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Workshop on 'Nutritional models of the developmental origins of adult health and disease'

Animal models for the study of the developmental origins of health and disease

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Human epidemiological studies have indicated that the risk of developing non-communicable diseases in later life may be related to exposures during the developmental period. Developmental life is a vulnerable period of the lifespan during which adverse environmental factors have the potential to disturb the processes of cell proliferation and differentiation or to alter patterns of epigenetic remodelling. Animal models have been instrumental in demonstrating the biological plausibility of the associations observed in human populations, providing proof of principle to the theory of the developmental origins of health and disease (DOHaD). A variety of large- and small-animal models have made important contributions to the field, providing strong evidence of a causal relationship between early-life exposures and metabolic risk factors in later life. Studies of animal models are continuing to contribute to improving the understanding of the mechanisms of the developmental origins of disease. All models have their advantages and disadvantages, and the model that is most appropriate for any particular study is hypotheses dependent. The present review aims to briefly summarise the contributions that animal models have made to the DOHaD field, before reviewing the strengths and weaknesses of these animal models. It is proposed that the integration of evidence from a variety of different models is required for the advancement of understanding within the field.

Programming: Nutrition: Animal models

Background to the developmental origins of disease

Chronic diseases such as CVD, cancer and diabetes are the leading cause of mortality worldwide, accounting for 60% of all deaths[1]. Disease outcomes represent an interaction between genetic background and a variety of lifestyle and environmental factors that act across the lifespan. Developmental life is a vulnerable period of the lifespan during which adverse environmental factors have the potential to disturb the processes of cell proliferation and differentiation or to alter patterns of epigenetic remodelling. In this way, environmental factors acting during critical periods of development may result in irreversible changes in tissue structure, gene expression patterns and physiological function, thus altering the risk of disease in later life.

Evidence from human populations

Human epidemiological studies have provided evidence to support the theory of the developmental origins of health and disease (DOHaD). Data obtained from historical

Abbreviation: DOHaD, developmental origins of health and disease.

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cohorts have demonstrated associations between early-life factors and a range of diseases in adulthood. Amongst men and women born in Hertfordshire (UK) between 1911 and 1931 it was reported that the risk of CHD mortality, blood pressure and type 2 diabetes is greater in those who are of lower birth weight[2–4]. Subsequently, a variety of studies have demonstrated similar associations between anthropometric measures at birth and risk of disease in later life[5–9]. A recent systematic review of such studies has demonstrated a 25% decrease in diabetes risk for every 1 kg increase in weight at birth[10]. Despite concerns about the use of anthropometric measures at birth as a proxy for maternal nutrient status, it has been suggested that maternal nutrition is the primary factor influencing fetal development and postnatal disease risk. Some studies have considered the direct associations between maternal nutritional status during pregnancy and disease risk in later life, many focusing on data from offspring born to mothers exposed to dietary restriction during the Second World War Dutch famine. Individuals who were *in utero* at the time of the famine were shown to have greater risk of obesity, glucose intolerance, hypertension and CHD than individuals born before and after the famine, with the timing of the exposure being crucial[11–13]. In studies of more contemporary populations blood pressure during childhood has been shown to be related to maternal Hb and body fatness during pregnancy[14]. Similarly, blood pressure in adult men has been shown to be positively related to maternal intakes of animal protein and inversely related to maternal intakes of carbohydrate[15]. In the USA blood pressure in infants was shown to be related to maternal Ca intake in pregnancy[16].

Although the human epidemiological studies are highly supportive of an association between maternal nutrition and postnatal disease risk, concerns have inevitably been raised about failures to adequately adjust for confounding factors and about the possibility of publication bias. Importantly, the use of anthropometric markers at birth as a marker for maternal nutrient status has been questioned. The link between maternal nutrition and fetal growth in well-nourished human populations is conflicting. Low intakes of animal protein in late gestation and high intakes of sucrose in early gestation have been reported to be associated with reduced birth weight and placental weight[17]. However, other studies have shown little or no association between maternal nutrient intakes and infant birth weight[18,19]. Ongoing prospective studies, such as the Southampton Women's Survey[20], are more directly investigating the association between maternal diet and postnatal health outcomes. However, experimental models are likely to remain necessary for investigating the causality of the associations observed and the underlying mechanisms and for the initial design of interventions.

The use of animal models

When designed within the context of evidence obtained from human populations, animal models are able to test specific hypotheses whilst overcoming the major limitations of epidemiological study designs. The use of animal models enables a strong level of control over confounding factors, the measurement of invasive end points and the characterisation of downstream events across the full lifespan and into subsequent generations. Animal models have been absolutely instrumental in demonstrating the biological plausibility of the associations observed in human populations, providing proof of principle to the theory of the DOHaD. A variety of large (e.g. sheep and pig) and small (e.g. mouse, rat and guinea-pig) animal models have made important contributions to the field, providing strong evidence of a causal relationship between early-life exposures and metabolic risk factors in later life. Much of the early research published in this field has been descriptive in nature, focusing on characterising the postnatal phenotypes associated with early-life interventions. These phenotypes have been essential in demonstrating proof of principle and developing suitable models of the human situation. Attention has since turned to identifying the mechanisms underlying the relationships observed and to the design of appropriate interventions.

Whilst some researchers will argue an overarching superiority of one model over another, there is a need for a more balanced realisation within the research community that the model that is most appropriate for any particular study is hypotheses dependent. All models have their advantages and disadvantages, which must be accounted for during experimental design and interpretation. The present review aims to briefly summarise the contributions that animal models have made to the DOHaD field, before reviewing the strengths and weaknesses of the animal models currently available. It is proposed that the integration of evidence from a variety of different models is required for the advancement of understanding within the field.

