

# The developmental origins of health and disease: current theories and epigenetic mechanisms

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The retrospective cohort studies of David Barker and colleagues during the late 1980s established the principle that the incidence of certain adult diseases such as stroke, type 2 diabetes and dyslipidaemia may be linked to *in utero* development. Later termed the “Developmental Origins of Health and Disease (DOHaD)” hypothesis, there have been several more recent attempts to explain this phenomenon. Although a general conceptual framework has been established to explain how mechanisms may have evolved to facilitate rapid adaptations to changing ecological conditions, it doesn’t identify the actual mechanisms responsible for such effects. Extensive covalent modifications to DNA and related proteins occur from the earliest stages of mammalian development. These determine lineage-specific patterns of gene expression and so represent the most plausible mechanisms by which environmental factors can influence development during the life course. In providing a contemporary overview of chromatin modifications during early mammalian development, this review highlights both the complexity and our current lack of understanding of how epigenetic alterations may contribute to *in utero* programming. It concludes by providing some thoughts to future research endeavours where the emphasis should be on bettering our understanding of epigenesis and devising more thoughtful experimental approaches that focus on specific environmental factors in appropriate animal and cellular models.

## Introduction

Clinical interest in the concept that late onset diseases can originate from events occurring *in utero* arose from the initial retrospective cohort studies of Barker and Osmond (1988) and Barker *et al.* (1989), who assessed relationships between size at birth, hypertension and ischaemic heart disease in adult humans. Further studies established inverse relationships between birth weight and the incidence of stroke, type 2 diabetes and dyslipidaemia, and these phenomena, often collectively referred to as Syndrome X or metabolic syndrome, led to the “fetal origins (later to be called developmental origins) of health and disease” or “DOHaD” hypothesis (Barker 1995). However, the central tenet of this hypothesis, that low birth weight is associ-

ated with elevated blood pressure or hypertension in adults, has since been challenged (e.g. Huxley *et al.* 2002). It transpires that reduced size at birth, which is only a proxy for sub-optimal *in utero* conditions, is not necessarily associated with increased disease risk in adults. Nevertheless, the general consensus based on an overwhelming body of epidemiological evidence among different human populations and from direct-interventionalist studies with animals supports the general concept that sub-optimal *in utero* conditions can predispose to late-onset disease in adults (Langley-Evans 2006).

In the decade or so that followed the initial observations of Barker and colleagues a number of hypotheses were proposed to explain this phenomenon. Many attempted to support this hypothesis by setting it in a broader 'evolutionary' context. Numerous animal-based studies have also been conducted to determine the effects of exposure to specific environmental factors, at different stages of gestation, on indices of long-term health. The purpose of the present article is not to provide an exhaustive overview of this topic, which has been provided elsewhere (McMillen & Robinson 2005), but rather to develop and critically assess some of the emerging concepts and related theories, whilst defining the role of heritable epigenetic modifications to DNA and associated proteins. Although studies in ruminants feature, the emphasis is on investigations which have provided unique conceptual insights into early mammalian development and some of the regulatory mechanisms where, in keeping with the original ideas of David Barker and colleagues, the bias is towards the effects of physiologically relevant alterations to maternal nutrition.

### **DOHaD: Current controversies and related concepts**

Experimentally induced exposure to a number of environmental factors during *in utero* development is known to modify post-natal physiology. Although studies have been conducted in the mouse, guinea pig, and domesticated species such as the pig and horse, the favoured animal models to date have been the rat and sheep. Studies with these species have revealed that maternal insults during pregnancy can alter subsequent cardiovascular and metabolic function in offspring in the absence of effects on birth weight (Table 1). Indeed, these observations have contributed to the current debate as to whether or not birth weight *per se* has any causal effect on subsequent physiological function in offspring (Lucas *et al.* 1999). An emerging concept from this debate is that a period of accelerated or 'compensatory' growth, that frequently accompanies episodes of growth restriction, may underlie many of the adverse effects on cardiovascular function and metabolism observed during adulthood (Singhal & Lucas 2004). Importantly, although initially proposed to defend the observation that catch-up growth during childhood in low-birth weight infants leads to cardiovascular disease (CVD) in adults, this hypothesis can be applied to any specific period during pre- and post-natal development.

#### *Thrifty phenotype and Predictive Adaptive Responses*

Much of the current thinking in the field (including that above) stems from the 'thrifty phenotype' hypothesis, originally proposed by Hales & Barker (1992), which attempts to explain the link between fetal growth and the metabolic syndrome. This hypothesis proposes that poor nutrition in early life (particularly during fetal development) leads to permanent changes in glucose-insulin metabolism. Thus offspring malnourished during pregnancy or infancy are ill-equipped to cope with high calorie diets in later life. This concept has been expanded by some to consider such adaptations as a means by which phenotypic modifications can be induced within a single generation in order to best accommodate prevailing or anticipated

