Dietary regulation of developmental programming in ruminants: epigenetic modifications in the germline

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Ruminants have been utilised extensively to investigate the developmental origins of health and disease, with the sheep serving as the model species of choice to complement dietary studies in the rat and mouse. Surprisingly few studies, however, have investigated delayed effects of maternal undernutrition during pregnancy on adult offspring health and a consistent phenotype, together with underlying mechanistic pathways, has not emerged. Nevertheless, when broad consideration is given to all studies with ruminants it is apparent that interventions that are initiated very early in gestation, and/or prior to conception, lead to greater effects on adult physiology than those that are specifically targeted to late gestation. Effects induced following dietary interventions at the earliest stages of mammalian development have been shown to arise as a consequence of alterations to key epigenetic processes that occur in germ cells and pluripotent embryonic cells. Currently, our understanding of epigenetic programming in the germline is greatest for the mouse, and is considered in detail in this article together with what is known in ruminants. This species imbalance, however, looks set to change as fully annotated genomic maps are developed for domesticated large animal species, and with the advent of 'next-generation' DNA sequencing technologies that have the power to globally map the epigenome at single-base-pair resolution. These developments would help to address such issues as sexually dimorphic epigenetic alterations to DNA methylation that have been found to arise following dietary restrictions during the peri-conceptional period, the effects of paternal nutritional status on epigenetic programming through the germline, and transgenerational studies where, in future, greater emphasis in domesticated ruminants should be placed on traits of agricultural importance.

Introduction

Ruminant species (predominantly sheep) have been used in what we now term a 'developmental programming' context since early in the 20th century. Indeed, in the first issue of The Proceedings of the Nutrition Society a paper was presented on 'The Influence of Diet on Pregnancy and Lactation in the Mother, the Growth and Viability of the Foetus, and Post-Natal Development'

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(Garry, 1944). At that time, the emphasis was on productive traits as influenced by maternal diet; that is, how to maximise the efficiency of meat production before, during and after the Great Wars. Slightly earlier, Joseph Barcroft was interested in how organs metabolised and handled gases in solution, particularly oxygen, and this led him to utilise pregnant ruminants, first the goat (Barcroft *et al.* 1934) and then the sheep (Barcroft & Barron 1936) to investigate aspects of fetal physiology; being a unique gaseous (hypoxic) environment. Since that time, thanks largely to the collective efforts of scientists such J Hammond, LR Wallace, R McCance & E Widdowson and, more recently, JJ Robinson, the pregnant ruminant has been extensively utilised as a model to investigate fetal physiology and responses to a variety of nutritional and non-nutritional stimuli.

Maternal diet and developmental programming in ruminants

Central to the current thesis are the ewe's predicted responses to a change in the supply of a variety of dietary micro- and macro-nutrients, which can influence maternal body composition at the onset of pregnancy, nutrient partitioning (e.g. amino acids and polyamines; Kwon *et al.*, 2004) and fetal development during pregnancy, and the ewe's subsequent lactational ability and lamb performance. Surprisingly, the first studies in sheep to truly embrace the concept originally proposed by Barker & Osmond (1986), that maternal nutrition during pregnancy could impinge on the long-term well being of offspring, didn't materialise until 11-12 years later, and these studies only considered the impact of variations in maternal diet on aspects of fetal growth and development (Trahair *et al.* 1997; Clarke *et al.* 1998; Gallaher *et al.* 1998; Warnes *et al.* 1998). A PubMed search (accessed May 21st 2010) for the MESH term "maternal nutritional physiological phenomena and sheep" returned 121 papers of which 87 were original contributions (not reviews). Of these, 35 were original research articles which investigated the delayed effects of maternal undernutrition on adult offspring. For example, Bloomfield *et al* (2003) described an association between 10 days severe undernutrition close to term in sheep and a greater pituitary (but not adrenal) response to stress in their offspring at 30 months of age.