Animal models: contributions to the field

A full review of the literature from animal models that have contributed to proof of principle of the DOHaD concept and informed current understanding of the underlying mechanisms is outside the scope of the present review, but a selection of the studies that have contributed to this literature will be outlined. Several excellent and more extensive reviews are available elsewhere[21–25].

Proof of principle

Within the context of the developmental origins of chronic disease, studies of nutritional programming using small-animal models have been ongoing since the early 1990s. The notion that interventions during critical periods of development could have permanent effects on long-term organ structure and function was not new, however, and the plasticity of tissues during the developmental period had previously been demonstrated using small-animal models. Treatment of newborn female rats with testosterone during the first few days of life, for example, had been shown to remodel the regions of the hypothalamus that control reproductive function, permanently rendering the treated animals sterile[26]. It was the rapidly-emerging human epidemiological evidence of associations between fetal growth restriction and postnatal CVD in the late

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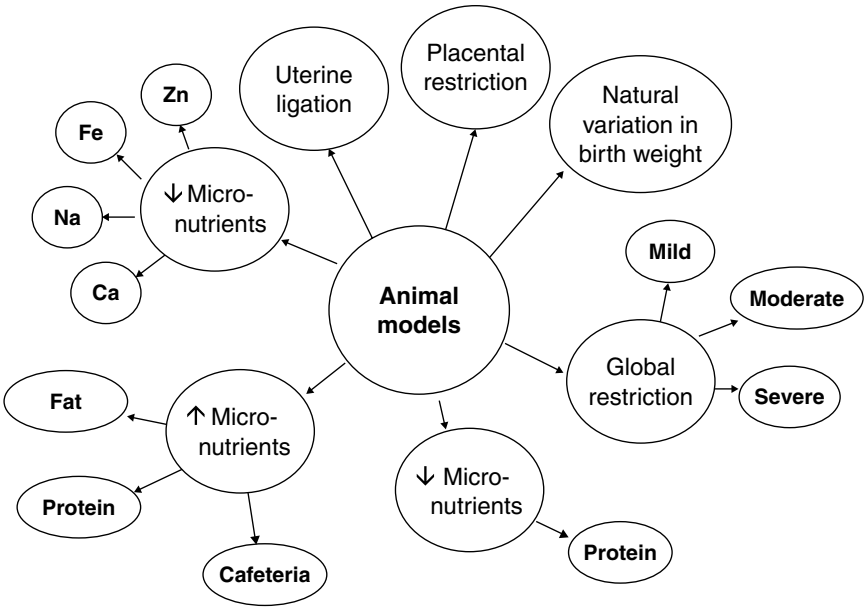

Fig. 1. An overview of the variety of tools used to modulate maternal and fetal nutrient status during pregnancy in small and large animals. ↓, ↑, Restricted and high levels respectively.

1980s and early 1990s[2–4] that marked a new era in the study of developmental programming. The quick generation time and relatively inexpensive costs of small-animal models make them ideal for testing proof of principle, i.e. demonstrating that factors acting during critical periods of development can have long-term consequences on postnatal physiological function and health status. A variety of models have been used to demonstrate that the associations observed in human epidemiological studies can be replicated under experimental conditions (Fig. 1). For example, protein restriction[27] and global nutrient restriction[28] in rats and uterine ligation in rats and guinea-pigs[29,30] were shown to restrict fetal growth and induce raised blood pressure in the offspring in postnatal life. More recently, Fe restriction[31] and high-fat feeding[32] during pregnancy in rats have also been shown to have similar effects on offspring blood pressure. Other outcomes associated with maternal nutrient restriction include effects on the fetal endocrine pancreas[33], altered muscle development[34], increased propensity for fat deposition during postnatal life[35,36], renal function[37,38] and altered offspring insulin sensitivity[39,40]. Importantly, these studies have demonstrated a direct effect of maternal nutrition on postnatal physiology and disease risk. Programming events occurring in response to alterations in maternal diet often occur without impacting on fetal size at birth, indicating that fetal growth restriction is not necessarily a component of the causal pathway between prenatal dietary exposures and postnatal outcomes.

Furthermore, the concept of nutritional programming was not new to the field of animal science and production. Proof of principle had already been demonstrated by investigating the impact of altered maternal nutrition or fetal growth restriction in large-animal species on offspring outcomes related to commercial benefits, including effects on offspring survival and growth rates, meat and fleece quality and reproductive function[41–46]. Such findings had relevance to the findings from the human epidemiological literature, particularly in relation to the impact of altered growth rates and muscle development on body composition and metabolic regulation in later life. In addition, the sheep had long been used as a model of fetal physiology, for reasons outlined later in the present review. Large-animal experimental paradigms with the potential to model the associations observed in human epidemiological studies were therefore readily available and became established within the literature by the late 1990s. Many studies have focused on the effects of reduced fetal nutrient delivery (by altering maternal diet, restricting placental growth or taking advantage of natural variation in birth size within litters), maternal body weight and composition and dexamethasone administration on fetal and placental development[47–53]. Similarly to small-animal models, although prohibited somewhat by the cost of long-term follow-up, large-animal models have also been used to investigate the long-term consequences of an altered prenatal environment. The offspring of sheep exposed to global nutrient restriction during early-to-mid pregnancy have been shown to exhibit increased blood pressure in some studies[54–56], but not others[57,58]. The timing of nutrient restriction varies between the first 30 d of pregnancy *v.* 30–80 d of pregnancy and could well explain the different blood pressure outcomes between studies. In pigs blood pressure at 3 months of age has been show to be negatively associated with birth weight[59], which is also associated with increased responsiveness of the hypothalamic–pituitary–adrenal axis to adrenocorticotropic hormone and insulin-induced hypoglycaemia[60]. Maternal nutrient restriction or fetal growth restriction have also been shown to be related to postnatal glucose homeostasis and insulin

