Imprinting of Genes and the Barker Hypothesis

Lorraine E. Young

Roslin Institute, Scotland

Several common adult diseases appear to be related to impaired fetal growth and this may be caused either by nutritional inadequacies at particular stages of pregnancy or by variation in alleles at specific growth loci. Little is known about the genes involved in the underlying mechanism. This review proposes that at least some of the effects have their origins at imprinted loci, genes that are unusual because they are expressed from only one parental allele. Many imprinted genes are crucial for fetal growth and determine birthweight. They can be disrupted in the early embryo by environmental influences and these disruptions can be inherited through many cell cycles into adult tissues. Their disruption can affect specific organs during fetal development and disruption could affect adult disease in a variety of direct and indirect means. Imprinted genes may be particularly vulnerable to disruption as they are functionally haploid and their expression is regulated by different means from the rest of the genome. Thus many imprinted genes provide plausible candidates for programming adult disease and warrant further study in this context.

There is now considerable evidence that growth-variations *in utero* correlate with major adult disease states such as hypertension, cardiovascular disease and diabetes. Several hypotheses have been suggested to account for the birthweight variation and the related fetal origins of adult disease. One proposes that these are due to genetic variations in growth-related loci (McCarthy, 1998). The Barker Hypothesis, arising from epidemiological data, predicts that disease effects are due to nutritional constraints at critical phases of key fetal organ development, effects now substantiated by several animal models (reviewed by Barker & Clark, 1997). Recent work from the field of livestock embryology has extended this hypothesis by demonstrating that very early events in the preimplantation embryo can also have significant impact on later development (reviewed by Young & Fairburn, 2000). These effects may be genetic and/ or epigenetic in nature and may also programme adult disease. Regardless of the origin, the mechanisms underlying fetal programming are largely unknown. This paper will briefly review the phenomenon of genomic imprinting and suggest that early developmental variations in this process may be the cause of at least some fetal growth variation and linked later life effects. As these hypotheses have not been tested, it is hoped that research in this area will be stimulated.

Gene nomenclature is confusing and often misused. For the sake of clarity, this paper will used the recommended human notation (http://www.gene.ucl.ac.uk/nomenclature/) for human and farm animal genes (Dolling et al., 1997), i.e. the use of italicized capital letters for the gene/ transcript and non-italicized capital letters for the corresponding protein. Mouse genes/ transcripts will be referred to by lower case italicized symbols, with lower case, non-italicized symbols for the protein, whereas upper case will be used for humans and livestock. Where the text refers to a general, non-species specific gene, the mouse notation will be used.

What is Genomic Imprinting?

Vertebrate genomes have a diploid set of genes, with a copy of individual loci inherited from each parent. While most genes are expressed from both parental loci simultaneously, there is a subset of genes in mammals that is only expressed from either the maternal or the paternal allele. This subset is known as the imprinted genes. Current estimates suggest there are between 100 and 500 present in the total human genome (see Murphy & Jirtle, 2000) of 100,000 genes. Some imprinted genes are expressed only from the maternal allele and some from only the paternal allele. The origin of the expressed parental allele does not appear to be related to positioning in the genome. To date around 50 imprinted genes have been identified (see http://www.mgu.har.mrc.ac.uk/imprinting/imprin-viewdatagenes.html) but many have not been fully cloned or still have unknown function. However, many of the imprinted genes with an identified function appear to act during fetal development, making them plausible candidates for fetal programming.

Imprinting is a dynamic process, both in forming the chromatin structure to mediate monoallelic expression (imprint formation) and in the manifestation of monoallelic expression throughout life (imprint maintenance; reviewed by Reik & Walter, 2001). Although imprints are generally maintained and somatically inherited through each cell cycle, some imprinted genes show monoallelic expression only at certain developmental stages or in specific tissues. Imprinting is a process that can also vary between mammalian species e.g. some genes that have been observed as showing monoallelic expression in mice, show biallelic expression in humans at equivalent stages of development or in the same tissue (John & Surani, 2000; reviewed by Young & Fairburn, 2000). Most of the imprinting studies to date

Address for correspondence: School of Human Development, Division of Reproductive Medicine, Queen's Medical Centre, Nottingham, NG7 2UH, UK. Email: Lorraine.Young@nottingham.ac.uk

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have focused on human and mouse and so there is only very limited comparative information in other mammals. Studies in sheep (McLaren & Montgomery, 1999) and marsupials (John & Surani, 2000), however, demonstrate that imprinting can occur in other mammals, but that the patterns of which genes are imprinted is not necessarily conserved.

Since imprinted genes are functionally haploid, they may be more vulnerable to inducing disease states when subjected to mutations or epimutations and thus their selection is somewhat puzzling. Current theories for evolution of genomic imprinting are reviewed by Hurst (1997). However the most cited theory at present suggests that there is asymmetry between parental contributions to the developing fetus and that imprinting has evolved to control this "parental conflict" (Moore & Reik, 1996). The mammalian fetus develops by removing nutrients from the mother, both from the placenta and during lactation. It is imperative for the fetus and it's littermates that the resources extracted from the mother are not detrimental to her health/ survival and it is advantageous to the mother that each pregnancy does not compromise the next (especially in non-monogamous species). However, the prevalence of mixed paternity in mammals results in selection for the paternally derived alleles in a fetus to demand relatively more resources from the mother as future pregnancies from the mother may be paternally-unrelated (Moore & Reik, 1996). Thus many imprinted genes have functions in fetal growth and/ or neonatal care and fetal size tends to be enhanced by paternally expressed genes and limited by maternally expressed genes.

