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J.R. Seckl Y. Christen (Eds.)

Hormones, Intrauterine Health and Programming

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Hormones, Intrauterine Health and Programming

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Foreword

In the last two decades a plethora of studies have extended the classical genes × adult environment paradigm of disease aetiology to include early life development (genes × development × environment). An early and powerful example comes from the epidemiological studies of Barker and colleagues linking low birth weight (a rather blunt marker of "something went wrong in utero") with a substantially increased risk of cardio-metabolic and neuropsychiatric disorders in later life. These findings have spawned a host of human observational and pre-clinical mechanistic studies to understand the link between the pre-natal environment and the programming (the hard wiring of structure, form and function) that might underpin later pathogenesis. A number of factors in the early environment have been invoked as causal including maternal malnutrition, inflammation, hypoxia and stress, in particular its glucocorticoid hormone mediators.

In this volume which gathers the contributions of the *Colloque Médecine et Recherche* organized by the Fondation IPSEN in December 2012 in Paris we address in particular the role of hormones and their links with other maternal environmental mediators in developmental programming. The crucial nature of the placenta as an interface and target between maternal and foetal environments is addressed. Emphasis is on the emerging science of epigenetics as a potential explanation for how environmental events that occur during brief windows of development may exert effects that impact upon somatic cells through many rounds of mitosis for much of the life-span of the subsequent organism. Some debate is also entered into on the role of inter-generational transmission of phenotype and epigenotype, at least for one further generation, a subject of topical interest beyond the immediate field and of substantial controversy.

The overall emphasis is on latest findings presented by global experts addressing from different viewpoints an area of rapidly emerging importance to health, well-being and scientific understanding of some of the biggest biomedical challenges facing our species.

Edinburgh, UK — Jonathan R. Seckl
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Glucocorticoids and Fetal Programming; Necessary and Sufficient?

Jonathan R. Seckl

Abstract Epidemiological evidence suggests that early life adversity, as marked by lower birth weight, associates with a substantially increased risk of cardiometabolic and neuropsychiatric disorders in later life, so called "fetal programming." Fetal overexposure to glucocorticoids is a possible basis for this association. Indeed glucocorticoid treatment or maternal stress may reproduce programmed phenotypes in inbred models where genetic differences are minimised.

The placenta and developing fetal organs highly express 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which catalyses rapid inactivation of cortisol and corticosterone thus forming a functional barrier to cellular glucocorticoid action. By pass, gene deletion or inhibition of 11β-HSD2 reduces birth weight and programmes lasting changes in cardiometabolic and behavioural parameters in the offspring in mammals including humans. In contrast, whilst maternal malnutrition similarly programmes the offspring and also reduces placental 11β-HSD2 levels, the effects appear to be mediated more by premature activation of the fetal hypothalamic-pituitary-adrenal axis than trans-placental passage of maternal glucocorticoids.

At a molecular level, epigenetic alterations, notably in methylation of specific cytosine deoxynucleotide residues in the promoters of target genes, may underpin persisting alterations in cellular gene expression. However, the inconsistency of patterns of methylation and related gene expression, notably in phenotypically similar progeny of a second programmed generation, imply understanding of such processes is far from complete. This emerging biology and its pathophysiological implications is a ripe avenue for future study.

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Developmental Programming

Low birth weight and other anthropometric parameters suggestive of exposure to an adverse intrauterine environment are associated with an increased risk of subsequent cardio-metabolic (Barker et al. 1993) and neuropsychiatric disorders (Seckl 2006). These relationships are largely independent of classical adult lifestyle risk factors and encapsulate the normal range of birth weights. To explain this association, the concept of developmental programming has been advanced (Barker et al. 1993; Edwards et al. 1993). Programming proposes that a stimulus or insult acting during critical developmental periods permanently alters tissue structure and function, producing effects which persist throughout life. Different cells and tissues are sensitive to influence at different developmental stages, so the effects of an environmental challenge will depend on its timing.

Whilst most of development is patently genetically determined, as evidenced by the close morphological, functional and pathological similarities between homozygous twins, studies in genetically inbred rodent models support the concept that early life environmental manipulations are indeed associated with later pathophysiology. These data imply some causation beyond classical inherited genetic processes (Drake and Walker 2004; Drake et al. 2005).