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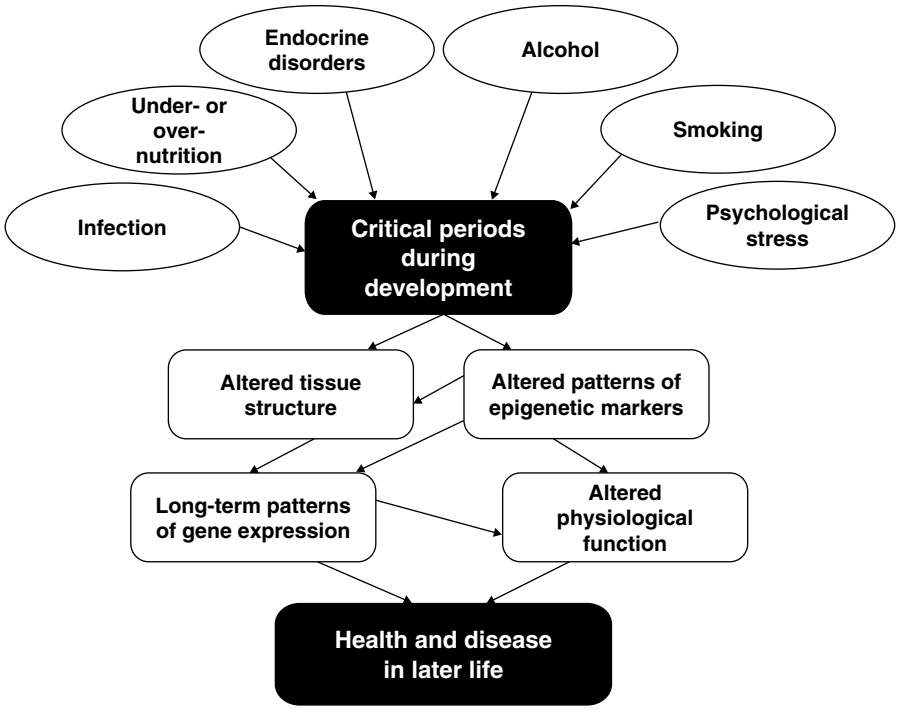

Fig. 2. A summary of current general concepts relating to the mechanisms underlying the developmental origins of health and disease. A variety of factors can act during fetal and early life to impact on tissues that are undergoing critical periods of development. This interaction may lead to altered tissue structure and/or altered patterns of epigenetic markers. These outcomes would be expected to impact on long-term gene expression patterns and physiological function, and thus health status in later life.

sensitivity in the pig and sheep[61–64], with a study in sheep suggesting that the capacity of β-cells to achieve compensatory increases in mass and function with ageing is impaired by exposure to an adverse fetal environment[64].

These experimental studies, amongst many others, have been critical in providing strong evidence of a causal relationship between maternal diet and postnatal disease risk. Importantly, long-term effects are observed across a number of species and in response to a range of physiologically-relevant factors, giving further support to the translation of the findings to the human situation. It must be noted, however, that the evidence of specific associations is not always consistent, perhaps reflecting differences in the species or strains of animals used and the composition of experimental diets[21,65]. There is also a clear interaction between prenatal exposures and postnatal environment, with some developmentally-programmed outcomes not becoming apparent unless the offspring are followed into the ageing period[66,67] or challenged in postnatal life[68–70], perhaps as a result of a mismatch between prenatal and postnatal diets[71].

Mechanisms of developmental programming

Small-animal models have been used extensively to investigate the mechanisms underlying the DOHaD. Their quick generation time and relatively inexpensive costs, together with the excellent availability of molecular tools for the mouse and rat, make them a useful model for testing specific hypotheses relating to biological mechanisms. Despite the time and cost constraints of large-animal models, sheep in particular have also been used to investigate the mechanisms that generate physiological changes in response to an altered maternal environment. Together, these studies have given rise to a number of general and interrelated mechanistic concepts (Fig. 2), which will be outlined.

Many animal studies have focused on identifying: (1) changes in physiological function that are associated with increased disease risk, e.g. altered cardiovascular function, renal function or insulin sensitivity; (2) alterations in expression and activity of the systems regulating such physiological function, e.g. the activity of the renin–angiotensin system or insulin signalling pathways. There has been a wealth of literature published within these two interrelated themes, which contribute greatly to the current understanding of the developmental origins of disease. However, the true initiating mechanisms by which maternal diet impacts on long-term physiology and health remain poorly understood; how do environmental factors act during development to have such long-term effects on metabolic regulatory systems? Changes in the expression or activity of regulatory systems observed during postnatal life could simply be secondary to the observed programmed phenotype, perhaps mediating its progression. After all, it is inevitable that tissues exhibiting altered

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physiological function, be it increased glomerular filtration rates in the kidney or insulin sensitivity in the muscle, for example, will also exhibit altered patterns of gene and protein expression. Alternatively, the changes in expression or activity observed may actually be drivers of the programmed phenotype. At present, theory suggests that such drivers could arise from the developmental period as a result of the impact of maternal diet on processes determining tissue structure or the pattern of epigenetic markers set down during the reprogramming of the embryonic genome.