**Table 1.** Examples of experimentally induced perturbations during pregnancy leading to altered physiology.

Intervention	Species	Stage of gestation	Physiological outcomes		Reference
			Pre-natal	Post-natal	
<b>A. Non-nutritional interventions</b>					
<i>(i) With intra-uterine growth restriction</i>					
Assisted reproduction (e.g. IVF)	Mice	< 20 %	↓ fetal:placental weight ratio	↑ growth, ↑ adiposity	Sjöblom <i>et al.</i> (2005)
Carunclectomy	Sheep	14 - 20 %	Hypoxia, hypoglycaemia	N/D	Owens <i>et al.</i> (1989)
Maternal hyperthermia	Sheep	25 - 95 %	Hypoxia, hypoglycaemia, ↓ ponderal index	↑ neonatal mortality	Anthony <i>et al.</i> (2003)
Utero-placental embolization	Sheep	80 - 100 %	↑ mean arterial bp	↓ mean arterial bp	Louey <i>et al.</i> (2000)
Glucocorticoid administration	Rat/Sheep	67 - 100 %	↑ ventricular wall thickness, - heart rate and bp,	↑ bp, hypertension, hyperglycaemia, hyperinsulinaemia	Seckle (2001)
Temporarily induced anaemia	Sheep	80 - 95 %	↓ haematocrit, ↓ blood O <sub>2</sub>	↓ adult weight, ↑ coronary conductance	Davis <i>et al.</i> (2002)
Between breed embryo transfer	Horse	0 - 100 %	↑↓ in placental and birth weights	Cross dependent ↑ arterial bp and baroreflex sensitivity	Giussani <i>et al.</i> (2003)
<b>B. Nutritional interventions</b>					
<i>(i) With intra-uterine growth restriction</i>					
Low protein diet	Rat	0 - 20 %	↓ blastomere number	↑ post-weaning growth, systolic bp (males)	Kwong <i>et al.</i> (2000)
<i>(ii) Without intra-uterine growth restriction</i>					
Low protein diet (9% casein)	Rat	0 - 33 %	↑ birth weight	↑ weaning weight, systolic bp (males)	Langley-Evans <i>et al.</i> (1996)
		34 - 67 %		↑ systolic bp	
		68 - 100 %		↑ weaning weight (males), ↑ systolic bp	
		0 - 100 %		↑ weaning weight (males), ↑ systolic bp	
Lard enriched diet	Rats	0 - 100 %	N/D	↑ systolic, diastolic bp (females)	Khan <i>et al.</i> (2003)
Global nutrient restriction	Sheep	-40 - 5 %	↑ arterial bp and rate pressure product in twins	N/D	Edwards & McMillien (2002)
		0 - 20 %	N/D	↑ pulse pressure, ↓ pulse pressure product, blunted baroreflex sensitivity to Ang II	
	Sheep	20 - 55 %	N/D	↓ nephron number, ↑ mean arterial bp	Gilbert <i>et al.</i> (2005)
	Sheep	70 - 80 %	N/D	Altered hypothalamic-pituitary-adrenal axis	

↑, ↓ = increase/decrease relative to Controls; N/D = not determined; bp = blood pressure; Ang II = angiotensin II

environmental circumstances (Bateson 2001; Gluckman & Hanson 2004). Citing numerous examples within the animal kingdom, these authors distinguish such responses (termed Predictive Adaptive Responses [PARs]) from those that confer an immediate advantage or arise from disrupted or teratogenic development as a consequence of severe environmental challenges either during pregnancy or infancy. This hypothesis draws some support from long term follow-up studies of human populations subjected to famine during the second world war, for example, in The Netherlands during the winter of 1944-45 (Ravelli et al. 1998) and during the siege of Leningrad from 1941 to 1944 (Stanner et al. 1997); and from animal studies, including some of those listed in Table 1.

#### *Intergenerational transmission, Lamarckian inheritance and epigenetics*

An apparent limitation of the hypothesis proposed by Gluckman & Hanson (2004) is that it is based on the premise that the *in utero* environment can provide the necessary cues for the fetus to predict future (i.e. post-natal) environmental conditions. Whilst it is understandable that appropriate *in utero* responses to circadian and seasonal cues may confer immediate post-natal advantages to the neonate, particularly for short-lived and altricial mammalian species, the longer term advantages, particularly for larger animals, are less apparent. Indeed, for long-lived species, such as the human and ruminant, such responses would seem improbable, given that they have to transcend seasonal fluctuations in day length, climate and nutrient provision. Nevertheless, one population based study in humans conducted with subjects in both the northern and southern hemispheres demonstrated a clear effect of season of birth on longevity (autumn born babies live longer), which the authors (Doblhammer & Vaupel 2001) attributed to *in utero* nutrition.

An attempt to explain the evolutionary significance of PARs was made by Kuzawa (2005), who considered the phenomenon in the context of life history theory. This is a branch of evolutionary biology which postulates that many physiological traits are linked to key maturational and reproductive characteristics that define the life course. Compelling evidence was presented that nutrient provision during pregnancy (particularly to the female fetus) conveys information reflecting the nutritional environments experienced by matrilineal ancestors. Termed 'Inter-generational Phenotypic Inertia' this hypothesis predicts that traits such as birth weight would have an inter-generational component; a prediction supported by data from the Dutch famine of 1944-1945 (Lumey 1992). However, such a mechanism is also expected to minimise the influence of immediate or short-term fluctuations in nutrient provision, typically associated with seasonal or other stochastic factors, in favour of longer-term strategic goals reflecting changing ecological conditions. That is, according to this model, the effects of PARs within a single generation would be minimal, but would accumulate across generations. Furthermore, the hypothesis predicts (and some evidence is presented) that the 'buffering effect' afforded by this mechanism primarily benefits the female fetus, so that the male fetus may be more susceptible to factors experienced by the mother during pregnancy (Kuzawa 2005). Indeed, in a nutritional re-alignment study with rats which had been offered a low protein diet for 12 generations the following observations were made during nutritional realignment: (i) there was a graded response in terms of increased birth weight and cognitive abilities which depended on the timing of protein re-alignment (*in utero* > from birth > from 4 weeks), and (ii) further incremental improvements in residual physical and behavioural outcomes were observed over the following two generations (Stewart et al. 1980). Importantly, the effects of both the initial 12 generations of protein restriction and of dietary re-alignment were greater in male offspring. The full significance of this gender-effect specifically in the context of DOHaD, however,

remains to be confirmed. The available evidence is inconclusive as unfortunately few experiments have considered long-term intergenerational effects, and experiments have often been conducted with insufficient power to determine gender effects within a single generation.