Other imprinted genes identified so far have diverse functions in behaviour, X-inactivation, cell cycle regulation, RNA splicing, etc. It may be significant that a large number of imprinted genes are expressed in the brain and pituitary. Thus Li et al. (1999) have suggested that in addition to imprinted effects on behaviour, there may be crucial effects on central, endocrine regulation of reproduction.

Discovery of Imprinted Genes.

The phenomenon of genomic imprinting was discovered in the early 1980's by two independent pieces of research. One group was performing a comprehensive genetic analysis of translocations in the mouse genome and discovered that specific regions of the genome produced reproducible phenotypes when one parental region was deleted or duplicated, but no effect when the other parental region was affected (Cattenach & Kirk, 1985). This was the first indication of non-equivalence of the two parental copies of specific genes. This work also demonstrated that the genes that were imprinted tended to be clustered on specific chromosomes and were not just randomly dispersed throughout the genome.

Simultaneously, another group (Surani et al., 1984) had used micromanipulation to create mouse embryos with either two female (gynogenotes) or two male (androgenotes) pronuclei to form diploid embryos with uniparental genomes. They found that the gynogenotes tended to have large placentae and smaller fetuses while the androgenotes produced larger fetuses with more normal placentae. These monoparental embryos only developed to about mouse E10 and none were able to survive to term, highlighting the importance of correct imprinting for normal development. More recently, clever experiments by Kono et al. (1996) have demonstrated that mouse gynogenetic embryos can develop 3 days later (to E 13.5) when the nuclei used arise from early-stage, non-growing oocytes that are known to be imprint-free.

Since these pioneering experiments, many new imprinted genes have been discovered by molecular methods used for differential genome screening, such as differential display, subtractive hybridization and restriction landmark genome scanning (Kamiya et al., 2000; Kaneko-Ishino et al., 1997).

What Regulates Imprinted Expression?

The full mechanism involved in regulating monoallelic expression from a gene is not fully understood and may act both at the genetic (sequence) and epigenetic (modifications which alter DNA structure/function rather than sequence) levels. Several sequence features of imprinted genes, including repeat sequences near imprinting control regions and antisense promoter sequences have been implicated in regulating the imprinting process (recently reviewed by Reik & Walter, 2001). Another functional consequence (or possibly effector) of genomic imprinting is asynchronous replication of alleles during mitosis, with earlier replication of the paternal allele (Bickmore & Carothers, 1995). However, the epigenetic DNA modifications that are ubiquitous mechanisms in cellular gene silencing, likely repress the silenced allele. These modifications include DNA methylation, histone acetylation and differences in chromatin structure. Some or all of these allele-specific features may confer imprinted expression by modulating allele-specific access to expressive or repressive transcription factors (Reik & Walter, 2001; Tilghman, 1999).

The methylation of cytosine residues in CpG dinucleotides almost invariably shows differential status between the expressed and silenced copies of DNA at imprinted loci. DNA methylation is a ubiquitous part of the mechanism used throughout the genome for silencing DNA in cells where expression is not required. This is thought to include regulation of tissue specific gene expression (by methylating the promoter control regions of silenced genes) and silencing of foreign DNA such as viruses and other parasitic sequences that have entered the mammalian genome throughout evolution (reviewed by Robertson & Wolffe, 2000). In imprinted genes however, allele-specific DNA methylation can occur at the promoter and/or other regions in the gene and can be involved in activating the expressed allele, as well as silencing the non-expressed allele, in a gene-dependent manner. The "differentially methylated regions" or DMR's of most imprinted genes examined in mouse transgenic studies are essential to maintain monoallelic expression. (Reik & Walter, 2001). In mice with null mutations for the DNA methylating enzyme, DNA methyltransferase 1 (*Dnmt1*), some imprints are lost, indicating a crucial role for DNA methylation in determining imprinted status of a gene (Li & Jaenisch, 1993). However, since there are at least two other methyltransferases (see Robertson & Wolffe, 2000), it is not yet possible to conclude that DNA

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methylation is not an important feature of imprinted genes that appear not to be affected in *Dnmt1* null mice.

Methylated promoters are transcriptionally repressed by a mechanism involving DNA methylation, CpG binding proteins (methyl binding domains or MBDs), as well Dnmt1 and histone acetylases (Robertson & Wolffe, 2000). Histones, the proteins that are interspersed with DNA to form it's 3D structure, are deacetylated by a deacetylase enzyme in inactive regions of the genome and acetylated in regions where the genes are expressed. Differential allelic acetylation of histones has been found for several imprinted genes (see Reik & Walter, 2001). Recently, two research groups identified a protein that directly links DNA methylation and histone acetylation and which presumably silences DNA on the inactive allele by preventing transcription factor access (Robertson & Wolffe, 2000). However, how these processes are linked in an allele-specific fashion remains to be established. Current thinking suggests that allelic DNA methylation patterns established in the germline (when the parental genomes are segregated) constitute the primary mark that distinguishes the two alleles at imprinted loci (see below). Presumably this eventually sets up differential chromatin structure and allelic expression patterns.

When are Genes Imprinted?