The impact of maternal diet on tissue structure. Several models have demonstrated a reduction in cell number or a change in the balance of cell types within tissues, which may account for their altered gene expression and physiological function. A reduction in nephron number, for example, has been observed in response to a prenatal low-protein diet in the rat[38,72,73] and mouse[74] and in uterine-ligation models in the guinea-pig[75] and rabbit[76]. A reduction in nephron number has also been observed in offspring of sheep exposed to reduced nutrition during early-to-mid gestation[77,78]. It has been proposed that a nephron deficit may predispose to an accelerated age-related decline in renal function and the onset of hypertension[79]. Similarly, in the pancreas a low-protein diet in the rat has been shown to reduce total pancreatic weight, islet cell mass and the relative contribution of β-cells to the islets[33,80,81], perhaps as a result of an altered balance of precursor cells during development that contribute to the α- and β-cell lineages[82]. Such structural changes within the pancreas may contribute to the subsequent impaired glucose homeostasis[40]. Maternal undernutrition during the critical proliferative period for muscle fibre development has also been shown to affect the numbers of secondary muscle fibres in the young offspring of a variety of species, including rats, guinea-pigs, sheep and pigs[46]. However, the long-term consequences remain unclear, as studies of adult offspring are limited and suggest that offspring have been able to compensate for the effects observed earlier in life[46]. In the brain a low-protein diet during pregnancy in the rat results in a reduced density of capillaries within the cerebral cortex[83] and lower densities of neurons expressing appetite regulatory systems[84]. Together these studies demonstrate that maternal diet can impact on the key processes of proliferation and differentiation, in a way that could drive disrupted physiology in later life.

Epigenetic programming. The term epigenetics has been defined as the study of heritable changes in genome function that occur without alterations to the DNA sequence[85]. Within DOHaD the study of epigenetic mechanisms that have the potential to link the intrauterine environment to postnatal outcomes is relatively 'young'. However, researchers within the field have harnessed the techniques available to determine whether maternal diet can influence the patterns of epigenetic markers within the genome, thus providing a mechanism by which maternal diet can permanently affect gene expression patterns[86]. Early evidence is emerging from small- and large-animal models that early-life nutrition may impact on both methylation and histone acetylation. Hypomethylation of the genes encoding the PPARα and glucocorticoid receptors has been demonstrated in the livers of rats exposed to a low-protein diet during pregnancy, which is associated with increased expression of both genes[87]. Low-protein offspring also exhibit changes in histone acetylation of the glucocorticoid receptor promoter that would further facilitate transcription. Hypomethylation of the proximal promoter of the type 1b angiotensin receptor has also been observed in the adrenal glands of low-protein rat offspring, and is associated with a persistent up-regulation of type 1b angiotensin receptor gene expression that may contribute to the development of hypertension[88]. Hypomethylation has also been observed in sheep that have been exposed to a diet deficient in methyl donors for a period of 8 weeks before conception and for the first 6 d of pregnancy. These sheep exhibit insulin resistance and elevated blood pressure in postnatal life. Restriction landmark genome scanning has shown that 4 % of 1400 CpG islands in the fetal liver are differentially methylated in a way that may cause overexpression of certain genes[89]. A recent study of human monozygotic twins has challenged the view that methylation patterns remain unchanged after establishment in early life[90]. However, the demonstrated effects of maternal diet on offspring methylation patterns provide a tempting theory to explain how maternal diet may bring about permanent changes in gene expression. In addition, epigenetic marks have been shown to be stably inheritable, and epigenetics therefore offers a plausible explanation of how environmental exposures in a single generation can impact on more than one subsequent generation[91,92].

Overexposure to maternal glucocorticoids. The mechanisms by which maternal diet acts on the developing tissues to cause long-term changes in tissue structure and gene expression patterns remains poorly understood. However, a large body of evidence suggests that imbalances in the maternal diet are associated with overexposure of the fetal tissues to glucocorticoids. This overexposure is thought to occur via reduced activity of placental 11β-hydroxysteroid dehydrogenase, which acts to convert maternal glucocorticoids to inactive forms, or by increased activity of the glucocorticoid receptor in fetal tissues. Studies of rodents and sheep have demonstrated that exposure to synthetic glucocorticoids can impact on renal development and subsequent blood pressure[93–97]. The similarities in phenotype associated with dietary and glucocorticoid exposures have led to suggestions that they may act through a shared mechanism, i.e. that maternal diet may act to increase exposure of the fetus to glucocorticoids. In sheep alterations in maternal cortisol in response to undernutrition appears to be dependent on the stage of gestation, being elevated or unchanged with late-gestation undernutrition[98,99] and reduced with early-to-mid-gestation undernutrition[100,101]. However, a reduction in placental 11β-hydroxysteroid dehydrogenase expression or activity has been observed in response to a low-protein diet in the rat[102,103] or global undernutrition in the sheep[104,105]. In addition, increased expression of the glucocorticoid receptor has been observed in a number of tissues in the neonatal sheep exposed to maternal nutrient restriction[105]. Giving further support to the theory of a common mechanism, inhibition of maternal glucocorticoid synthesis using the pharmacological agent metyrapone has been shown to prevent the nephron deficit and raised blood

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pressure observed in the low-protein rat model[106], suggesting that these outcomes are dependent on overexposure of the fetus to maternal glucocorticoids. The precise mechanisms and gene targets by which glucocorticoids impact on development and subsequent health remain under investigation. During development glucocorticoids act through a number of mechanisms to alter the balance between tissue proliferation and differentiation[107], with many genes containing glucocorticoid response elements. Overexposure of the fetus to glucocorticoids may therefore explain some of the effects of maternal diet on tissue structure and subsequent function.

Small-animal models

Advantages of working with small-animal models

Short duration of gestation and lifespan. A major advantage of working with small-animal models is their short gestation and lifespan, and the relatively inexpensive costs of maintaining large cohorts of animals. These factors enable studies across the lifespan and into subsequent generations. Ageing studies have been particularly effective in demonstrating the long-term sequelae of events that occur in response to dietary interventions in early life. Rats exposed to a low-protein diet during fetal development show little evidence of metabolic abnormalities at 9 months of age, although blood pressure is elevated from early life. By 18 months of age, however, the rats are hypertriacylglycerolaemic and hypercholesterolaemic and have developed hepatic steatosis[66]. Gene-expression changes observed in early adult life are often transient, and secondary changes in gene expression and tissue function observed later in life form an important part of the life course of the disease process. Lifespan studies have also been possible in rodent models and shortened lifespan has been observed in rats[108,109] and mice[70] exposed to a low-protein diet during the fetal development.