Nevertheless, such phenomena evoke the ideas of the French naturalist Jean-Baptiste Lamarck (1744-1829), who proposed that the inheritance of acquired characteristics is the hereditary mechanism by which changes in physiology acquired during the lifetime of an organism are transmitted to its offspring. Previously refuted in favour of Darwin's theories on natural selection, the emerging field of epigenetics, whilst in no way contradicting the laws of natural selection, has provided a potential mechanism for Lamarck's ideas on the inheritance of acquired traits. The environmental lability of acquired epigenetic modifications, particularly during the earliest stages of development (discussed later), provides a mechanistic basis for predictive adaptive responses and transgenerational inheritance.

#### *Effects on fertility*

Investigations into the long-term consequences of the war-time famine in The Netherlands revealed that undernutrition during gestation can lead to a number of disease states in later life, the nature and severity of which are dependent on the stage of gestation at the time of insult (Painter *et al.* 2005). A striking observation from studies with this cohort, however, was that the subsequent fertility of offspring undernourished during pregnancy was unaffected (Lumey 1998). Although there are reported effects on ovulation rate among non-obese adolescent girls born small for gestational age (effects associated with central adiposity and dyslipidaemia (Ibáñez *et al.* 2002)), it remains to be seen if these effects persist to normal reproductive age. Furthermore, whilst it is clear that ovulation rate and/or litter size in adult ewes can be reduced by undernutrition during fetal development (Rae *et al.* 2002) or during the pre-pubertal period (Rhind *et al.* 1998), there seems to be little effect on male fertility and no effect on the incidence of barrenness among ewes; that is, it seems that fertility is not affected.

These observations are consistent with current theories on PARs and the transgenerational effects discussed earlier. Teleologically, it makes sense that a polyovular species, faced with a long-term decline in nutrient provision, would attempt to reduce ovulation rate. Failure to ovulate, which impedes the initiation of pregnancy, could only be an emergency measure of short-term significance, unsustainable in the longer term. It would also seem probable for long-lived species faced with several generations of nutritional impoverishment that, in addition to a reduction in mature size (Kuzawa 2005), puberty would be delayed and reproductive senescence advanced. Although there is a considerable body of evidence to support a delay in the onset of puberty under such circumstances in humans (Frisch 1994), at present there is limited evidence to support the notion that growth restriction during late gestation or during early childhood advances the onset of menopause (Cresswell *et al.* 1997; Hardy & Kuh 2002). Regrettably, this is an area where there appears to be a dearth of suitable animal models (Roof *et al.* 2005; Danilovich & Ram Sairam 2006). Given the evolutionary theories concerning ageing discussed next it would seem that rodents are not best suited for the study of reproductive senescence in humans.

#### *Evolutionary theories of ageing*

Many of the existing evolutionary theories on ageing intricately link fecundity to mature size and lifespan, and so go some way to underpin many of the current hypotheses on the origins of adult health and disease (Holliday 2005). The 'accumulated mutations' theory first proposed

by PB Medawar in 1952, for example, proposes that deleterious genetic mutations that are manifest at a young age would be selected against whereas those displayed in late (post-reproductive) life would not, and so would accumulate across generations. A related theory, the 'antagonistic pleiotropy' theory (proposed GC Williams in 1957), suggests that genes exist which confer an advantage early in life (e.g. increased fecundity or cancer resistance) but which later advance the onset of age-related senescence. To date, however, this has been mainly supported from studies with the fruit fly and nematode with only limited evidence from the mouse (Leroi *et al.* 2005). More recently a third theory has attracted greater interest. The 'disposable soma' theory links the energy costs of maintenance to extrinsic mortality (e.g. predation), fecundity and longevity (Kirkwood 2005). The theory considers trade-offs between maintenance and reproduction, so that small mammals that develop and reproduce early, and have larger litters, have shorter lifespans. In contrast, in the absence of factors contributing significantly to extrinsic mortality, natural selection favours species that are better adapted to their environment, so that mature size increases, reproductive rates are reduced, reproduction occurs later and longevity is increased. This theory explains the rather paradoxical scenario where caloric restriction throughout life enhances longevity; a feat achieved through reductions in fecundity. The effect in rodents (a species utilised extensively in investigations into the DOHaD hypothesis) has been estimated to be 10 times greater than that in humans (Phelan & Rose 2005); demonstrating the extent to which these small mammals invest in reproduction (or annual fecundity rate), also calculated to be 10 times greater in rodents than in humans.

#### *In pursuit of immortality: the germline and the soma*

Primordial germ cells (PGCs) in mammals are derived from the inner-cell mass and are segregated from the somatic lineage during the early stages of gastrulation (Matsui & Okamura 2005). Destined to differentiate into gametes, these specialised cells are unique in that they give rise to the lineage responsible for the transfer of the genome from one generation to another. Relieved from the responsibility of transgenerational inheritance at an early stage in metazoan evolution, the surrounding soma was able to specialise into the many other cell types found in multicellular organisms but, in so doing, these cells incurred a cost; that is they became expendable. In his 'deprivation syndrome paradigm', Heininger (2002) explained that during early metazoan evolution a common strategy in response to environmental stress was to redirect resources from the soma towards the germline (e.g. the dying fruiting body and germ-like spores of *Dictyostelium*). Ageing, it was argued, evolved as a counter measure on the part of the soma to postpone its inevitable demise.

It follows that the distinction between the germ line and the surrounding soma in higher organisms, and the conflict that exists between them, is central to the current thinking on the evolution of ageing and age-related disease, under-pinning the ideas presented earlier about the trade-off between somatic maintenance and reproductive effort in a world of finite resources (Kirkwood 2005). This evolutionary conflict led Heininger (2002) to state that "death is no altruistic suicide but is imposed on the soma by the "enemy within", the germ cells".