Imprinting is a dynamic process but it is generally considered that "primary" imprints that mark the alleles as male or female are established in the germline (reviewed by Kono, 1998). The nature of the primary imprint is unknown but is likely to involve DNA methylation, either as an initiating effect or secondary to another epigenetic modification such as allele-specific protein binding. Precisely when primary imprints are established during gametogenesis is still unknown. The period after fertilization before the pronuclei are fused may also provide an opportunity for some imprints to be established (Moore & Reik, 1996). At least in the mouse, there are considerable differences in genome wide methylation events in the male and female pronuclei. Imprinted genes then may be further modified ("secondary imprints") in a sex-specific manner during preimplantation development and after implantation to ultimately confer imprinted gene expression in the target tissue, a phenomenon which appears to occur after implantation in the mouse (Latham et al., 1994) but that can occur in the preimplantation embryo in human (Lighten, 1997). For "mature" imprints to be read, i.e. translated into monoallelic expression, it may be that cell-type specific transcription factors need to be present as well as allele-specific epigenetic modifications (Reik & Walter, 2001).

Imprinting methylation marks often persist into the adult. This is consistent with the view that DNA methylation patterns are stably inherited through many cell cycles. Since at least some mature imprints are clonally inherited into adult tissues, even if imprinted expression no longer occurs, the sperm and oocyte DNA contributed from each parent will contain imprints. Segregation of maternal and paternal diploid DNA during meiosis will result in half of the gametes from both sexes inheriting their paternal copy of DNA and half inheriting their maternal copy of DNA. Thus all inherited imprints must be erased in the gametes to ensure that each offspring's sperm or egg cells all contain only male or female imprints, respectively (Reik & Walter, 2001). This occurs in both germlines at around E12-13 in the mouse as part of a genome-wide demethylation event (see Reik & Walter, 2001).

Imprints are then re-established at different times of development, depending on the gene and probably also on the species. However, information on this aspect is limited to only a proportion of imprinted genes so far identified and is also only available for the mouse. In the mouse male germline, allele-specific methylation occurs before meiosis. However, in the female imprints begin to be established during the phase of oocyte growth after first meiotic arrest (Reik & Walter, 2001). Imprints have been examined in various mouse genes during the oocyte growth phase, using diploid parthenogenetic embryos containing one pronucleus from a non-growing oocyte and one from a fully grown oocyte (see Kono et al., 1998). These experiments have established that specific loci are imprinted in different ways, in some cases maternally expressed genes are imprinted by repressive modifications during spermatogenesis, other maternally expressed genes activate the maternally-expressed allele during oocyte growth, while a further group of paternally expressed genes are imprinted by maternal repression during oocyte growth (Kono et al., 1998). Furthermore, it seems that different genes also establish secondary imprints at different times. Some genes have more than one DMR that is important for imprinting. The *Igf2r* (insulin-like growth factor 2/ mannose-6- phosphate receptor) gene, for example, has one DMR in the second intron that seems to act as a mark to distinguish the alleles and is already differentially methylated (but biallelically expressed) in the mature oocyte (Neumann et al., 1997). The other *Igf2r* DMR surrounds the promoter and is essential for monoallelic expression of the gene. The promoter DMR is differentially methylated after fertilization but expression is not monoallelic until after implantation. If the *Igf2r* intronic primary imprint is not present, monoallelic expression does not occur even if the promoter is differentially methylated. Imprints of other genes are fully established prior to fertilization and some are not established until implantation or later. Furthermore, the timing of all of these events may well vary at an individual locus between species.

DNA methyltransferase 1 has distinct transcript forms that are expressed during oogenesis and spermatogenesis, in addition to the somatic form. Recent evidence has suggested that the oocyte specific form, which is localized in the cytoplasm during most of preimplantation development, transiently enters the nucleus at the 8 cell stage to methylate at least some imprinted alleles (Howell et al., 2001). It is not yet known whether other DNA methyltransferases have oocyte or sperm specific forms.

Imprinted Genes and the Barker Hypothesis

The Barker Hypothesis suggests that coronary heart disease, stroke, hypertension and non-insulin dependent diabetes originate through impaired growth and development during fetal life and infancy and that other adult disease states such a cancer may also be related (Barker & Clark, 1997).

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The rationale is that these diseases may be consequences of 'programming' whereby a stimulus or insult at a critical, sensitive period of early life results in long-term changes in physiology or metabolism. The proposed mechanism is via the fetus adapting to lack of nutrients or oxygen by slowing its rate of cell division, possibly changing the distribution of cell types, hormonal feedback, metabolic activity and organ structure. As fetal nutrition relies not only on maternal dietary intake and nutrient stores, but also on placental nutrient delivery and transfer capacity, there are a number of possible routes for fetal nutrient restriction to occur. Taken to it's extreme, the Barker Hypothesis predicts that the diversity and form of human newborns is essentially determined by the intrauterine environment rather than the fetal genome (Barker & Clark, 1997). Regardless of whether the correlation between birthweight and adult disease turns out to be mostly genetically or environmentally determined, imprinted genes may have plausible roles through a variety of means, some of which are outlined here.

Imprinted Genes Determining Fetal Growth and Development.

Imprinted genes have diverse functions and it is important to emphasize that it is likely more imprinted genes will be identified. Below are highlighted some of those that are known to have a role in fetal growth and development.