The short generation times have also enabled studies of the transgenerational effects of maternal diet. A recent study has demonstrated that the low nephron number and raised blood pressure observed in first-generation offspring of low-protein-fed rats is also observed in the second generation[92]. Interestingly, the programmed phenotype is transmitted down both the maternal and paternal lines, indicating that epigenetic reprogramming of the germ lines may have taken place. Similarly, exposure to dexamethasone during pregnancy leads to elevated phosphoenolpyruvate carboxykinase and disrupted glucose homeostasis in first- and second-generation offspring[91], with transmission through both the maternal and paternal lines. In both studies the effects of maternal protein restriction or dexamethasone administration were shown to have been resolved by the third generation. This important insight into the transgenerational effects of factors that impact on fetal development has really only been feasible because of the availability of small-animal models.

Control over genetic and environmental variability. A major advantage of working with rodent models is the high extent of control over genetic and environmental variability. Outbred strains of rat (such as the Wistar or Sprague Dawley) are the predominant model within the DOHaD literature. Genetic variation does exist within outbred strains and each individual is genetically unique. However, outbred colonies are considerably less variable than human populations as a result of being maintained for many generations in relatively-small closed colonies. In contrast, inbred strains of rat are isogenic, each exhibiting a unique set of phenotypic characteristics and providing an absolute control over genetic variability. The level of control over biological variability increases the efficiency of research and reduces the number of animals required[110]. In addition to the control over genetic variability, the ability to tightly control environmental factors (light, temperature, food and water intakes etc.) in modern small-animal housing facilities further reduces the contribution of extraneous factors to any differences observed between treatment groups.

Availability of GM models. In the wider literature studies of rodent models that under- or overexpress a particular gene have made major contributions to the elucidation of gene pathways involved in metabolic disease. The application of such models to test novel hypotheses within the DOHaD field offers similar potential, but has been a little-used resource to date. For example, a variety of target genes have already been suggested to be involved in mediating the effects of nutritional or glucocorticoid exposures on developmental processes and future health (e.g. encoding the angiotensin receptors or PPARα), but their involvement is yet to be assessed using GM models in this context. Several studies have, however, used knock-out mice with particular cardiovascular phenotypes to demonstrate effects of the prenatal environment on postnatal disease outcomes, whilst maintaining a high extent of control over genetic variability. The effects of maternal hypercholesterolaemia on postnatal vascular function have been assessed using an LDL receptor-knock-out mouse model[111]. The LDL receptor is involved in clearing lipoproteins from the circulation and mice lacking a functional LDL receptor develop hypercholesterolaemia and arteriosclerosis. Female and male LDL receptor-knock-out mice and their wild-type counterparts were cross-bred to produce heterozygous pups that developed in a hypercholesterolaemic mother (i.e. maternally-derived mutation) or normal wild-type mother (i.e. with a paternally-derived mutation). Despite being genomically similar, heterozygous offspring that developed in a hypercholesterolaemic mother were found to have abnormal vascular function in comparison to those that developed in a normal wild-type mother. A similar approach has been taken with the ApoE-knock-out mouse to demonstrate increased total cholesterol levels and incidence of atherosclerosis in offspring born to hypercholesterolaemic mothers compared with genomically-similar offspring born to wild-type mothers[112]. Abnormal vascular function has also been observed in heterozygous offspring born to mice lacking the endothelial NO synthase gene in comparison with those born to wild-type controls[113]. Such experimental designs enable investigation of the contribution of the uterine environment *v.* that of parental genetics.

The ApoE*3-Leiden mouse differs from the ApoE-knock-out mouse in that impaired clearance of lipoproteins

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from the circulation and development of atherosclerosis only occur when the mice are fed diets rich in cholesterol[114]. This model has therefore enabled investigators to evaluate the influence of maternal diet on the development of atherosclerosis. Exposure to a prenatal low-protein diet was shown to increase the extent of dyslipidaemia and severity of atherosclerotic lesions in mice fed an atherogenic diet in postnatal life when compared with mice exposed to a control diet prenatally[115]. Importantly, this study demonstrates interactions between genotype, prenatal environment and postnatal diet, with evidence of gene–nutrient interactions at an early stage of development influencing responses made to dietary challenges in later life.

Genetic modifications also offer the potential for the more detailed analysis of mechanisms potentially involved in developmental programming. For example, transgenic Tie2-GFP mice express green fluorescent protein under the direction of the endothelial-specific receptor tyrosine kinase promoter. Endothelial cells expressing green fluorescent protein can be visualised via fluorescent microscopy. The use of this transgenic model has enabled investigation of the effects of a prenatal low-protein diet on the placental vasculature by assisting with visualisation of blood vessels during analysis of immunoreactivity[116]. This study has shown that low-protein-fed dams exhibit perturbation of vascular endothelial cadherin and β-catenin, regulators of junctional integrity, permeability and quiescence.

Ease of manipulation of diet. Small-animal models of developmental programming have utilised a wide variety of nutritional interventions, including both under- and over-nutrition (Fig. 1). The ease of manipulation of rodent diets is an advantage because it allows the controlled evaluation of very specific changes in dietary composition. This approach has enabled investigation of specific nutrient deficiencies and problems within the human population. Globally, Fe-deficiency anaemia is the most prevalent micronutrient deficiency[117]. The feeding of an Fe-deficient diet before and during pregnancy in the rat leads to alterations in cardiac development and elevated blood pressure in their offspring[118,119]. Offspring of Fe-deficient rat dams also exhibit perturbed fatty acid metabolism in later life[120]. The most widely used nutritional intervention during pregnancy is protein restriction, with the offspring of protein-restricted rats and mice exhibiting a shorter lifespan[108,110], elevated blood pressure[27,92], disturbed glucose homeostasis[40,67], vascular dysfunction[121], impaired immunity[122] and an increased propensity to develop obesity[36]. Availability of protein varies considerably between regions of the world and an estimated 65 % of the world population are at risk of protein intakes below the UK reference nutrient intake for pregnancy[22].