#### *The case for DOHaD*

Some of the hypotheses described in this section predict that, at least in viviparous species, environmental influences from the earliest stages of development can alter key physiological processes during the life course. Some of these modifications may be adaptive responses, some may not, but all could contribute either directly or indirectly to DOHaD. Set in the

context of life history theory, the hypothesis of 'trans-generational phenotypic inertia' further predicts that these effects accumulate gradually over successive generations. The 'disposable soma' theory links reproductive effort to extrinsic mortality and resistance to 'environmental stress', and forms the basis of the concept that ageing, and age-related diseases, are an inevitable consequence of the efforts of the soma to evade death; which in an evolutionary context is seen as a 'germ-cell triggered' event.

The importance of understanding these latter hypotheses are two-fold. Firstly, it may yet transpire that germ cells are uniquely resistant to *in utero* 'programming' effects, which have so clearly been demonstrated in somatic lineages; so that traits such as fertility may be unaffected by factors such as maternal nutrition during pregnancy. In this regard, the onset of reproductive senescence will be an important future area of study. Secondly, as many of the post-natal health-related outcomes programmed *in utero* are displayed during 'post-reproductive' life, so they may be intricately linked to the mechanisms that extend life; that is, these mechanisms may also be 'programmed' *in utero*.

Although compelling, evidence supporting many of these hypotheses is largely correlative and the mechanistic basis for the observed effects remains to be firmly established. The period of *in utero* development, however, would appear to be particularly sensitive to alterations in the maternal environment. This is perhaps not surprising given that, of the estimated 43 cell divisions that occur during the course of human development, 37 have been predicted to take place *in utero* (Freitas 1999), with 8 occurring during the pre-implantation period. It follows that as each round of cell division is accompanied by a round of DNA replication, so the mechanistic basis for *in utero* programming of adult health and disease may reside in subtle alterations to ongoing chromatin or epigenetic modifications taking place during the normal processes of cellular differentiation and lineage commitment.

### Epigenetics: the chemical basis for change

The modern use of the term epigenetics is to describe heritable changes in gene function that occur without an alteration in DNA sequence. These changes arise principally as a consequence of specific covalent modifications to DNA and associated histone proteins which act in concert with chromatin structure to define the transcriptome associated with a specific cell lineage. Epigenetic inheritance is therefore defined as the transmission of such modifications from a cell (or multicellular organism) to its descendants without any alteration in nucleotide sequence.

#### *Histone modifications*

The nucleosome is the functional unit of chromatin and consists of an octomeric complex of core histone proteins (H2A, H2B, H3 and H4), around which is wrapped 146 bp of DNA. A single histone H1 polypeptide interacts with an additional 20 bp of DNA as it enters and leaves the nucleosomal core. The negatively charged DNA is usually very tightly associated with the positively charged N-terminal histone tails which protrude from the core. Specific amino acid residues on these tails are targets for a number of enzyme-catalysed post-translation modifications which affect their charge and function (Table 2). This, in turn, can affect the configuration of the chromatin resulting in either a tightly bound heterochromatic configuration or a more open euchromatic configuration. These modifications can also serve to direct the binding of transcription factors and DNA damage repair proteins.

**Table 2.** Examples of post-translational histone modifications in mammalian cells.

Histone modification	Histone and residue	Modifying enzyme	Demodifying enzyme	Function	Species	Reference
Acetylation	H3K14	GCN5/PCAF	HDAC1	Transcriptional activation	Hn	Yamagoe <i>et al.</i> (2003)
	H4K?	HAT1/GCN5	HDAC 1, 2, 3, 7	Activation/Repression	Bov	McGraw <i>et al.</i> (2003)
Phosphorylation	H3S10	Aurora-B-Kinase	?	Transcriptional activation	Ms	Fischle <i>et al.</i> (2005)
	H3T11 H2AX	Dlk/ZIP Kinase PI3 Kinase	? PP2A	Mitosis DNA damage repair	Hn/rat Hn	Preuss <i>et al.</i> (2003) Chowdhury <i>et al.</i> (2005)
Methylation	H3K4	Set9	LSD1	Transcriptional activation	Ms/Hn	Francis <i>et al.</i> (2005) Shi <i>et al.</i> (2004)
	H3K9	SUV39HI	?	DNA methylation/ heterochromatin	Hn	Snowden <i>et al.</i> (2002)
	H3R17	CARM1	PAD4	Transcriptional activation	Hn	Wang <i>et al.</i> (2004)
	H4R3	PRMT1	PAD4	Transcriptional activation	Hn	Wang <i>et al.</i> (2004)
Ubiquitylation	H2AK199	Ring1b	?	X-inactivation in trophoblast	Ms	Fang <i>et al.</i> (2004)
Sumoylation	H4	UBC9	ULP proteases?	Repression via HDAC and HP1	Hn	Shiio & Eisenman (2003)

Histone (H); lysine (K); arginine (R); serine (S); threonine (T); human (Hn); mouse (Ms); bovine (Bov)

The post-translational modifications that occur to these histone proteins have been best studied in yeast (*S. cerevisiae* and *S. pombe*), the fruit fly (*D. melanogaster*) and in *Arabidopsis* (Bannister & Kouzarides 2005). Of these modifications histone acetylation is perhaps the best understood. This modification involves acetyl substitution of the  $\epsilon$ -amino group of lysine (K) (e.g. H3K9 or H4K12) which results in a more acidic (less positive) state. These reactions are catalysed by members of one of five families of histone acetyltransferases (HATs) (Kouzarides 1999); the reverse reactions being catalysed by histone deacetylase (HDAC) of which there are 17 known mammalian members belonging to three classes.