Fetal growth is largely controlled by the complex insulin/ insulin-like growth factor (IGF) system. In particular, several growth–related imprinted genes are related to the expression and function of the potent fetal growth factor, insulin-like growth factor II (Igf2; reviewed by Young & Fairburn, 2000). The *Igf2* gene is paternally expressed in most fetal tissues of the mouse, human and sheep (McLaren & Montgomery, 1999). Mice with null mutations for this gene or patients with chromosomal loss in this region exhibit reduced fetal growth. Loss of imprinting of *Igf2* results in biallelic expression, excess production of *Igf2* transcripts and increased levels of Igf2 protein. In both experimental loss of imprinting of *Igf2* in mice and in human Beckwith Wiedemann syndrome, this increase in Igf2 results in fetal overgrowth (see Hastie, 1997). In some cases of Beckwith Wiedemann syndrome, the adjacent *H19* gene, which is maternally expressed to inhibit maternal expression of *Igf2*, is also disrupted. Levels of Igf2 protein can also be regulated by the Igf2 receptor (Igf2r), a multifunctional receptor that is not involved in Igf2 signal transduction but apparently acts to clear Igf2 from the circulation. Experimental deletion of the expressed maternal allele of *Igf2r* results in mice born 20-30% larger than normal, with an associated increase in circulating Igf2. However large sheep fetuses, born after *in vitro* culture of fertilized eggs to the blastocyst stage, showed a decrease in *IGF2R* transcript and circulating IGF2R protein in late gestation, but no corresponding increase in circulating IGF2 or tissue *IGF2* expression (Young et al., 2001). Thus Igf2r may also affect fetal growth in Igf2-independent mechanisms. That Igf2 has local growth promoting effects in the fetal tissues is demonstrated in *H19* null mice (Eggenschwiler et al., 1997). Although pups with a maternal *H19* deletion are 30% overgrowth at birth, Igf2 ligand is only elevated in the tissues and not in the circulation. *Meg1*/*Grb10* (growth factor receptor bound protein 10) is another maternally expressed gene that may inhibit transduction of the IGF's as well as insulin to direct fetal growth (Murphy & Jirtle, 2000; Reik & Walter, 2001). In both mouse and human the insulin gene is imprinted and can have fetal growth consequences. It is chromosomally adjacent to the *Igf2* gene and is also paternally expressed in the placenta, but not normally in the pancreas (Giddings et al., 1994). *Peg3* (paternally expressed gene 3) null mice exhibit fetal growth retardation, suggesting that this gene can also stimulate growth. Furthermore *Peg1*/*Mest* (paternally expressed gene 1/ mesoderm specific transcript) has a growth-promoting role and a deletion of *Snrpn* (small nuclear ribonucleoprotein polypeptide N) and its flanking sequence yields smaller progeny (Tilghman, 1999; Vrana et al., 1998). However, it is not yet known whether these imprinted genes act via the insulin/ IGF system or via another mechanism to influence fetal growth.

The IGF system regulating fetal growth is complex, involving some imprinted and some non-imprinted genes. For example, none of the Igf binding proteins (Igfbps) identified to date have been found to be imprinted, nor is *Igf1* (insulin-like growth factor 1), *Igf1r* (insulin-like growth factor 1 receptor) or *Insr* (insulin receptor). Other genes such as *Gpc3* (glypican 3) that may bind Igf2 and affect its biological action, also exhibit fetal growth phenotypes when mutated in both mice and humans (Cano-Gauci et al., 1999). *Gpc3*, although not an imprinted gene, is likely to be subjected to haploid dosage control as it is situated on the X chromosome. Intrauterine growth retardation (IUGR) is a common feature of mice with a deletion of *Tsix*, the antisense gene that is reciprocally imprinted to the X inactivation gene, *Xist* (Lee, 2000). Thus, since there are many potential layers of regulation of the fetal growth promoting effects of IGF2, there are likely to be many mechanisms, variations in which could contribute to the wide variation in fetal growth observed in human populations. Of interest is also that the phenotypic effects of *Tsix* disruption were inherited across multiple generations (Lee, 2000), an observation that may be of relevance in interpreting the Dutch Famine study (Roseboom et al., 2001).

In addition to direct effects on fetal growth, imprinted genes could mediate perinatal weight by acting on nutritional delivery via the placenta or also during lactation. IUGR term placentas have higher levels of *IGF2* compared with normal term placentas (Abu-Amero et al., 1998). In addition to the IGF2 related genes, *Mash 2* (mammalian achaete-scute homologue 2) *Peg1/Mest* and *Xist*/ *Tsix* (Guillemot et al., 1994; Kaneko-Ishino et al., 1995; Lee et al., 2000) are candidates that are imprinted in the placenta. Other imprinted genes have been implicated in lactation and neonatal maternal care (Lefebvre et al., 1998). The perinatal period has not been well studied as a timepoint correlated with disease in later life but may prove to be important also if some of the disease effects are imprinting-related.

Imprinting and the Fetal Origins of Adult Disease

Barker and Clark (1997) suggest that metabolic adaptations to undernutrition are linked to changes in the concentra-

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tions of fetal and placental hormones such as insulin and the insulin-like growth factors. Since several key fetal/ placental growth hormones are either imprinted in fetal tissues or are regulated by imprinted genes (see above), imprinting variations could account for variability in fetal growth-related organ development and inherited disease states. Within the context of the cardiovascular and diabetes-related diseases implicated, there is convincing evidence that restricted development of specific fetal organs could alter later physiology. Organs affected by poor fetal growth include a reduced number of pancreatic beta cells and hence reduced capacity to make insulin, reduced skeletal muscle resulting in insulin resistance, impaired liver growth resulting in permanent impairment of cholesterol and blood clotting, as well as changes in vascular structure resulting in raised blood pressure (Barker & Clark, 1997).