In developed countries maternal overnutrition is a more relevant concern. Consistent with models of undernutrition, maternal high-fat feeding in rats is associated with elevated blood pressure, vascular dysfunction[32,123], obesity and glucose intolerance[124]. Similarly, cafeteria feeding during pregnancy has been shown to programme adiposity and altered feeding behaviour in the rat[125,126]. In the mouse diet-induced obesity leads to elevated blood pressure, adiposity and insulin resistance in the offspring[127]. Exposure to a high-protein diet during pregnancy has also been linked to increased risk of obesity[128]. Collectively, these studies in small-animal models have been critical in demonstrating the long-term effects of a wide range of nutritional manipulations relevant to the human situation.

Disadvantages of working with small-animal models

Large litter size. The large litter sizes observed in rats and mice are a major disadvantage of working with rodent models of prenatal programming. Space sharing within the uterus results in each pup developing in a slightly different environment. Mice that occupy a position at either end of a uterine horn, for example, receive more nutrient-rich maternal blood and have heavier birth weights than their littermates[129]. The same is true for rat pups that develop at the cervical end of the uterus. Fetal development within litters may also be influenced by exposure to different levels of hormones, depending on whether the fetuses are positioned next to male or female littermates[130]. These intrauterine exposures may contribute to variability in phenotype between offspring within a litter. Controversy exists over how to select offspring from a litter for postnatal study, with some investigators using random selection and others selecting pups closest to the median birth weight. Litter size may also contribute to variability between litters, with litter size generally positively correlated with average birth weight[131]. Whilst variability in litter size cannot be controlled for during the prenatal period, it is common practice for the number of pups per litter to be reduced to a set number at birth, reducing variation in post-suckling nutrition[132].

The hierarchal statistical design of experiments with litter-bearing species has been the centre of debate in the past[133,134], and it is essential that differences in variation within and between litters are accounted for in the statistical analysis[135,136]. In many studies the use of one male and/or one female offspring per litter circumvents this issue, but it is not uncommon to see studies published in which a low number of animals within each treatment group have been obtained from an even lower number of litters. The statistical robustness of such studies should be questioned at the manuscript review stage and the advice of a statistician sought.

Rodents are born immature. When comparing the outcomes of interventions during development between different animal models and when extrapolating conclusions to man, it is important to remember that the timing and trajectory of developmental processes differs between species. A particular limitation of small-animal models is that they are generally altricial species and are therefore relatively immature at birth in comparison with man and large-animal models (e.g. sheep and pig). An exception is the guinea-pig, a precocial species that give birth to neuroanatomically-mature young. However, the most commonly used small-animal species, mice and rats, are born with a poorly-developed central nervous system and autocrine system and the development of organs implicated in the programming of disease (e.g. the pancreas and kidney) continues into postnatal life. The periods of vulnerability of the developing systems therefore differs between

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species and interventions directed at the same stage of gestation cannot be considered comparable. For example, nephrogenesis is complete by 32–34 weeks of gestation in man (term approximately 40 weeks) and by 130 d of gestation in the sheep (term approximately 145–150 d). In contrast, nephrogenesis in the rat continues into the postnatal period and is not complete until approximately 8 d after birth. It is therefore important that the mechanisms of impact of dietary interventions during early life are discussed within the context of their timing in relation to developmental processes rather than the stage of gestation *per se*. For example, glucocorticoid administration has been shown to be most effective in programming hypertension when administered to sheep at 26–28 d of gestation[95] and rats at 15–16 d of gestation[97]. Although representing very different stages of gestation in sheep and rats, these time points are similar in terms of the stage of renal development, when increased apoptosis of mesenchymal cells could affect nephron formation and thus final glomerular number[137]. The altricial nature of small-animal models can also be an advantage. For example, the critical window for islet development is postnatal in the mouse and rat, and is therefore more readily accessible for experimental manipulation than in species in which the critical window lies earlier in gestation.

Diets fed to rodents are not the equivalent of human dietary patterns. As discussed previously, small-animal models of developmental programming have used a wide variety of nutritional interventions, including both under- and overnutrition. In the early animal studies primarily aimed at demonstrating proof of principle it could be argued that the precise composition of the experimental diets was of lesser importance. These studies were effective in demonstrating independent effects of maternal diet on offspring metabolic function and disease risk, and the consistency in the effects of a wide range of types and extents of nutritional intervention gave strong support to the general DOHaD theory. Having demonstrated proof of principle, the research priorities have since turned to more closely modelling human disease pathways, with the ultimate aim of contributing scientific knowledge that will help to predict the outcomes of dietary behaviours and interventions in the human population. The hypotheses generated therefore require animal models to reflect the human situation as closely as possible, thus strengthening the ability to translate the findings back to the study of human subjects. Although the ease of manipulation of rodent diets is an advantage because it allows the controlled evaluation of very specific changes in dietary composition, the experimental diets fed do not necessarily reflect the heterogeneity of the human diet or levels of exposure that are physiologically relevant. Cafeteria feeding overcomes these issues to some extent and provides a useful alternative to the feeding of purified high-fat diets, inducing persistent hyperphagia and increased energy intakes[138,139] as a result of the variety and novelty of the foods available. The dietary pattern observed with a cafeteria feeding system more closely reflects that of the 'non-prudent' human subject[140,141] and avoids the very high intakes of specific and potentially-biologically-active fatty acids that are observed in other models of high-fat feeding. It may therefore be an effective tool for modelling the effects of 'non-prudent' dietary patterns in human subjects.