Histone methylation occurs on non-acetylated lysine and arginine residues, and the methylation of H3 and H4 has attracted most attention where the outcomes can either be stimulatory or inhibitory depending on the particular lysine and arginine residue modified. Methylation of lysine and arginine residues is directed by histone methyltransferase (HMT) and protein arginine methyltransferase (PRMT) family members respectively; the former can direct S-adenosylmethionine (SAM) mediated methylation of lysine residues (Lee *et al.* 2005). In contrast to histone methyltransferases, the identification of histone demethylases is comparatively recent, and so our understanding of how these enzymes operate is incomplete (Bannister & Kouzarides 2005). A key feature of these post-translational modifications, however, is that they seldom act in unison but there exists considerable 'cross-talk' between these processes (Fischle *et al.* 2003). What's more, these histone modifications are intricately linked to covalent DNA modifications to collectively define the transcriptome. Details of such interactions are only now emerging but they highlight a complex bi-directional interplay, for example, between histone acetylation, methylation and DNA methylation (Fuks 2005).



### DNA methylation

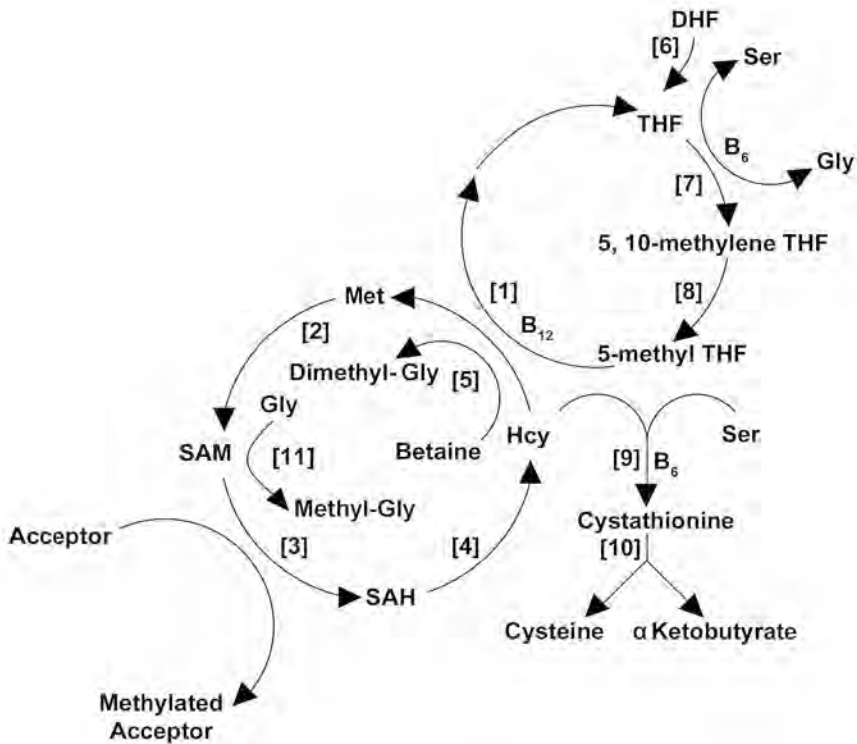
DNA methylation involves the addition of a methyl group to the number 5 carbon atom of the cytosine pyrimidine ring. This covalent modification is targeted to CpG dinucleotides which are recognised by DNA methyltransferase enzymes (DNMTs) (Goll & Bestor 2005). It has been estimated that around 75% of CpG dinucleotides in the human genome are methylated and are to be found in non-translated and heterochromatic regions; the remaining 25% unmethylated sites being located in specific 1 to 2 kb regions known as CpG islands (Caiafa & Zampieri 2005). These CpG islands, in turn, are frequently found in the promoter regions of genes. Among its numerous roles, DNA methylation is thought to serve in tissue-specific gene expression, X-chromosome inactivation, genomic imprinting and the silencing of transposable elements which constitute around 45% of the human genome (Lander *et al.* 2001). A key feature of DNA methylation, currently thought to distinguish it from many other chromatin modifications, is that it is normally faithfully restored during each cell cycle so that methylation patterns established during the earliest stages of development can persist in adult somatic cells. At present, the temporal means by which DNA methylation is targeted to specific regions of the genome is poorly understood, although it is thought to involve protein-protein interactions between the DNMTs and transcriptional repressor proteins, chromatin insulators and RNA-mediated interference (Klenova *et al.* 2002; Klose & Bird 2006). Methylated DNA in turn attracts methyl-CpG-binding proteins and these interact with other repressor proteins, including HDACs, to form transcriptional repressor complexes.

Although DNA methylation is thought to be an irreversible reaction, catalysed exclusively by members of the DNMT family of methyltransferases, it has long been recognised that active-demethylation of paternally-derived single copy genes takes place in the mammalian zygote (Oswald *et al.* 2000); and there is emerging, albeit controversial, evidence that DNA methylation in other cell types may also be fashioned by demethylase activity (Santos *et al.* 2002; Vairapandi 2004). The universal methyl donor SAM has been shown to inhibit demethylase activity (Detich *et al.* 2003), and so intracellular depletion of SAM may lead to a reduction in DNA methylation by reducing the provision of labile methyl groups and by increasing DNA demethylase activity.

