the underlying mechanisms and signalling pathways involved is established. Whilst the mechanisms and pathways involved in postnatal programming of adult health following adverse preimplantation embryo environments are likely to be complex and involve several interacting pathways, epigenetic changes occurring during germline and preimplantation development may be one critical mechanism through which the early-life environment can affect subsequent disease susceptibility, and even result in the transmission of environmentally induced phenotypic change between generations. However, further studies are still required for a better understanding of how the mammalian embryo responds to its immediate environment and the potential long-term programming effects that can occur. Whilst our understanding of certain physiological systems are well characterised (cardiovascular), areas such as digestive function, the immune system and cognative functions are understood to a lesser extent. It is also essential that further development of existing animals models occur if we are to define the limits of the early mammalian embryo, and design improved conditions for the culture and manipulation of human embryos. As most ART children are still relatively young, a wider analysis of postnatal phenotype focusing on cardiovascular and metabolic profiles, as well as substantiation of those effects already well characterised from animal studies is required in these children.

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