Rodents do not develop the same disease profiles as human subjects. Rodent models are limited in their ability to model associations with CVD. In comparison with human subjects, they manifest relatively low levels of total cholesterol and LDL-cholesterol and high levels of HDL-cholesterol and are resistant to the development of hypercholesterolaemia and atherosclerosis[142]. The use of monogenic or transgenic animals has yielded models that exhibit circulating lipid profiles and atherosclerotic end points similar to those of human subjects. However, their use for testing interventions risks the possibility that results arise from the model's genetic background rather than the exposure of interest. The literature demonstrates consistent evidence of moderate hyperglycaemia, hyperinsulinaemia and hypertriacylglycerolaemia in response to high-fat feeding[142]. This outcome is associated with increased number and size of adipocytes[143,144], decreased skeletal-muscle insulin sensitivity[145] and hepatic steatosis[146], all key components of the metabolic syndrome. It is therefore possible to induce a metabolic phenotype in rodents that is similar to that observed in the human subject, despite not exhibiting progression to an atherosclerotic state. However, they remain limited in their ability to directly model associations with specific CVD outcomes relevant to the human situation.

Large-animal models

Research into the developmental programming of tissue structure and physiological function is of interest to animal scientists and developmental biologists aiming to maximise growth and development in large-animal species for production purposes. However, within the context of DOHaD there is the potential to extrapolate findings from large-animal models to the human situation. The present review will consider three large-animal models: an established model (sheep); an emerging model (pig); an ethically-restricted model (non-human primate). Given the physiological disparity between these three models, the benefits and disadvantages of each model will considered separately. This position is in contrast to the most commonly used small-animal models (rats and mice) that have considerably more similar anatomy and physiology.

Clearly, man is closer, in evolutionary terms, to primates. However, the other large mammals have some important similarities and differences that can be utilised for experimental purposes (Table 1). Human subjects usually bear one offspring and, depending on breed, sheep may have one to three lambs. The average birth weight in human subjects is also similar to that of a singleton lamb (approximately 3·5 kg). In contrast, pigs are litter bearing and the birth weight within a litter exhibits a 'U'-shaped curve.

The gastrointestinal system of the sheep is of the ruminant type and sheep breakdown the plant products they consume via commensal bacteria in the gut, producing volatile fatty acids as their major source of energy. In contrast, pigs, human subjects and primates can consume a

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Table 1. Comparison of characteristics of large animals *v.* man[147,148]

	Placentation	Offspring number	Birth weight (kg)	GI system	Postnatal nutrition
Man	Discoid	1	3·4 (UK average)	Single stomach	↓Protein ↓Fat ↑Sugar
Sheep	Cotyledonary	1, 2 or 3	3–5	Ruminant	↑Protein ↑Fat ↓Sugar
Pig	Diffuse	Litter bearing	0·6–3	Single stomach	↑Protein ↑Fat ↓Sugar
Non-human primate	Discoid	1 or 2	1–2	Single stomach	↓Protein ↓Fat ↑Sugar

GI, gastrointestinal; ↓, ↑, restricted and high levels respectively.

varied omnivorous diet[147]. Differences also exist in terms of postnatal nutrition, with sheep and pig milk containing markedly more fat and protein than human milk[148]. All the large species presented have a functioning hypothalamic–pituitary–adrenal axis before birth, unlike rats and mice that are altricial. As the size of the animal model increases, the cost of housing and feeding the animal increases. With more intelligent animals the requirement for environmental enrichment also increases. Work with non-human primates is often limited by ethical concerns and there is little work on these animals within the DOHaD field in the UK.

Similarly to small-animal models, a variety of experimental procedures have been used to generate models of developmental programming in large-animal species. Placental restriction has been employed by several groups using the sheep as a model[149]. The sheep has a cotyledonary placenta and removal of a percentage of attachment sites produces an overall reduction in the delivery of nutrients to the fetus. The natural variation in birth weights within pig litters can also be used to model associations with birth weight. The large litters produced comprise piglets with birth weights conforming to a 'U'-shaped relationship and both extremes of birth weight can therefore be examined[62,150]. There are fewer studies of the effects of overnutrition in larger-animal models compared with rodents, but there have been studies in the sheep and pig[151–153]. Undernutrition during pregnancy is the area that has received most interest in large-animal models, most notably in the sheep[154,155].

The major disadvantages for all large-animal models are the time and financial implications of offspring generation and postnatal follow-up, particularly when investigating intergenerational effects. The average gestation length of the sheep is approximately147 d and individual housing, feeding and monitoring a pregnant ewe during this period is very costly. Once the time periods to sexual maturity (6 months–1 year) and for postnatal follow-up are factored in, the cost and time constraints can be prohibitive. Securing funding for studies of large-animal models therefore requires a sound argument for their superiority over less-costly alternatives. Despite these issues, long-term follow-up studies have been done. The influence of prenatal glucocorticoid infusion on offspring has been investigated up to 7 years of age[156]. However, this study is the exception and very few studies have investigated true long-term effects on programmed offspring in large-animal models. Some researchers might argue that the 'normal' lifespan of these domestic species is rarely past sexual maturity, especially for non-breeding males, as these breeds are all utilised commercially in food production.

An established model: the sheep

The sheep has been used in fetal and neonatal physiology since the seminal experiments in the 1960s that demonstrated the crucial role of glucocorticoids in the preparation of the fetus for birth[157]. The sheep has a number of benefits for DOHaD work including: the generation of a large singleton offspring (in most breeds); expression and activation of uncoupling protein 1 (sheep have brown adipose tissue) and maintenance of homeothermy by non-shivering thermogenesis at birth; ability to tolerate fetal catheterisation; housing can be simple outdoor pasture.