Two major causal hypotheses have been proposed to explain the relationship between the intrauterine environment, measures of fetal growth and later pathophysiology: fetal malnutrition (Barker et al. 1993) and overexposure of the fetus to stress and, specifically, its glucocorticoid hormone mediators (Edwards et al. 1993). Here some recent advances in the role of glucocorticoids in fetal programming are reviewed. More general reviews are available (Seckl and Holmes 2007).

Glucocorticoids and Fetal Development

Glucocorticoids clearly impact on fetal growth and tissue maturation (reviewed in Seckl 1998). Thus glucocorticoid treatment during pregnancy, albeit typically with substantial and repeated dosing, reduces birth weight in animals and in humans (Reinisch et al. 1978; Ikegami et al. 1997; Nyirenda et al. 1998; Newnham et al. 1999; French et al. 1999; Bloom et al. 2001). Blood cortisol levels are increased in human fetuses with intrauterine growth retardation or in pregnancies complicated by pre-eclampsia, indicating a potential role for endogenous cortisol in fetal growth retardation (Goland et al. 1993, 1995).

Glucocorticoids act largely via their two intracellular receptors, members of the nuclear hormone receptor family of ligand-activated transcription factors. Glucocorticoid receptors (GRs) are expressed in most fetal tissues from early embryonal stages (Cole et al. 1995; Speirs et al. 2004). In contrast, expression of the higher affinity mineralocorticoid receptor (MR) has a more limited tissue distribution in

development and is only present at later gestational stages (Brown et al. 1996a, b), at least in rodents. Additionally, GRs are expressed in the placenta (Sun et al. 1997) where they mediate metabolic, anti-inflammatory and parturition effects. The presence of significant densities of MR in placenta is less clear-cut, but there are some MR ligand-specific effects implying potential functional importance (Gennari-Moser et al. 2011) In humans, expression of GR mRNA has been identified in the embryonal metanephros, gut, muscle, spinal cord and dorsal root ganglia, periderm, sex chords of the testis and adrenal by 8–10 weeks and the lung by 12 weeks of gestation (Condon et al. 1998). Thus, systems mediating glucocorticoid actions exist from early developmental stages. Their complex cell-specific patterns of expression are presumed to underlie tissue variations in sensitivity to corticosteroids, which, being highly lipophilic, are assumed to readily penetrate most cells (Speirs et al. 2004).

Glucocorticoid therapy, using synthetic steroids such as dexamethasone and betamethasone that readily access the fetus, have been used clinically for decades in preterm labour. They very effectively accelerate maturation of the neonatal lung (Crowley 2000). Indeed a preterm rise in endogenous glucocorticoid levels is one of the mammalian fetuses' endogenous signals for cells to enter terminal differentiation in preparation for birth and independent life. Many of the late maturational changes in organs, including the lungs, heart, liver, gut and kidneys (Bian et al. 1982), are glucocorticoid dependent and can be induced prematurely by exogenous glucocorticoid administration, underpinning their widespread therapeutic use in threatened preterm labour (Ward 1994). However, there may be significant differences between the effects of fetal exposure to endogenous glucocorticoids and those of synthetic glucocorticoids. For example, while endogenous glucocorticoids can bind both GR and MR, and the effects in tissues such as brain may be mediated by both of these receptors, synthetic glucocorticoids typically used in obstetric practice are more selective for GRs. Similarly, there may be differences in local tissue concentration governed by differences in transport in blood (binding to/release from corticosteroid binding globulin or CBG), ability to cross physiological barriers (e.g., the blood-brain barrier, itself comprising ABC pumps that use some corticosteroids as substrates) and metabolism of endogenous and synthetic glucocorticoids (McCabe et al. 2001).