After excluding descriptive reports of tissue and sex-specific molecular changes in offspring due to variations in maternal nutrition, there are approximately 17 papers that reported a characterised phenotype in adult offspring (Table 1). The physiological outcomes described within these papers are variable, reflecting (1) the different breeds of sheep used, (2) different nutritional interventions at (3) different gestational time-points and (4) different post-natal ages. Thus, we know that reduced nutrient intake by pregnant dams can influence appetite and tissue energy oxidative capacity (Sebert *et al.* 2009; Jorgensen *et al.* 2009), glucose-insulin metabolism (Gardner *et al.* 2005; Burt *et al.* 2007; Ford *et al.* 2007; Poore *et al.* 2007; Todd *et al.* 2009; Rhodes *et al.* 2009), hypothalamic-pituitary-adrenal axis function (Bloomfield *et al.* 2003; Gardner *et al.* 2006; Chadio *et al.* 2007), cardiovascular and renal function (Gardner *et al.* 2004; Williams *et al.* 2007; Chan *et al.* 2009; Cleal *et al.* 2007; Gopalakrishnan *et al.* 2005), behavioural responses (Dwyer *et al.* 2003; Erhard *et al.* 2004; Hernandez *et al.* 2009) and immune function (Sinclair *et al.* 2007; Eckersall *et al.* 2008). In summarising these studies it is clear that variations in maternal nutrition interact with maternal body composition to alter cellular function within the organs of offspring; for example, corticotrophin/adrenocorticotrophin sensitivity within the pituitary/adrenal with respect to programming of the HPA axis or the density/activity of insulin signalling proteins in peripheral tissues with respect to programming of glucose-insulin dynamics. However, a consistent phenotype has not emerged and whilst the other 18 papers demonstrating descriptive effects invariably infer mechanistic insights to one or other of the physiological systems programmed, a clear mechanistic pathway remains elusive.

Table 1. Studies investigating the effect of maternal nutrient restriction on adult offspring physiological function in sheep

Dietary paradigm	Period of nutrient restriction before or during gestation (days) -60 0 30 80 147	Observed physiological phenotype	Reference
10-15% wt loss (-61 to 30 days)		↓ (10%) glucose tolerance (10mth)	Todd *et al* 2009
10-15% wt loss (-61 to 30 days)		altered behavioural responses	Hernandez *et al* 2009
30% ME (-60 to 7 days)		↑ fetal BP (3-8±2.5 mmHg)	Edwards & McMillen 2002
MD diet (-56 to 6 days)		↓ insulin sensitivity (males) at 2yr ↑ BP (11 ± 2 mmHg)	Sinclair *et al* 2007
30% ME (0 to 30 days)		↑ HPA axis in female *vs.* male	Chadio *et al* 2007
50% ME (1 to 30 days)		↑ adult PP at 1 yr (5 ± 1 mmHg)	Gardner *et al* 2004
50% ME (1 to 30 days)		↓ baroreflex sensitivity to AII	Gardner *et al* 2006
50% ME (1 to 95 days)		↑ adult BP at 3 yr (10 ± 2 mmHg)	Gopalakrishnan *et al* 2004
50% ME (1 to 95 days)		altered behavioural responses	Erhard *et al* 2004
50% ME (28 to 78 days)		↓ (~ 15%) glucose tolerance at 8 mth	Burt *et al* 2007
50% ME (28 to 78 days)		↓ (~ 15%) glucose tolerance at 8 mth	Ford *et al* 2008
50% ME (28 to 80 days)		↓ BP (6 mth , 8 ± 3 mmHg). ↓ (40 ± 12 %) nephron number	Gopalakrishnan *et al* 2005
30% ME (31 to 100 days)		little to no effect	Chadio *et al* 2007
35% ME (28 to 147 days)		↓ ewe-lamb bonding behaviour at birth	Dwyer *et al* 2003
30% ME (65 to 125 days)		↓ immune response to vaccination	Eckersall *et al* 2008
50% ME (110 to 147 days)		↓ (first phase) insulin sensitivity at 1 yr	Gardner *et al* 2005

Key: % ME, percentage reduction in metabolisable energy intake fed to ewes; MD diet, a methyl deficient diet; mth, months; Day 0, conception (147 days in the sheep); BP, blood pressure; PP, pulse pressure; HPA, hypothalamic-pituitary-adrenal axis; yr, year; RAAS, renin-angiotensin-aldosterone axis; GTT, glucose tolerance test; p24, postnatal age in months.