That nutrition can affect imprinted growth factors such as Igf2, Insulin and *H19* has been well established in animal experiments (see Barker & Clark, 1997), as has the variable effects of imprint disruptions on specific organ development. Disruptions at imprinted gene loci, including *H19*, *Igf2* and *Igf2r*, have all been demonstrated to significantly disrupt allometric growth and development of specific relevant organs such as liver, heart, muscle, kidneys and pancreas (Sinclair et al., 2000; reviewed by Young & Fairburn, 2000). Many imprinted genes are cell cycle-regulators and so could easily affect the rate of cell division and possibly distribution of cell types in a key organ. Furthermore, it has been well established that alterations in at least the DNA methylation component of imprints are stably inherited and so can have long lasting effects (Reik & Walter, 2001).

Can Disease Programming Also Originate From the Oocyte/ Preimplantation Embryo?

Until recently, the dogma has been that nutritional influences on fetal development that correlate with adult disease status arise from nutritional fluctuations specifically at the time of critical organ development, for example when pancreatic beta cell numbers are being determined. However recent studies in livestock species suggest that both the preimplantation period and even the earlier phase of oocyte growth may be also highly susceptible to fluctuations in nutritional status and result in long term programming of both the fetus and adult.

Studies in sheep have demonstrated that mothers entering pregnancy with low nutritional body stores suffered marked impairment of fetal and placental growth if exposed to a further period of undernutrition during mid-pregnancy, whereas mothers well-nourished at the time of conception responded to mid-term dietary restriction by inducing placental hypertrophy (Robinson et al., 1994). Other studies in sheep (see McEvoy et al., 1997) have also indicated that the nutritional status of mother in the peri-conception period can programme preimplantation embryo development and this is mirrored in the increasing infertility problem observed in dairy cattle now highly selected for milk yield. There is also evidence that poor nutrition around the time of conception can influence birthweight in the human (Wynn & Wynn, 1998). Experiments in sheep examining effects of transient embryo transfer into uteri that were 3 days advanced in pregnancy stage with regard to the uterine environment, have demonstrated environmentally-induced fetal growth effects programmed in the early embryo that appear to be progesterone-mediated (reviewed by Young et al., 1996). These observations highlight the importance of a carefully regulated uterine environment in very early pregnancy and also the plasticity of mammalian preimplantation embryo development.

More direct evidence for nutritional programming of early preimplantation embryo development has been obtained using an embryo culture model. Sheep zygotes exposed to serum-containing culture media for 6 days (until blastocyst development), often resulted in the birth of considerably larger offspring after transfer to a surrogate mother than zygotes developed in serum-free culture (Sinclair et. al., 1999). In addition to fetal overgrowth in LOS, key organs such as the heart, liver, kidney's and skeletal muscle showed altered size and development.

A genetic component to this "Large Offspring Syndrome" (or LOS) effect was ruled out by distributing full-sibling zygotes between treatments as far as possible and in fact recent studies have implicated epigenetic change in DNA methylation in at least one imprinted locus (*IGF2R*; Young et al., 2001). Furthermore, there is now an increasing body of evidence from mouse studies that the preimplantation embryo is particularly sensitive to epigenetic modifications that may have programming consequences (Dean et al., 1998; Reik et al., 1993).

That the oocyte can also be predisposed to the LOS-inducing effect of serum is also suggested by the observation that full-sibling embryos cultured in the same serum-containing culture drop resulted in both developmentally normal and abnormal offspring being obtained (Sinclair et al., 2000). However, in this case an interactive later effect induced by the uterine environment into which cultured blastocysts were transferred, cannot be ruled out. The existence of an oocyte component to programming of fetal development has also recently been suggested by a mathematical model predicting that the rate of apoptosis in human preimplantation embryos is determined prior to fertilization (Hardy et al., 2001).

In addition to *in vitro* embryo culture effects on the fetal development phase in livestock, postnatal effects have been observed (although few studies have addressed this issue). Both increased musculature compared with bovine half siblings bred *in vivo*, as well as abnormally large hearts have been reported, even although post-natal growth compensation resulted in no overall difference in body weight at slaughter age (see McEvoy et al., 2000). These studies indicated that the effects of embryo culture *in vitro* on the development of vital organs could persist into later life.

In the rat, a recent study has now confirmed that *in vivo* dietary restriction can affect preimplantation embryo development, fetal growth and adult organs. Maternal low protein diet fed only during the preimplantation period of development before return to a control diet for the remainder of gestation, induced programming of altered birthweight, postnatal growth rate, hypertension and organ/body weight ratios in offspring at up to12 weeks of age (Yee Kwong et al., 2000). Blastocysts showed a slower rate of cell

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proliferation. Reduced insulin and essential amino acid levels and increased glucose levels were observed in maternal serum by day 4 of development. These metabolic effects are postulated to induce early metabolic stress on the embryo, thus restricting early embryo cell proliferation and underlying fetal programming. The basis for postnatal programming appears to reside in the altered protein content of the diet during preimplantation development. Although this has no effects on blastocyst rates, number of implantation sites formed or litter size, more subtle effects on growth-related criteria are indicated rather than severe compromise of fetal viability and pregnancy termination (Yee Kwong et al., 2000). The involvement of insulin, cell-proliferation and fetal growth effects all suggest imprinted genes as plausible candidates for mediating the nutritional effects.