11β-Hydroxysteroid Dehydrogenase Type 2: A Feto-Placental Glucocorticoid "Barrier"

Fetal glucocorticoid levels are normally much lower than the levels in maternal circulation (Campbell and Murphy 1977) These lower levels are thought to be caused by placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), an NAD-dependent microsomal enzyme which catalyses conversion of active glucocorticoids (cortisol and corticosterone in humans and most mammals,

corticosterone alone in rats and mice) to inert 11-keto forms (cortisone and 11-dehydrocorticosterone; Chapman et al. 2013) which do not bind to receptors. 11β-HSD2 is located at theinterface between maternal and fetal circulations, in the syncytiotrophoblast in humans (Brown et al. 1996a, b) and the labyrinth zone in rodents (Waddell and Atkinson 1994; Waddell et al. 1998). Here it is proposed to prevent maternal glucocorticoids from accessing the fetus (Lopez Bernal and Craft 1981; Benediktsson et al. 1997; Lopez-Bernal et al. 1980). In guinea pigs, the decrease in placental 11β-HSD2 at term associates with increased transfer of maternal cortisol to the fetus (Sampath-Kumar et al. 1998). Similar late gestational falls in placental 11β-HSD2 activity occur in mice and rats. However, in primates, including humans, activity is largely maintained to near term, presumably because the fetal adrenals contribute more to the endogenous glucocorticoid surge that prepares the fetus for extra-uterine life.

The placental glucocorticoid "barrier" is incomplete. Thus, 10–20 % of maternal cortisol crosses the human placenta to the fetal circulation (Benediktsson et al. 1997). In rodents, the circadian peak of plasma corticosterone crosses the placenta intact to an appreciable extent (Venihaki et al. 2000).

Reduced placental 11β-HSD2 may result in overexposure of the fetus to maternal glucocorticoids; this has been proposed as a mechanism for the programming of later disease risk (Edwards et al. 1993). In humans, deleterious mutations of the gene encoding 11β-HSD2 (*HSD11B2*) associate with reduced birth weight (Dave-Sharma et al. 1998). 11β-HSD2 nullizygosity lowers birth weight in mice congenic on the C57Bl/6 strain background (Holmes et al. 2006a, b).

Within a species, the activity of placental 11β-HSD2 near term varies considerably (Benediktsson et al. 1993; Stewart et al. 1995). Lower placental 11β-HSD2 activity at term in rats associates with the smallest fetuses (Benediktsson et al. 1993), a finding reproduced in some (Stewart et al. 1995; McTernan et al. 2001; Shams et al. 1998; Murphy et al. 2002), but not all (Rogerson et al. 1996, 1997), human studies.

And there is a further complexity. Not only do GR and MR show complex ontologies in fetal tissues, 11β-HSD2 is also expressed in the mid-gestation mammalian fetus (Brown et al. 1996a, b; Diaz et al. 1998). Its expression is strikingly turned off in a cell- and tissue-specific pattern during the last third of fetal development. Indeed, it appears to be extinguished as cells enter terminal differentiation, indicating a role in determining the timing of tissue sensitivity to the late maturational effects of glucocorticoids. In the cerebellum, there is postnatal development that is sensitive to glucocorticoids. The postnatal loss of the external granular layer is preceded by turn off of 11β-HSD2, which coincides with increased tissue sensitivity to glucocorticoid remodelling (Holmes et al. 2006a, b). Manipulation of the enzyme confirms this role.

Glucocorticoid Programming

So glucocorticoids are key endogenous factors determining fetal cellular maturation. But does this alter structure and function in the longer term? Fetal glucocorticoid exposure can be increased (1) by maternal administration of synthetic glucocorticoids, such as dexamethasone and betamethasone, which are poor substrates for 11β-HSD2; (2) by inhibiting feto-placental 11β-HSD2 using liquorice or its derivatives such as carbenoxolone (Chapman et al. 2013); and (3) by chronic maternal stress, which elevates maternal glucocorticoid levels that may overcome the placental 11β-HSD2 barrier. Indeed, in a potential double hit, maternal stress also reduces placental 11β-HSD2 in some models (Mairesse et al. 2007) and in some studies in humans (O'Donnell et al. 2012).