However, the sheep is a ruminant and does not metabolise energy in the same way as single-stomach species (man, pig). Care must therefore be taken when extrapolating any metabolic findings. In particular, glucose tolerance tests may be much less meaningful in the sheep as ruminants do not utilise glucose in the same manner as human subjects. Instead, they rely on volatile fatty acids generated in the rumen and the composition of any ruminant diet will alter the volatile fatty acid profile[158]. Of ruminant glucose requirements <10% are absorbed from the small intestine, with the remaining 90% produced by gluconeogenesis[158,159].

A further difficulty of working with sheep is that it is impossible to determine whether ewes are carrying twins or singletons until scanning takes place, which often occurs after the start of a nutritional intervention. Given the cost of large-animal trials and the unpredictability of twinning rates, it is often not feasible to include sufficient animals to overcome imbalances in twins:singletons between treatment groups. To some extent this imbalance can be overcome by using breeds that have strong tendencies to produce singleton or twin pregnancies, but a certain unpredictability remains. A mismatch between the number of twin and singletons within experimental groups can

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therefore act as a confounding factor[160], although statistical correction for differences in twins : singletons between groups is a possibility. Similar criticism has been made of studies that do not indicate the gender dynamics of groups. Numerous studies have indicated gender-specific effects in response to an altered prenatal environment[161] and researchers must ensure adequate statistical power to determine gender effects before embarking on a study. However, this requirement may increase the number of animals substantially, with further cost implications.

An emerging model: the pig

As described earlier, farmers have long been aware that runt piglets grow less efficiently and produce a carcass with more fat and less muscle than a normal-birth-weight littermate. The physiological and molecular mechanisms leading to this costly adaptation are now being investigated under the auspices of the DOHaD hypothesis. Despite being a litter-bearing species, there are some key benefits of the pig in DOHaD research. Indeed, it is the differences between pig and man that provide the main use for this species. Pigs display a 2–3-fold difference in body weight amongst littermates, providing a natural model of differential fetal growth. The pig is relatively large at birth and is a precocial single-stomach species that can readily become obese, diabetic and hypertensive.

As with all models, there are some drawbacks in the use of the pig. The production of up to twenty piglets in one litter is in stark contrast to the singleton pregnancy observed in most human pregnancies. The controversy described for selection of rodent offspring within litters can also affect porcine studies; investigators must be explicit in the description of their experimental design and statistical analyses, highlighting exactly how many piglets are selected from each litter and the position of the piglet within the birth-weight hierarchy. This problem is negated when selecting for small-, normal- and high-birth-weight offspring. Similar cost and husbandry implications exist for the pig as for the sheep.

An ethically-restricted model: the non-human primate

Little DOHaD research on non-human primates is carried out within the UK because of moral and ethical concerns, but an active research group in the USA have utilised the model to demonstrate a number of molecular and physiological changes in response to different prenatal challenges.

The main advantages of using a non-human-primate model are clear from Table 1. The baboon (*Papio* spp.) is the most widely studied species and exhibits similar placentation, offspring number, metabolism and milk nutritional content to the human subject as well as a relatively long dependent-infant state. Despite the obvious benefits of the non-human primates, there are important financial, moral and ethical implications to be considered and work on these animals continues to be highly contentious[162]. In their natural environment baboons live in social groups characterised by dominance hierarchy that affects their likelihood of mating. Baboon species in captivity require substantial environmental enrichment and an environment conducive to the social order of the species; this requirement poses certain problems for monitoring food intake in pregnant animals. One group in the USA have overcome this problem[163]. The relatively long prepubertal development in non-human primates means that long-term follow-up studies are costly; a macaque (*Macaca* spp.), for example, does not reach puberty until approximately 4 years. Investigators must forward plan to a great extent in order to cover the costs involved in husbandry, housing and investigations. However, the working group report on the use of non-human primates in research mentions fetal development and disease in later life as an area that may have a role for use of non-human primates in UK research in the future[162].

Novel models

Future research into the DOHaD area may not lie entirely in the small- and large-animal models that have been most commonly used to date and presented in the present review. Work has been reported from novel species such as the spiny mouse (*Acomys cahirinus*) and zebra finch (*Taeniopygia guttata*), which have specific attributes that make them a suitable model for testing specific hypotheses. The spiny mouse is a rodent adapted for life in desert regions and its reduced nephron number and cortex : medulla are thought to allow the production of highly-concentrated urine during periods of dehydration[164]. Compared with laboratory breeds of mice the spiny mouse has a longer gestation, fewer offspring and is relatively mature at birth, e.g. nephrogenesis is complete[164]. This adaptation may provide a useful model that bridges the gap between small and large animals; the spiny mouse is small and reproduces in a short time frame allowing transgenerational effects to be investigated, but displays a trajectory of renal development that is more similar to that of man. Dexamethasone treatment of the spiny mouse at a stage of gestation coinciding with the early stages of the nephrogenesis leads to a reduced nephron number in the offspring, although an elevation in blood pressure is not apparent[165]. Avian species also provide a novel model in which to investigate the effects of glucocorticoid exposures during the developmental period, independently of maternal postnatal influences. In an altricial bird species, the zebra finch, postnatal exposure to glucocorticoids has been shown to influence the response of the hypothalamic–pituitary–adrenal axis to stress in later life[166]. Precocial bird species exist and may provide an additional avian model.

Conclusions

Animal models have been essential in providing proof of principle of the DOHaD concept, by providing strong evidence of a causal relationship between a variety of environmental challenges during development and disease risk in later life. Studies of animal models continue to be essential to the evolving theory of the mechanisms of the developmental origins of disease. A range of small- and large-animal models have been developed, each with their own inherent advantages and disadvantages. None of these animal models can be considered superior for all aspects

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of research in this field, and the choice of model will differ according to the specific hypotheses. Collaboration between those researchers working with different small- and large-animal models will provide the DOHaD field with stronger hypothesis-driven research outcomes with benefits for animal and human health.

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