In recent years, a few studies with sheep have experimentally tested the developmental programming hypothesis by combining a post-natal nutritional challenge after exposure to a pre-natal nutritional insult (Table 2). We asked the question whether moderate adult obesity would exacerbate the effects on the offspring of maternal global energy restriction, and tested the hypothesis by longitudinally sampling the offspring in a lean and then obese condition (Rhodes *et al.* 2009). We found little evidence of programming by maternal global energy restriction *per se*, but some support for the hypothesis that development of obesity would reveal a deleterious phenotype (altered glucose-insulin handling), albeit very mild. The only other studies to have used a similar approach, but with an entirely different experimental paradigm (i.e. 2x2 factorial arrangement; prenatal undernutrition [1-31 days gestation] x postnatal undernutrition [pair-fed to achieve 85% growth of control sheep]) found that post-natal intervention had the greater effect on adult physiology (e.g. a 5-6 mm Hg greater increase in mean arterial pressure to Angiotensin II) (Cleal *et al.* 2007)) and a ~ 30% increase in insulin appearance after a glucose tolerance test in male offspring only (Poore *et al.* 2007).

Peri-conceptional diet and developmental programming in ruminants

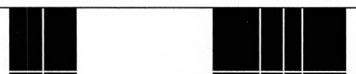
Taken together, *a priori* assumptions about the expected adult phenotype when utilising either postnatal overnutrition (greater deterioration of metabolic control) or postnatal undernutrition (improvement of metabolic control) are not clear cut and further work in this area is warranted. Nevertheless, when broad consideration is given to all studies conducted in ruminants it is apparent that interventions that are initiated very early in gestation (and/or prior to conception) lead to greater effects on adult physiology than those that are specifically targeted to late gestation. This is most evident in the study of Sinclair *et al* (2007) in which dietary methyl group deficiency for 8 weeks before and 6 days into gestation (i.e. embracing the periods of oocyte growth and maturation, and pre-implantation embryo development) revealed a 11 mm Hg increase in mean arterial pressure and a 30% increase in the pressor response to infused angiotensin II in two-year-old male offspring. Responses such as this at the earliest stages of mammalian development hint at the importance of key epigenetic processes that occur in germ cells and pluripotent embryonic cells, and that subsequently determine offspring health.

The present article, therefore, will provide a contemporary overview of these molecular processes drawing, where necessary, on information acquired from mice and other mammalian species, before reviewing our current state of knowledge with respect to ruminants. Finally, current limitations and outstanding issues are indentified, and consideration given to future priorities for research funding.

DNA methylation programming in the germline

The aforementioned processes of development are regulated in a temporal and spatial manner by a series of carefully orchestrated alterations to the transcriptome which arise as a consequence of covalent modifications to DNA and associated histone proteins that act in concert with chromatin structure in a cell-lineage specific manner. Over the last 20 years there has been an exponential increase in activity in the field of epigenetics and mammalian development with more than 7000 research articles and 2000 reviews dedicated to the topic. For a contemporary overview of some of the broader aspects of this area the reader is directed elsewhere (Reik, 2007; Petronis, 2010). Instead, attention in the current article is directed towards epigenetic programming in the germline, with consideration given to covalent modifications to components of the nucleosome (the functional subunit of chromatin) and, in particular, to DNA.

Table 2. Studies investigating variations in dam and lamb nutrition on adult offspring physiological function in sheep.

Dietary paradigm	Period of nutrient restriction during or after gestation (months) 0 1 T P3 P6 P12 P18 P24		Reference
Dam: 50% ME (0-1 mth) Lamb: 85% growth (3-6mth)		↓ (30%) insulin sensitivity	Poore *et al* 2007
Dam: 50% ME (0-1 mth) Lamb: 85% growth (3-6mth)		↑ RAAS response (5-8mmHg) to furosemide at 2.5yr	Cleal *et al* 2007
Dam: 50% ME (1-2.5 mth) Lamb: Ad lib (3-12 mth)		↓ renal function at 1 yr	Williams *et al* 2007
Dam: 50% ME (1-2.5 mth) Lam: Ad lib (3-12 mth)		↑ BP (10 ± 2 mmHg) at 1 yr	Chan *et al* 2009
Dam: 50% ME (1-2.5 mth) Lamb: Ad lib (3-12 mth)		Subtle effects on energy balance	Sebert *et al* 2009
Dam: 30% ME (2-4.3 mth) Lamb: 1.5M (18-24 mth)		↑ peak insulin to GTT at 1.5yr	Rhodes *et al* 2009
Dam: 50% ME (0-1 mth) Lamb: high-fat (0-6mth)		↓ (40%) mitochondrial activity & VO_{2max}	Jorgensen *et al* 2009