Intriguingly in terms of an imprinting component, Yee Kwong et al. (2000) observed that there might be gender-specific susceptibility to programming in the nutrient-restricted embryo. Female embryos responded differently to males, with only the former showing reduced birthweight. Both sexes showed growth overcompensation compared with controls during early postnatal development, while only male offspring showed significant increase in postnatal systolic blood pressure and disproportionate liver and kidney growth. This is reminiscent of the impairment of only maternal transmission of an IUGR phenotype in mice mutated at the paternally expressed antisense *Tsix* locus that imprints the X-inactivation gene, *Xist* (Lee, 2000). In addition to IUGR, affected mice also show reduced fertility in the adult.

Imprints and Adult Disease — Is there a link?

Imprinted loci are now known to be susceptible for inducing neurobehavioral disorders and developmental disorders (reviewed by Murphy & Jirtle, 2000). In terms of adult disease, however, so far imprinting has been mainly studied in terms of cancer.

Since they are functionally haploid, imprinted genes are more vulnerable than non-imprinted loci to being inactivated or overexpressed and have been likened to a plane only having one engine (Hurst, 1998). As many imprinted loci have either tumour suppressor function or protooncogenic potential, both mutations and epimutations are commonly detected in a variety of tumours (reviewed by Murphy & Jirtle, 2000). For example, the *IGF2R* locus is inactivated in a variety of human tumours. Although the gene is imprinted in post-implantation tissues of the mouse, it's imprinting in human is polymorphic, with only a minority of people showing monoallelic, maternal expression. However, since over 50% of patients with Wilm's tumour of the kidney exhibit at least partial imprinting of *IGF2R* (Xu et al., 1997), these patients may exhibit increased vulnerability as only one 'hit' rather than two may be needed for oncogenesis (Murphy & Jirtle, 2000). This may illustrate a wider mechanism for disease susceptibility as a consequence of disrupted imprinting. Furthermore, since imprinted genes tend to be clustered, any regional disruptions e.g. in an imprinting control center, could disrupt more than one gene and thus lead to either a complex disease phenotype or to increased risk of tumour formation.

In addition to cancer, however, there are suggestions of imprinted gene involvement in other adult disease states. Relevant to both atherosclerosis and hypertension are the observations that human atherosclerotic plaques show reactivation of *H19*, which is not normally expressed in the adult (Han et al., 1995). As *H19* is normally down regulated during terminal differentiation of vascular smooth muscle during prenatal blood vessel formation, adult reactivation appears to mimic the fetal status and may well involve the mechanisms that normally regulate imprinted expression in the fetus.

Giddings et al. (1994) have also suggested that increased risk for insulin- dependent diabetes may be linked to the insulin gene through a paternally inherited effect and propose an imprinting-linked mechanism for loss of insulin-self tolerance and immunodestruction of pancreatic beta cells. More recently, an imprinted gene has been described in humans that regulates transcription of pituitary adenylate cyclase activating peptide (PACAP), a potent insulin secretagog in the pancreatic islet (Kamiya et al., 2000). The paternally expressed gene, *ZAC/PLAG1*, encodes a zinc finger DNA binding protein known to regulate apoptosis and cell cycle arrest. *ZAC/PLAG1* effects on PACAP could thus induce permanent diabetes by mediating beta cell apoptosis as well as by altering PACAP expression in the islet. As a cell-cycle regulator, *ZAC/PLAG1* is also another imprinted gene with tumour suppressor characteristics.

Finally, since several imprinted genes affect brain development, effects on e.g. the hypothalamus or pituitary may affect conditions such as hypertension in later life through endocrine disruption.

How Could Imprints Be Affected?

Imprinted genes are susceptible to classical mutations, as well as epimutations and either of these could lead to either biallelic silencing or expression of a disease-relevant locus (Reik & Walter, 2001). Genetic effects on imprinted loci are possible that do not affect imprinting mechanisms such as DNA methylation, but that affect expression through the existence of multiple alleles, parent-specific karyotypic defects such as uniparental disomy of the affected region, or microsatellite instability. In terms of epimutations, any disruption to primary or secondary imprinting, or to maintenance of a mature imprint would result in somatic inheritance of the defect and induction of under/overexpression in later development. Mann and Varmuza (1994) have suggested that imprints form late-replicating chromosomal domains that respond in different ways depending on the cellular milieu. So even if full imprints are maintained in the adult, they may not be read in normal tissues. Thus imprinted genes, that are normally biallelically silenced or expressed in the diseased adult, may show inappropriate expression patterns due to indirect environmental influences on relevant transcription factors, rather than effects on the imprints themselves.

In theory, imprints or imprinted genes could be disrupted during gametogenesis, in the embryo, in the fetus or at any later time. Erasure of imprints in the germline may normally ensure erasure of any epimutations inherited during the lifetime of the parent (Reik & Walter, 2001) but this

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process may not be foolproof, setting up susceptibility loci in the egg or sperm. Since imprinted genes are the only loci that are not demethylated in the early embryo, they may also be particularly vulnerable to error or environmental influences at this stage, particularly as many secondary imprints are being set up at this time. In somatic cells imprints may also be particularly susceptible to disruption as their expression/ repression occurs by different mechanisms to the rest of the genome (e.g. antisense transcripts) and furthermore imprints may be established/ maintained by different enzymes from the rest of the genome.