### Methyl cycle metabolism

The metabolic cycles which supply methyl groups for DNA and histone methylation involve a complex series of interactions between a number of different intracellular metabolites (Stipanuk 2004) (Fig. 1). The two principal methyl donors in animal metabolism are betaine (trimethyl glycine), a metabolite of choline, and SAM, a metabolite of methionine. SAM is a universal donor of methyl groups providing one-carbon moieties for phospholipid biosynthesis, creatine production and protein synthesis in addition to DNA methylation. In donating its methyl group to an acceptor, SAM is converted to S-adenosyl homocysteine (SAH). The subsequent loss of the adenosine residue produces the non-protein amino acid homocysteine (hcy), a central intermediate in the metabolism of sulphur in all animals. Homocysteine in turn can either be catabolised to cystathionine in the trans-sulphuration pathway or remethylated to form methionine. In mammals, two separate enzymes catalyse the remethylation step to complete a cycle which enables other molecules, notably serine and glycine, to act as methyl donors. One enzyme (*methionine synthase*, MTR) utilises a derivative of folic acid, N5-methyltetrahydrofolate as the methyl donor. The reaction is mediated by the coenzyme, methylcobalamin, derived from dietary vitamin B12 (cyanocobalamin). The activity of this

reaction is therefore determined by the availability of two B vitamins, namely folic acid and cyanocobalamin. An alternative re-methylation reaction using betaine as the cofactor is also available to convert hcy to methionine. As betaine is derived from the breakdown of choline, the activity of this enzyme (*betaine-hcy methyl transferase*; BHMT) is partly determined by the availability of choline in the diet.



**Fig. 1.** Combined methionine and folate cycles. Metabolites: Methionine (Met), S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH), homocysteine (Hcy), dihydrofolate (DHF), tetrahydrofolate (THF), serine (Ser), glycine (Gly), methylcobalamin (B12), pyridoxal phosphate (B6). Enzymes: *methionine synthase* [1], *methionine adenosyl transferase* [2], SAM-dependent methyltransferases [3], *SAH-hydrolase* [4], *betaine-homocysteine methyltransferase* [5], *dihydrofolate reductase* [6], *serine hydroxymethyl transferase* [7], *5,10-methylene THF reductase* [8], *cystathionine b-synthase* [9], *cystathionine g-lyase* [10], *glycine methyltransferase* [11].

In contrast to rats, the total capacity of *choline oxidase* and BHMT is much lower than MTR in the adult sheep (Table 3). This reflects differences in the metabolism of the two species. Adult ruminants derive only a limited quantity of the precursors of transmethylation from the diet as those that are present are degraded by rumen microbial action. The transition from pre- (neonatal) to post-ruminant states in ruminants results in a decline in choline and creatine availability leading to a modification in single carbon metabolism. Nevertheless, both human subjects and sheep exhibit strikingly similar rates of methionine recycling, despite differences in dietary methyl group provision (Lobley et al. 1996).

**Table 3.** Methyl cycle enzyme activity (adapted from Snoswell & Xue, 1987)

Enzyme	Specific activity (pmol/min/mg protein)	
	Sheep	Rats
<i>Choline oxidase</i>	294	7980
<i>Betaine Hcy methyl transferase</i>	130	725
<i>Methionine synthase</i>	203	55
<i>Glycine methyltransferase</i>	25	7098

Metabolic responses to methyl nutrients are also known to differ between individuals as a result of polymorphic variation in at least four enzymes involved in these cycles. For example, the 677C to T polymorphism in *methylene tetrahydrofolate reductase* (*MTHFR*) reduces enzyme activity and is highly prevalent among Caucasians (5-15% for the TT and 40-50% for the CT genotypes respectively). Such individuals require high intakes of folic acid to reduce plasma hcy concentrations (de Bree *et al.* 2003). Given the central role of MTR in ruminant single-carbon metabolism, it is interesting to note that the A to G polymorphism at position 2756 of this gene is common in human subjects (~ 40% and 5% for AG and GG genotypes respectively; Sharp & Little 2004).

### Epigenetic programming during development

Given that the genetic code does not vary between cell types, the transition to multicellularity and the division between the germline and the soma necessitated the evolution of epigenetic mechanisms (such as the methylation of DNA) that would determine lineage-specific gene expression (Jablonka 1994). Recent studies using restriction landmark genome-scanning (RLGS) have identified numerous tissue-specific differentially methylated regions (DMRs) within CpG islands in both germ and somatic cells of differing lineages in mice (Shiota *et al.* 2002; Kremensky *et al.* 2003). The temporal complexity of the epigenetic mechanisms that direct somatic cell lineage specification was further illustrated by Rupp *et al.* (2002) who considered the interactions that exist between myogenic regulatory proteins and chromatin modifying enzymes during the differentiation of skeletal muscle. Both *MyoD* and *Myf5* are expressed during somitic myogenesis under the influence of Sonic hedgehog and Wnt-1, and serve to induce mesodermal precursor cells to differentiate into myoblasts, as well as activating downstream target genes such as myogenin (Maltin *et al.* 2001). *MyoD* expression is associated with a loss of methylation at a conserved distal enhancer element known to mediate its activation. The DNA binding activity of *MyoD* is regulated by both HATs and HDAC and, together with *Myf5*, *MyoD* is believed to initiate target gene transcription by recruiting mammalian SW1/SNF complexes (members of the trithorax group of proteins associated with euchromatin). It is interesting, therefore, that temporal shifts in *Myf5* protein expression in proliferating ovine fetal muscle cells should be induced by temporarily exposing Day 3 pre-implantation embryos to an advanced uterine environment (Maxfield *et al.* 1998); suggesting that events occurring during the earliest stages of post-fertilisation development can alter down-stream gene expression and cellular proliferation (discussed later).

Epigenetic mechanisms are also known to participate in the specification and formation of primordial germ cells (Allegrucci *et al.* 2005a). Crucially, these cells undergo additional dramatic epigenetic modifications during gametogenesis that serve to erase epimutations, re-establish totipotency and establish sex specific imprints. Yet further important modifications

occur following fertilisation in the period leading up to zygotic gene activation; modifications which may herald the initiation of the genetic conflict that is thought to exist between the sexes (Moore & Reik 1996) and/or that are associated with zygotic activation and preparation for implantation.