Key: % ME, percentage reduction in metabolisable energy intake fed to ewes; MD diet, a methyl deficient diet; mth, months; T, term (147 days in the sheep); BP, blood pressure; PP, pulse pressure; HPA, hypothalamic-pituitary-adrenal axis; yr, year; RAAS, renin-angiotensin-aldosterone axis; GTT, glucose tolerance test; p24, postnatal age in months.

DNA methylation

DNA methylation involves the covalent addition of a methyl group to the number 5 carbon atom of the cytosine pyrimidine ring and is targeted to CpG dinucleotides which are recognised by DNA methyltransferases (DNMTs) (Goll & Bestor, 2005). Unlike single-copy genes, repeat sequences in the genome are CpG rich and are usually highly methylated. This is thought to confer chromosomal stability and to regulate the extent of recombination, but it also serves to silence transposable elements which constitute around 45% of the human genome (Lander *et al.*, 2001). In contrast, single-copy genes are usually deficient in CpGs, which tend to cluster as 'CpG islands', and co-localise with the promoters of most constitutively expressed genes (Illingworth & Bird, 2009). Around 40% of mammalian genes contain CpG islands in or close to their promoters. Whilst the majority of these are hypomethylated, tissue-specific differences in the extent of CpG island methylation exist. Indeed, it has been estimated that tissue-specific differentially methylated sequences constitute at least 5% of total CpG islands in the genome (Song *et al.*, 2005), and these serve to regulate gene expression, lineage specification and differentiation during development. In the case of imprinted genes CpG islands can, in addition, reside distally to the promoter at so-called differentially methylated (DMR) or imprint control (ICR) regions. However, non-CpG island methylation and indeed non-CpG methylation are known to exist in the mouse and human. Emerging data, using 'next generation' deep sequencing and array based technologies, indicate that the former may represent a more important determinant of tissue-specific gene expression, whereas the latter appears to be specific to embryonic stem cells (Lister *et al.*, 2009). Using comprehensive high-throughput array-based relative methylation (CHARM) analysis, Irizarry *et al.* (2009) found that tissue differentially methylated regions (t-DMR), which are functionally related to gene expression, do not occur in promoters or in CpG islands but in adjacent (i.e. within 2kb) lower density sequences which they termed "CpG island shores". Other recent studies (e.g. Ball *et al.*, 2009; Maunakea *et al.*, 2010) support the notion that t-DMR at non-CpG islands within gene bodies may be more important in regulating highly expressed genes than DNA methylation in 5' CpG island promoters, where covalent modifications to histones may have a more prominent role.

The mouse germline

Much of what we know about changes to DNA methylation in the germline pertains to studies conducted in the mouse (Lees-Murdock and Walsh, 2008; Sanz *et al.*, 2010) with only limited data available for ruminants and other species (discussed later). Briefly, between days 11.5 and 12.5 *post coitum* in the mouse, single copy imprinted and non-imprinted genes, together with repeat sequences, are synchronously demethylated in both male and female genomes. However, in contrast to imprinted and single-copy genes, repeat sequences are only partially demethylated, a process hypothesised to minimise transposition and stabilise chromosome integrity (Hajkova *et al.*, 2002). At the same time X-chromosome reactivation is initiated. This involves a loss of methylation at the DMR regulating X-inactive specific transcript (*Tsix*) and X (inactive)-specific transcript, antisense (*Xist*) expression (Boumil *et al.*, 2006). *De novo* methylation occurs later during gametogenesis and, in contrast to demethylation, occurs asynchronously, both between and within various sequence classes and between male and female genomes. The timing of methylation acquisition in the female gamete coincides with the stage of oocyte growth leading up to the re-initiation of meiosis and is associated with increased expression of genes (i.e. *Dnmt3a*, *Dnmt3b* and *Dnmt3L*) involved in *de novo* methylation (Lucifero *et al.*, 2007).