In a range of animal models, these manipulations have shown that prenatal stress or glucocorticoid excess exert persisting effects on the adult offspring, including higher adult blood pressure, glucose and insulin levels, altered behaviours reminiscent of anxiety/depression and changes in the activity of the hypothalamic-pituitary-adrenal (HPA) axis. These effects are seen in many species, including rats, mice, guinea pigs, sheep and pigs (Nyirenda et al. 1998; Benediktsson et al. 1993; Gatford et al. 2000; Dodic et al. 1998, 1999, 2002; Jensen et al. 2002; Levitt et al. 1996; Sugden et al. 2001; Lindsay et al. 1996a, b). Similar persisting programming of these systems is observed in non-human primates exposed to small doses of glucocorticoids (de Vries et al. 2007; Nyirenda et al. 2009). The main window of sensitivity to glucocorticoids appears to be the last half of gestation.

Maternal exposure to stress, glucocorticoids or 11β-HSD2 inhibitors might act via effects on the mother's physiology, the placenta and/or the fetus. However, the key role of feto-placental 11β-HSD2 has been shown using a 11β-HSD2 knockout mouse model. Crossing healthy 11β-HSD2$^{+/-}$ heterozygous mice results in the presence of wild-type, heterozygous and null offspring in the same pregnant heterozygous female, since placental 11β-HSD2 is determined by the fetal not the maternal genotype. Thus the same mother has offspring with intact, half and absent placental (and fetal) 11β-HSD2. Importantly, birth weight and adult anxiety-like behaviours (the adult forebrain has no 11β-HSD2 so the phenotype is not due to any adult CNS effects) follow the feto-placental genotype; 11β-HSD2 null offspring have the lowest birth weight (Holmes et al. 2006a, b). Heterozygote offspring show birth weights intermediate between wild type and 11β-HSD2$^{-/-}$ littermates, suggesting that variation and not only complete absence of feto-placental 11β-HSD2 determines fetal growth and, plausibly, programming.

The detailed biology of organ-specific programming by fetal glucocorticoid/stress exposure has been reviewed recently (Harris and Seckl 2010). Suffice it to state here that programmed effects involve both permanent changes in cell number (nephrons, beta cells in the islets of Langerhans) and in gene expression in individual cells. Both processes are highly locus specific. For instance, the GR is a key target for permanent changes in its expression. However, even within the brain it is persistently down-regulated in hippocampus but up-regulated in the nearby

amygdala by late gestational glucocorticoid exposure. Similar locus—specific effects are reported in the hepatic acinus, pancreatic islets, etc. The magnitude of changes in cell number and gene expression and their resulting functional impacts are relatively slight when compared with the major developmental changes from manipulation of key developmental genes. Nonetheless, such small changes appear to underpin differences in the set point of important physiological variables such as the HPA axis, insulin-glucose homeostasis, blood pressure regulation, and control of mood and intellectual variables.

Is 11β-HSD2 at the Hub of Fetal Programming Beyond Stress and Liquorice?

Maternal malnutrition (notably a low protein diet) also reduces fetal growth and causes programming of adult physiology in rodents, ruminants and many other species. The adult phenotypes are very similar to those observed after prenatal stress/glucocorticoid overexposure. Intriguingly, a maternal low protein diet in pregnancy rather specifically substantially reduces placental 11β-HSD2 in a variety of model organisms. This finding has spawned the notion that loss of the placental glucocorticoid barrier is a common mechanism for fetal programming.

To test this idea, the heterozygous 11β-HSD2 null cross mouse model has been used to explore the effects of an isocaloric low protein diet and the level of expression of 11β-HSD2 in the placenta. Low protein diet fed throughout gestation elevated fetal glucocorticoid levels in midgestation, then subsequently reduced placental growth and finally decreased fetal weight near term (Cottrell et al. 2012). The implication is that the placenta and fetus are able, in part, to compensate for the increased glucocorticoid load. Late fetal growth correlated closely with fetal glucocorticoid levels. While low protein diet reduced placental 11ß-HSD2 activity near term, as previously reported, earlier in gestation placental 11β-HSD2 activity was unexpectedly increased. Crucially, 11ß-HSD2$^{+/-}$ crosses showed that, while both low protein diet and 11β-HSD2 deficiency reduced fetal growth, low protein diet acted independently of feto-placental 11β-HSD2 levels and vice versa. Instead, low protein diet induced the fetal HPA axis, elevated fetal glucocorticoid levels with premature activation of hypothalamic corticotropin-releasing hormone (CRH) and adrenal 11β-hydroxylase, the rate-limiting enzyme for glucocorticoid biosynthesis. Thus maternal protein malnutrition and placental 11β-HSD2 deficiency act via distinct processes to retard fetal growth, both involving feto-placental over-exposure to glucocorticoids but from distinct sources: the fetal HPA axis or the maternal circulation.