#### *Epigenetic modifications following fertilisation*

Epigenetic programming during pre-implantation development has been extensively studied in the mouse, where the paternally derived genome is actively (in the absence of DNA replication) demethylated during the first cell cycle, consistent with early male pronuclear and zygotic gene transcription in this species (Schultz, 2002). Recent studies using immunofluorescent staining targeted to heterochromatic regions in one-cell mouse embryos have revealed that, as the sperm nucleus decondenses, it acquires H3-K9 acetylated and mono-methylated histones as the DNA becomes progressively demethylated (Santos *et al.* 2005). In contrast, the maternal pronucleus retains high levels of H3 di- and tri-methylated K9 and K27, which these authors suggest may serve to protect DNA methylation in the maternal genome at this time.

Whilst similar patterns of global DNA demethylation of the paternal genome have been observed in the rat, cow and human zygote, there are significant differences in the rabbit and sheep zygote (Beaujean *et al.* 2004a). Through the use of interspecies intracytoplasmic sperm injection, these authors were able to demonstrate that the more extreme loss of paternal demethylation in the mouse compared to the sheep male pronucleus arises as a consequence of (1) the enhanced resistance of sheep sperm to demethylation and (2) the greater demethylating capacity of the mouse ooplasm (Beaujean *et al.* 2004b). Subsequent cell divisions during the early pre-implantation period are associated with further losses of DNA methylation which again are more extreme in the mouse than either the cow or sheep genomes (Beaujean *et al.* 2004a). Although the functional significance of these species-related differences in DNA methylation are unclear, they may partly be due to pre-existing levels of methylation in the male pronucleus at the time of syngamy, or to differences in the timing of embryonic gene activation (discussed by Young & Beaujean, 2004). There are also species differences in the expression patterns of *DNMTs* in oocytes and pre-implantation embryos (Golding & Westhusin 2003; Vassena *et al.* 2005) which could partially account for these effects.

#### *Epigenetic modifications in germ cells*

Primordial germ cells in mammals are believed to be specified from the epiblast during the early stages of gastrulation. Most extensively studied in the mouse, their specification has been found to involve complex interactions between maternally inherited and zygotically induced molecules in a multi-step process (Matsui & Okamura 2005). In addition to epigenetic modifications associated with germline specification during this period, PGCs undergo further unique epigenetic alterations believed to coincide with their migration across the genital ridge (reviewed by Allegrucci *et al.* 2005a). From around 10.5 days *post coitum* in the mouse, single copy imprinted and non-imprinted genes are actively demethylated, thereby ensuring that epigenetic modifications that accumulate in somatic cells with age are erased through the germline. In contrast, the demethylation of repetitive elements is more protracted, variable and generally less complete; a process hypothesised to be a protective mechanism designed to minimise transposition and aberrant expression of adjacent genes (Hajkova *et al.* 2002).

Sex-specific remethylation of PGCs then takes place in the gonad. However, the timing of *de novo* methylation differs between the male and female germlines, at least in the mouse. Remethylation in the male, and the re-establishment of gametic imprints, commences in mitotically dividing (pre-meiotic) spermatogonial stem cells (Rousseaux *et al.* 2005). In contrast, the timing of *de novo* methylation in the female germ cell is less certain, but methylation acquisition at a number of imprinted loci is known to generally commence later, during the growth phase of oocytes arrested at the diplotene stage of the first meiotic division (Lucifero *et al.* 2002). Importantly, these events are known to be immediately preceded by an increase in transcript expression for key DNMTs (La Salle *et al.* 2004).

### Epigenetic basis of DOHaD

Existing theories provide a conceptual framework to explain how, in viviparous species, mechanisms have evolved during the course of evolution to facilitate rapid adaptations to changing ecological conditions; and that these may form the basis for the developmental origins of adult health and disease. From the preceding discussion it is apparent that extensive covalent modifications to DNA and related proteins occur from the earliest stages of mammalian development to determine lineage-specific patterns of gene expression; and so these represent the most plausible mechanisms by which environmental factors such as diet can influence development during the life course. Indeed, both the nature and extent of the modifications which occur normally during gametogenesis and early embryogenesis suggest that these may be particularly vulnerable periods during which epimutations can be induced. However, it is also apparent that our understanding of how these epigenetic processes may underpin the environmentally-induced phenotypic modifications discussed earlier is far from complete. The following discussion highlights some of the major findings of recent years with some thoughts on future directions.

#### *Inducing epigenetic modifications*

In addition to the epigenetic modifications that occur as a part of normal development, changes can also be induced by environmental factors although often the mechanisms by which these effects arise are unclear. For example, short term exposure of mammalian zygotes to non-physiological *in vitro* culture environments can lead to epigenetic alterations in global DNA methylation in mice (Shi & Haaf 2002) and genomic imprinting in sheep (Young *et al.* 2001). Recently, transient exposure of gestating female rats to specific endocrine disrupting compounds during the period of fetal gonadal sex determination was shown to decrease spermatogenic capacity and increase male infertility in offspring; effects associated with altered DNA methylation at several loci (Anway *et al.* 2005). Also, in altricial mammals, infant licking and grooming by the mother during the first week of life can reversibly modify DNA methylation and histone acetylation at the glucocorticoid receptor promoter, thereby altering glucocorticoid receptor (GR) expression (Meaney & Szyf 2005). Dietary induced epigenetic effects have also been reported in mice following weaning. For example, synthetic diets and diets deficient in folic acid, vitamin B12, methionine and choline offered to weanlings each caused a persistent loss of imprinting of *Igf2* in the kidney relative to controls (Waterland *et al.* 2006).