Epigenetic events during syngamy are again best studied in the mouse where the paternally derived genome is actively (in the absence of DNA replication) demethylated during the first

cell cycle. The identification of a putative demethylase, however, had remained elusive for many years (Ooi & Bestor, 2008). Recently, however, key roles for Activation-Induced cytidine Deaminase (AID) (Popp *et al.*, 2010; Bhutani *et al.*, 2010) and components of the elongator complex (Okada *et al.*, 2010) in paternal DNA demethylation have been demonstrated in mouse primordial germ cells and zygotes, although the precise mechanisms of their action remain to be determined. The maternal pronucleus and paternal ICRs are able to maintain their hypermethylated state during this period due, in part, to the actions of PGC7/Stella (Nakamura *et al.*, 2007), a maternal factor essential for early development, which can protect these regions from demethylation although, once again, the precise mechanism of protection has not been established. The protected state of the maternal genome at syngamy may also be related to the methylation status of lysine residues K9 and K27 on histone H3 which are thought to guard against active demethylation (Santos *et al.*, 2005). In contrast to the paternal genome, the maternal genome is passively demethylated with each cell cycle and round of DNA replication during early pre-implantation development. DNA methylation imprints, however, are maintained. Recent evidence indicates that this is largely achieved through the actions of maternally and zygotically derived Dnmt1, which are speculated to specifically target DMRs by some unknown mechanism (Hirasawa *et al.*, 2008).

By the blastocyst stage global differences in DNA methylation are evident between the extraembryonic and embryonic lineages in the mouse (Morgan *et al.*, 2005). In contrast to the trophectoderm (TE), there is clear evidence of extensive *de novo* methylation within the inner cell mass (ICM). The *de novo* methylase Dnmt3b is known to preferentially localise to the ICM in mouse blastocysts (Watanabe *et al.*, 2002). Also, specific histone modifications such as the aforementioned trimethylation of K9 and K27 on histone H3 are enriched in the ICM relative to the TE (Erhardt *et al.*, 2003). Over-expression of the histone methyltransferase (RMT4/CARM1), that methylates arginine 26 of Histone H3, has been shown to upregulate Nanog and Sox2 expression in individual blastomeres of 4-cell embryos. The expression of these factors is believed to ultimately lead to the preferential allocation of blastomeres to the ICM (Torres-Padilla *et al.*, 2007). Although somewhat controversial, these observations indicate that epigenetic modifications can influence cell fate determination and lineage commitment during the earliest stages of mammalian development.

The ruminant germline

In contrast to the mouse strikingly little is known about epigenetic modifications to DNA and associated proteins during gametogenesis in ruminants. However, broadly in keeping with the mouse, global DNA methylation (determined using a 5-methylcytosine antibody and quantified by fluorescence microscopy) has been shown to increase in growing oocytes from the late pre-antral through to the large-antral stage of follicle development (Russo *et al.*, 2007). As yet, nothing is known about how acquisition of DNA methylation may differ between the various sequence classes.

The process of active demethylation during syngamy, described earlier for the paternal genome in the mouse, appears to differ between species (Fulka *et al.*, 2008). Similar to the mouse, a dramatic loss of cytosine methylation from the male pronucleus is observed in the rat, human and cow, but not in the rabbit and sheep. Through the use of interspecies intracytoplasmic sperm injection, Beaujean *et al.* (2004a) were able to demonstrate that the more extreme loss of paternal DNA methylation in the mouse compared to the sheep pronucleus arises as a consequence of the enhanced resistance of sheep sperm to demethylation and the greater demethylating capacity of the mouse ooplasm. The molecular basis for these differ-

ences is currently not known. In the mouse, histone H3K9 acetylation and methylation status is mechanistically linked to DNA methylation but, although a similar configuration for this histone exists in the sheep zygote, it is not related to the level of DNA methylation in the male pronucleus (Hou *et al.*, 2008). The functional significance of these species differences in demethylation following fertilisation is also unclear. They may merely reflect pre-existing levels of methylation in the male pronucleus at the time of syngamy, which are comparatively low in sheep (Beaujean *et al.*, 2004b).