Variations observed in adult disease phenotype could result from direct or indirect effects on an imprinted gene at varying times. The timing of disrupting imprints may be a critical factor. If these were affected in the preimplantation embryo they might be expected to affect fetal growth and several stages of later fetal and adult development. If the disruption occurs prior to critical organ formation may not only affect that but also induce specific changes in later life. Furthermore, disruptions induced after the time of critical organ formation may still induce adult disease states by pleiotrophic effects rather than through the route suggested by the Barker Hypothesis.

Variable effects in the adult could also be due to chimaerism. Even in normal circumstances, embryos can be chimaeric for cells with and without imprinting. For example, only 85-90% of murine extraembryonic cells actually show imprinted inactivation of the paternal X chromosome via expression of the *Xist* gene (see Lee, 2000). Furthermore, in at least one imprinting syndrome, altered imprinting occurs in only a subset of cells (Mann & Bartolomei, 2000). Variability in growth responses may also be due to variation in the number of genes affected, depending on the duration or nature of the nutritional effect (Moore & Reik, 1996).

Although there are few studies on disruptions at imprinted loci in non-cancer adult disease states, insights of how imprinted genes could be affected can be derived from general disease models. Weatherall et al. (2001) discuss the emerging evidence that many monogenic diseases have very variable clinical features even if they are associated with mutations at a single locus. This variability could be associated with environmental effects that could act either on the primary disease locus, or could be due to secondary modifiers which affect its' function or to tertiary loci which do not affect the primary locus but may modify the disease through other means such as effects on co-selected genes (reviewed by Weatherall, 2001). Imprinted genes could also be affected at these three levels.

1. Direct Disruptions in Imprinted Genes

First of all there could be a direct mutation or epimutation in a fetal growth-related gene that had effects on one or more specific organ. This could either result directly in an adult disease state or effect adult disease due to pleiotrophic effects of the gene imprinted in the fetus. If the gene affected in the embryo or fetus encoded a metabolic effector hormone or growth factor, such as insulin for example, both effects on fetal growth and adult metabolism may result. Some directly affected genes may also have indirect effects on fetal growth and organ development e.g. the role of *Igf2r* in effecting placental neovascularisation (see Young & Fairburn, 2000). Altered levels of this and other IGF-related genes (such as *Gpc3*, *Grb10* etc) may furthermore affect fetal growth by influencing expression of other members of the IGF-related growth axis, including levels of Igf2 and Igfbps (Murphy & Jirtle, 2000; Young & Fairburn, 2000).

Alternatively, adult disease states could be due to pleiotrophic effects of imprinted genes, where the gene function in the fetus is different from that in the adult. Pleiotrophic effects have been described for the *Peg1/Mest* locus that affects both embryonic growth and adult maternal behaviour (Lefebvre et al., 1998). So far, however, most imprinted genes have been studied only during fetal development and their full effects in the adult are not known. Genes that are important for regulating normal fetal growth and development may have more deleterious roles in the post-reproductive adult, where there is less selection pressure. This type of effect has been well described for reproductive hormones, which are essential for reproduction but cause deleterious aging effects in older women (Kirkwood & Austad, 2000). As there is no corresponding conflict in parental imprinted loci in the adult, functional imprints would not be expected to persist in the adult (Hurst, 1996). Although alleles may still be differentially marked at some loci, it may well be that imprinting and allele-specific expression of imprinted genes may not persist in all tissues throughout adult life. Latham et al. (1995) suggest this may be due to loss of parent-specific methylation differences in some tissues but it is also possible that differential methylation is maintained and adult gene expression is regulated at the level of transcription factors.

Some genes that show imprinted expression in the fetus are entirely biallelically expressed or repressed in the adult. For example, *IGF2* exhibits a developmental switch in humans and sheep, from imprinted expression in most fetal tissues to a general tissue silencing in the adult, except for biallelic expression in the liver (McLaren & Montgomery, 1999). However, *Igf2* is not expressed at all after birth in the normal mouse. Thus reactivating even one repressed allele in a normally silenced adult tissue may be sufficient to induce a disease state (c.f. the earlier reference to *H19* and atherosclerosis). In terms of fetal versus adult effects therefore it seems that Igf2 is not required to mediate postnatal growth and general tissue cell proliferation in the same way that it regulates fetal growth. The growth effects of *Grf1* are also abolished after termination of weaning (Hurst, 1998). Localised reactivation of such mitogens may therefore have deleterious consequences in later life. As imprinted or biallelic expression of *Igf2* within a mammalian species appears to involve a complex useage of developmental stage and tissue-specific alternative promoters and transcript splice forms, there may be some promoters that are particularly sensitive to disruption at critical stages in development or in particular tissues (see Sinclair et al., 2000).

2. Imprinted Genes Modifying Other Loci

Disruptions in imprinted genes may also result in adult disease states indirectly through 'modifier' effects on other gene loci that may or may not be imprinted. Variation in the

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expression of modifier genes provides a plausible mechanism for inducing the variability in fetal growth that is associated with later-life disease. *H19* is an example of an imprinted gene that can modify the allelic expression pattern of another imprinted gene, *Igf2*.