Of particular relevance to the DOHaD hypothesis are the observations that alterations to maternal diet during pregnancy and/or lactation can also lead to epigenetic modifications in DNA methylation associated with varied phenotypic outcomes. For example, viable yellow

agouti (*Avy*) mice harbour a transposable element (responsible for ectopic *agouti* transcription) at the *agouti* locus, the epigenetic status of which is metastable. Recently, Waterland & Jirtle (2003) demonstrated that dietary supplementation with the methyl donors folate, vitamin B12, choline and betaine throughout gestation and lactation can increase CpG methylation at this site, modifying the expression of the *agouti* gene which lies adjacent to the transposable element. Significantly, these authors observed that the *A<sup>vy</sup>* methylation status of tissues derived from the three germ layers of the early embryo were affected indicating that the effects are likely to have been induced during early embryo development. Subsequent studies with genistein (the major isoflavone in soy) offered to gestating female mice at physiological levels also found alterations in coat colour of heterozygous viable *agouti* offspring toward the mottled *agouti* phenotype (Dolinoy *et al.* 2006). Moreover, these effects, which were associated with a similar increase in methylation at the *agouti* locus, persisted into adulthood and reduced the incidence of obesity, a phenotype commonly associated with ectopic *agouti* expression. Significantly, the increase in DNA methylation at this locus occurred in the absence of any effect on either SAM or SAH (Fig. 1), indicating a dietary-induced epigenetic mechanism independent of single carbon metabolism.

Recent studies in the rat, using protein restricted diets similar to those employed in previous studies with this species (Table 1), assessed the methylation status and expression of the GR and peroxisomal proliferator-activated receptor (PPAR) genes in weanlings (Lillycrop *et al.* 2005). Methylation-sensitive PCR revealed a significant reduction in CpG methylation in the promoter regions of both PPAR $\alpha$  and GR which, in each case, was associated with a significant increase in gene expression. Importantly, the inclusion of 1mg/kg folic acid in the protein restricted diet reversed the effects of protein restriction on DNA methylation and the expression of both genes. Although epigenetic mechanisms were not determined, Jackson *et al.* (2002) was able to demonstrate that the non-essential amino acid glycine (which plays a key role in methionine and SAM metabolism [Fig. 1] *via* the actions of *glycine methyltransferase* [Table 3]) was able to reverse the effects of protein restriction during pregnancy on offspring blood pressure in Wistar rats. These studies serve to further demonstrate that epigenetic effects may be involved in changes of phenotype due to the macronutrient composition of the maternal diet.

#### *Transgenerational epigenetic inheritance*

The most significant observation from the study of Anway *et al.* (2005), cited earlier, was that both epigenetic and phenotypic effects observed in the F1 generation persisted in all subsequent generations studied (i.e. F1 through to F4) indicating that, in the absence of genetic mutations, phenotypic effects can be transmitted to successive generations *via* epigenetic inheritance. Clues that such inheritance patterns may exist in mammals were provided earlier when the altered phenotypes and patterns of gene expression observed in mouse nucleocytoplasmic hybrids were found to be present in their offspring (Roemer *et al.* 1997). There is also evidence of epigenetic transgenerational inheritance at the viable yellow *agouti* (*Avy*) locus where, in the absence of alterations to the nutritional environment, the phenotype of the offspring has been found to resemble that of the dam; indicating incomplete erasure of epigenetic modifications through the female germline (Morgan *et al.* 1999). Evidence of paternal as well as maternal transmission of epigenetic modifications in mice was later provided for the *axin-fused* (*Axin<sup>F1</sup>*) allele (involved in determining embryonic axis formation in vertebrates) which is also associated with a retrotransposon; the methylation state of this allele in mature sperm closely resembled that of somatic cells of the animal suggesting this also was not completely erased through the germline (Rakyan *et al.* 2003).

The significance of these observations is that they establish the principle of transgenerational epigenetic inheritance in mammals, previously only observed in plants and fission yeast (Chong & Whitelaw 2004). None of these examples, however, could be considered to meet the criteria of PARs described by Gluckman & Hanson (2004) or the inter-generational effects predicted by the phenotypic inertia model of Kuzawa (2005). Nevertheless, they demonstrate that environmentally induced epigenetic modifications, which may lead to adult disease, can be transmitted to future generations through the germ line.

#### *Future directions*

The foregoing discussion highlights both the complexity of epigenetic mechanisms and our current lack of understanding of how they may contribute to *in utero* programming. It does, however, provide some guidance for future research endeavours. The evidence contained within this article indicates that both nutritional and non-nutritional factors contribute to the DOHAD. To date, the former has relied largely on two experimental paradigms that involve either calorie or protein restriction. In contrast, ongoing collaborative investigations between the authors' laboratories are assessing the effects of deficiencies in specific micronutrients (i.e. methionine, choline, folate, vitamin B12 and B6) that are known to directly influence single carbon metabolism. The clinical significance of these nutrients is that their consumption is acknowledged to be highly variable in most Western societies and also in developing countries (e.g. Stabler & Allen 2004). The focus in future, therefore, should be directed towards understanding the effects of specific dietary nutrients and environmental factors. Further detailed studies should recognise (i) that the epigenetic regulatory mechanisms involved in environmentally induced gene dysregulation encompass complex interactions between non-transcribed RNAs and covalently altered proteins as well as DNA, and (ii) that the susceptibility of individuals to environmentally induced epimutations may have a genetic base. The significance of this latter point is that the metabolic responses which can epigenetically modify chromatin vary between individuals as a consequence of polymorphic variation in relevant enzymes. Although it will be necessary to establish such relationships in model animal species, direct studies in human cells will be of greatest value. To this end we have begun to identify key polymorphisms in enzymes directing the combined methionine/folate cycles within human embryonic stem cells, which serve as a model of the human embryo (Allegrucci *et al.* 2005b). Complementary studies in appropriate animal models will allow such investigations to take place at later stages of gestation and confirm their significance with respect to the onset of adult disease. They would also permit the investigation of transgenerational inheritance.

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