The subsequent loss of DNA methylation during the early pre-implantation period is also more extreme in the mouse than either the cow or sheep genomes. Indeed, only at the blastocyst stage is demethylation apparent in the sheep TE, whereas cells of the ICM remain methylated (Beaujean *et al.*, 2004b). Therefore, whilst the outcome of methylation differences at the blastocyst stage is similar for the mouse and sheep (hypermethylated ICM and hypomethylated TE), the manner in which these comparable states are reached differs between the species. This may reflect differences in the timing of key developmental stages, such as embryonic transcription activation which, in ruminant species, occurs later than in the mouse at around the 8 to 16-cell stage. Nevertheless, despite these species differences it is evident that sweeping epigenetic changes to both DNA and associated proteins occur through the germline, rendering germ cells and pluripotent embryonic cells vulnerable to environmentally induced epigenetic dysregulation.

Dietary manipulation of epigenetic programming in the germline

With the foregoing discussion in mind it is surprising to note that there has been little attempt to investigate nutritional induced epigenetic programming in the germline. Most studies with ruminants and other species have tended to focus on the epigenetic consequences of procedures used in assisted reproduction, such as ovarian stimulation, intra-cytoplasmic sperm injection and embryo culture (Grace and Sinclair, 2009), or reprogramming during somatic cell nuclear transfer (Niemann *et al.*, 2008). These studies clearly highlight the vulnerability of gametes and pluripotent embryonic cells to environmentally-induced epigenetic dysregulation. Those investigators that have assessed aspects of dietary induced epigenetic programming have tended to focus on the regulation of specific metabolic pathways in rodents exposed to some form of global nutrient restriction throughout *in utero* development (Burdge and Lillycrop, 2010).

Some of our early investigations into the mechanistic basis of the 'Large Offspring Syndrome' (LOS; Sinclair *et al.*, 2000a), however, suggested that the composition of the diet of embryo donor ewes could influence both the incidence and severity of the observed LOS phenotypes. At least in the sheep, the aberrant fetal phenotype is associated with a loss of imprinting and expression of the gene encoding the type 2 insulin-like growth factor receptor (*IGF2R*) which, in turn, arises as a consequence of a loss of methylation on the second intron DMR of the gene (Young *et al.*, 2001). Elevated plasma urea concentrations in zygote-donor ewes in subsequent studies were induced by feeding high-nitrogen diets from the onset of oestrous synchronisation and ovarian stimulation, two weeks prior to AI and zygote recovery. Plasma urea concentrations were negatively correlated to *IGF2R* expression in the fetal heart and kidney which, together with the positive correlation between plasma urea and conceptus mass, confirmed an involvement of nitrogen/urea metabolism in the aetiology of the LOS (Powell *et al.*, 2006).

In cattle we have shown that the feeding of specially formulated diets that lead to elevated plasma ammonium and urea concentrations can impair post-fertilisation development of oocytes (Sinclair *et al.*, 2000b). Related studies in the mouse revealed that elevated ammonium concentrations during culture can alter the expression of the imprinted gene *H19* and impair fetal

development (Lane and Gardner, 2003); although similar levels of ammonium during oocyte growth in a long-term follicle culture system did not alter the methylation status of DMRs in three imprinted genes (*Snrpn*, *Igf2r* and *H19*) in MII oocytes (Anckaert *et al.*, 2009). In the rat, the feeding of a LPD for just the first 4 days of gestation led to post-natal hypertension in male offspring (Kwong *et al.*, 2000), and a reduction in *H19* and *Igf2* transcript expression in fetal male, but not female, livers (Kwong *et al.*, 2006). However, *H19* transcript expression was not associated with altered methylation at its DMR in that study.