Many imprinted genes are epigenetically modified in an allele-specific fashion after fertilization as well as during gametogenesis. The male and female pronuclear genomes undergo extensive epigenetic remodeling after fertilization, perhaps creating a vulnerable period for epigenetic disruptions to occur. Since modifying the epigenetic component of genomic imprinting appears to be subject to the same genetic variability as any other trait (discussed by Latham & Sapienza, 1998), differences between different individuals and even between different lineages may result in variations in birthweight correlated with adult disease. Components of the egg cytoplasm confer some of these modifications and it is known from mouse studies that variation in ooplasm modification of the sperm genome can be genetically determined. Nucleocytoplasmic interactions during early development are known to have a significant effect on fetal growth rates and this may occur by DNA methylation variations at imprinted, growth-related loci (Hurst, 1998; Moore & Reik, 1996).

As many adult disease phenotypes can also be dependent on genetic background, genetic modifiers are possibly responsible throughout life, directing variation such as severity and age of onset (Nadeau, 2001). Alternative alleles and environmental factors could also act to influence imprinted modifier genes or perhaps alleles promoting or limiting fetal growth confer specific responses to environmental influences during adulthood (such as alcohol, diet, exercise, smoking etc) and are coincidental to the fetal growth effect rather than there being a causal relationship.

If indeed imprinted genes have pleiotrophic effects at different developmental stages, genetic modifiers of pleiotropy will result in different combinations of traits on different genetic backgrounds. Although no human study of modifier effects on imprinted genes (or imprinted genes as modifiers) has been undertaken, there are examples of adult cardiovascular disease susceptibility genes for modifier gene variation (Hobbs et al., 1989).

Potter (2001) reviews the existence of both susceptibility and protector alleles in modifier genes and this is reminiscent of the oncogenic potential/ tumour suppressor roles of imprinted genes in tumours. In both coronary heart disease and in non-insulin dependent diabetes, protector alleles have been demonstrated and ultimately the genetic susceptibility to adult disease may involve susceptibility that requires environmental triggers for activation. A classic example of a susceptibility locus that could be involved in both determining size at birth and risk of cardiovascular disease and type II diabetes in later life is the human insulin gene (Ong et al., 1999). This effect appears to be mediated through the adjacent VNTR locus, the imprinting status of which is not known. As this locus is close to the imprinted cluster containing both the *Ins* and the *Igf2* genes, it may confer variation in allelic expression at these or other adjacent loci or may itself be subject to regulation by an imprinted modifier gene.

3. Imprinting Disruptions Affecting Non-related Loci

Due to the clustering of imprinted genes, it is also possible that nutritional effects may be transmitted to several genes, especially if an imprint control center is affected. This may exhibit fetal growth effects due to disruptions of one gene and induce adult effects through another, simply through adjacent location. Such proximity effects are known to influence the variable phenotypes often observed in the human imprinting disorder, Beckwith Wiedemann syndrome (Hastie, 1997).

Can Nutrition Affect Imprints?

Once again one can postulate many ways in which environmental influences, including nutrition, may directly affect the imprinted loci. There may be indirect effects on expression, action or accessibility of DNA methyltransferases that regulate or maintain imprints, although the enzymes involved in specifically methylating imprinted loci are not well understood. There may also be indirect effects on availability or accessibility of transcription factors that maintain the normal tissue-specific pattern of allelic expression. However, perhaps of most interest to the Barker Hypothesis, Murphy and Jirtle (2000) suggest that imprinted genes are potential targets for disruption by epigenetic toxicants that may modify DNA methylation and histone acetylation. They suggest, "because the imprinting of genes varies between species, individuals, tissues, cells and stages of development, disease susceptibility due to alterations in genomic imprinting represents a substantial epidemiologic and genetic issue that must be addressed".

At least in terms of DNA methylation, there is now a large body of evidence supporting the link between nutrition and epigenetic modification. Many dietary components are known to be important for methyl group supply to methyltransferases. Diets deficient in methionine, folate, vitamin B12 and choline can result in cancer in animal experiments (Choi & Mason, 2000; Ehrlich, 2000). This is associated with DNA hypomethylation and also cell death, as well as over-proliferation of specific cell types in liver. Several of the enzymes involved in methyl group donation are known to be polymorphic in humans. These enzymes affects methyl group donation via a pathway involving conversion of homocysteine to cysteine and increases in dietary homocysteine correlates with susceptibility to several types of vascular disease as well as cancer (Kimura et al., 2001). Thus plasma homocysteine determination is now used as a clinical test for proneness to cardiovascular disease (Refsum et al., 1998). Although a genetic factor, methyl donor enzyme polymorphisms are known to act via environmental means. Interestingly, alcohol is a risk factor that can compound folate deficiency and so adult disease may be predisposed in the fetus or embryo, but may only be initiated in some cases under certain environmental conditions in the adult.

Despite the accumulating evidence that supports the Barker Hypothesis, human interventional studies of maternal nutrition during pregnancy have led to the view that fetal development is little affected by changes in maternal nutrition, except in circumstances of famine. Deficiencies in methionine and choline only occur in the context of severe

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malnutrition and so are unlikely to be a common mechanism in Barker effects. Folate deficiency, on the other hand, is a more common event (Choi & Mason, 2000) and may be more likely to influence cellular DNA methylation.

Unfortunately, none of these nutritional effects have been studied specifically at imprinted loci and since these loci may be more vulnerable to methylation changes than the rest of the genome, (particularly at critical phases of development when imprints are established, reinforced or modified) such a study could be of considerable clinical interest. Thus, the jury is still very much out on the question of whether nutrition or genetics (or, of course, both) determine human birthweight and predispose adult disease. In either case, imprinted genes may play a role.

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