Sticking with the theme of imprinting, human subjects who were prenatally exposed to famine during the Dutch Hunger Winter in 1944-45 had, as aged adults, reduced *IGF2* DMR methylation compared with their unexposed, same-sex siblings (Heijmans *et al.*, 2008). Significantly, this only applied to those subjects who were exposed during the peri-conceptional period and not during late gestation. Whilst this epigenetic modification in DNA methylation was not related to transcript expression, these data provide the first evidence in a human population for the epigenetic basis of the developmental origins of adult disease. More recently, peri-conceptional folic acid use was shown to increase the level of *IGF2* DMR methylation of DNA extracted from whole blood of infant children (Steegers-Theunissen *et al.*, 2009). Interestingly, *IGF2R* DMR methylation in that study was correlated with the concentration of S-adenosyl methionine (SAM) in maternal blood.

As an initial step to provide a link between maternal diet and epigenetic alterations to DNA methylation we investigated aspects of the linked methionine-folate cycles in human embryonic stem cells (Steele *et al.*, 2005), somatic cells and oocytes from the bovine ovary, and bovine pre-implantation embryos (Kwong *et al.*, 2010). Analyses revealed that transcripts for most of the enzymes and transporters involved in these cycles were present in all cell types from both species. There were, however, some noticeable similarities and differences both between the two species and between cell types within species. For example, in contrast to cells of the spleen, transcripts for the folate receptor *FOLR2* were absent in human and bovine embryonic cells, and were also not present in oocytes and somatic cells within the bovine ovarian follicle. Methionine-adenosyl transferase (MAT) (EC 2.5.1.6) is an enzyme that catalyses the activation of methionine by ATP to form SAM. The isoenzyme encoded by *MAT1A* exists as two proteins with moderate to high K_m values for methionine, but transcripts for *MAT1A* were either absent or very poorly expressed in both human and bovine embryonic cells, and all cells within the bovine ovary, indicating that these different cell types are not accustomed to metabolising high concentrations of methionine so typical of contemporary cell and embryo culture media. Most strikingly in the cow was the absence of transcripts for the enzyme betaine-homocysteine methyltransferase (EC 2.1.1.5) in all somatic cells of the bovine ovary, as well as in the bovine oocyte and pre-implantation embryo. This may reflect species-specific differences in the importance of this enzyme for these cell types. It certainly indicates that these cycles function differently between species, and also between cell type within a species.

With the foregoing characterisations in mind we sought to assess if restricting the dietary supply of specific methyl-cycle metabolites (i.e. vitamin B_{12}, methionine and folate) in the diet of embryo donor ewes could lead to epigenetic modifications to DNA methylation in offspring with long-term implications for animal health. Methyl deficient or control diets were offered from 8 weeks before, until 6 days after, conception by AI. This duration of exposure ensured that the critical periods of DNA methylation programming in the germline of sheep (Beaujean *et al.*, 2004b; Russo *et al.*, 2007) were incorporated. Embryos were recovered and transferred singly to normally fed surrogates. Neither pregnancy rate nor birth weight was affected in this study but adult offspring were heavier and fatter, elicited altered immune responses to antigenic challenge, were insulin resistant and had high blood pressure (Sinclair *et al.*, 2007). In keeping

with studies in the rat (Kwong *et al.*, 2000; Kwong *et al.*, 2006) these effects were most evident in male offspring. The altered methylation status of 4% of 1,400 CpG islands examined by restriction landmark genome scanning (RLGS) in the fetal liver revealed compelling evidence of a widespread epigenetic mechanism associated with this nutritionally programmed effect. Furthermore, more than half of the affected loci were specific to males. These intriguing observations point to sexually dimorphic epigenetic programming during early pre-implantation development and merits further investigation.

Current limitations, outstanding issues and future priorities

Genome maps in ruminants have been under development for the last decade. Whilst the bovine genome has been fully sequenced and is largely annotated (Elsik *et al.*, 2009), sheep and goat maps are still under construction. The lack of genomic sequence information in the sheep has proved a significant barrier to progress and required us to use RLGS as a non-selective screening technique for the methylome to define the proportion of genes affected by peri-conceptional maternal nutrition (Sinclair *et al.*, 2007). In contrast, the power of 'next-generation' DNA sequencing technologies to globally map the epigenome was recently demonstrated in human pluripotent embryonic stem cells and terminally differentiated fetal fibroblasts. Lister *et al.* (2009) published the first complete DNA-methylation map of the human genome at single-base-pair resolution and their analyses challenges a number of existing theories in the field. For example, as eluded to earlier, whereas in fetal fibroblasts almost all DNA methylation occurs at CpG dinucleotides, around 25% of methylation sites in pluripotent stem cells do not occur at CpGs but on cytosines that neighbour other bases, in particular adenosine. The function, significance and enzymes involved in non-CpG methylation, however, remain to be identified. Nevertheless, large-scale analysis of genome-wide DNA methylation by deep sequencing of bisulphite-treated DNA represents a future advancement to current RLGS protocols and methods that array immunoprecipitated methylated DNA; the former is limited by methylation sensitive restriction endonucleases available, and uses radiolabelled phosphorus, whilst the latter is biased towards CpG-rich sequences and exhibits poor sensitivity for low CpG dense regions (i.e. those outside CpG islands) (Beck & Rakyan, 2008; Thu *et al.*, 2009).

Several outstanding issues emerge from the studies cited earlier in this article. For example, the fact that the effects of peri-conceptional nutrient restriction manifest more in male than female offspring in contrasting species (i.e. rat and sheep), and that this is consistent with sexually dimorphic epigenetic alterations to DNA methylation (at least in the sheep), is of profound importance. Sexual dimorphism in the development and metabolism of pre-implantation embryos is well established in ruminants and other mammalian species (Gutiérrez-Adán *et al.*, 2006). Epigenetic differences between male and female bovine blastocysts have also recently been reported (Bermejo-Álvarex *et al.*, 2008). Using a methylation-sensitive PCR technique, these authors assessed the methylation status of 6 genomic regions in male and female bovine blastocysts and found one region, close to a variable number tandem repeat sequence, to be differentially methylated. The authors concluded that there may be genomic region-specific differences in epigenetic status between male and female bovine blastocysts which may apply to other single-copy genes involved in regulating full-term development. Although this remains to be determined, the potential exists to identify loci in the pre-implantation embryo that, in terms of epigenetic programming, are altered by maternal diet in a sexually dimorphic manner.

A neglected area of research concerns the effect of paternal nutritional status on epigenetic programming through the germline and the consequences for offspring health and well being. Indirect evidence that environmentally induced defects programmed into the male gamete can

alter offspring health and fertility come from transgenerational studies in the rat investigating the effects of maternal protein restriction during pregnancy (Harrison & Langley-Evans, 2008) and pregnant rats exposed to the agricultural fungicide vinclozolin (Guerrero-Bosagnna & Skinner, 2009). In both cases phenotypic defects were transmitted via the male germline, at least to the F2 generation, indicating that defects programmed into the male gamete can alter offspring health. Furthermore, the studies with vinclozolin were extended to demonstrate epigenetic alterations to DNA methylation in epididymal sperm which persisted to F3 offspring.

There also needs to be greater emphasis in the future on assessing the effects of specific components of the maternal diet during clearly defined periods of gestation. To that end due care and consideration should be given to the processes of nutrient storage, transport and function in the pregnant animal offered a nutrient deficient diet, as this will lead to the onset of body tissue depletion followed by deficiency, dysfunction and ultimately disease (Sinclair and Singh, 2007). In the past the timing and extent of nutrient restriction has frequently been inadequately monitored, greatly hindering data interpretation. Finally, and returning to the interests of the pioneering scientists listed at the beginning of this article, greater consideration should be made in future towards understanding the programming of traits of agricultural importance.

Conclusions

The foregoing discussion highlights the need to fully sequence and annotate the genomes of domesticated animal species, in particular the sheep, so that full advantage can be made of the contemporary molecular tools available to provide the mechanistic insights required in the field of developmental programming of health and disease. This is best exemplified by the extent of knowledge in epigenetic programming through the germline in the mouse compared to other mammalian species. Large animals, such as the sheep, represent a more appropriate model to study the developmental origins of health and disease because their mature size, and associated reproductive rate, metabolism and physiology are more similar to that of humans. Domestic ruminants are also species of commercial interest, so that in the future perhaps more emphasis should be placed on studying traits of economic importance where animals are offered more thoughtfully formulated diets that facilitate the study of specific micro-and macro-nutrients.

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