Testing this finding further, maternal adrenalectomy causes premature activation of the fetal HPA axis (presumably to compensate for the lack of steroids from the maternal circulation, which normally afford a minority, but not an insignificant one, of fetal glucocorticoids). Feeding low protein diet to adrenalectomised dams has no

effect on fetal growth (Cottrell et al. 2012), presumably because the fetal HPA axis is already maximally activated. These data put glucocorticoids firmly at the heart of fetal programming but allow a complex matrix of maternal-transplacental and fetal sources to drive these effects. The role of fetal tissue sensitivity, notably fetal tissue 11β-HSD2, in modulating this further is unexplored but merits attention.

Glucocorticoid Programming in Humans

Glucocorticoids are used as immunosuppressants and are used extensively in obstetric practice, primarily to accelerate lung maturation in cases of threatened preterm labour occurring in up to 10 % of pregnancies. Whilst there is no doubt that such synthetic glucocorticoids enhance lung maturation and reduce mortality in preterm infants (Crowley 2000), recently 98 % of British obstetric departments prescribed repeated courses of antenatal glucocorticoids (Brocklehurst et al. 1999), though there is little evidence for the safety and efficacy of such approaches. In addition, women at risk of bearing fetuses at risk of congenital adrenal hyperplasia (CAH) may receive low-dose dexamethasone from the first trimester to suppress fetal adrenal androgen overproduction. Birth weight in such infants has been reported as normal (Forest et al. 1993; Mercado et al. 1995); however, programming effects of antenatal glucocorticoids are seen in non-human primates models in the absence of any reduction in birth weight (de Vries et al. 2007; Nyirenda et al. 2009; Moss et al. 2001).

In a non-randomized cohort of 14 year olds, those given antenatal glucocorticoids therapy had higher mean blood pressures (Doyle et al. 2000). They also had a higher cortisol:cortisone ratio in umbilical venous cord blood, which may reflect reduced placental 11β-HSD2 activity, associated with higher systolic blood pressure at age three (Huh et al. 2008), and antenatal glucocorticoids in preterm births, associated with a reduction in glomerular filtration rate in young adulthood. These data suggest prenatal glucocorticoids programme the kidney and blood pressure in humans. Similarly, in a double-blind, placebo-controlled, randomized trial of a single course of antenatal betamethasone (534 individuals followed over 30 years), antenatal exposure to glucocorticoids caused insulin resistance in adults, albeit mild (Dalziel et al. 2005).

The human HPA axis appears to be programmed by the early life environment. Programming of the HPA axis appears to occur in various populations (Phillips et al. 2000) before the onset of overt adult disease (Levitt et al. 2000). Higher plasma and urinary glucocorticoid levels are found in children and adults who were of lower birth weight (Clark et al. 1996; Phillips et al. 1998). HPA responses to ACTH stimulation are exaggerated in those of low birth weight (Levitt et al. 2000; Reynolds et al. 2001). HPA activation is associated with higher blood pressure, insulin resistance, glucose intolerance and hyperlipidaemia (Reynolds et al. 2001).

Mechanisms of Early Life Programming

The programming effects of prenatal glucocorticoid overexposure, and other challenges, may be underpinned by alterations in cell number and/or permanent changes in cellular gene expression. Both processes have been demonstrated in a variety of models.

There has been much recent interest in the role of epigenetic changes in the early life origins of disease. Epigenetic modifications include DNA methylation and the recently discovered hydroxymethylation of cytosine, a host of histone modifications and the effects of non-coding RNAs. Whilst many of these are causes or markers of short-term gene transcriptional control, DNA methylation and perhaps hydroxymethylation exert more persistent impacts upon access of transcription factors and other proteins that determine gene expression in the longer term.

An increasing number of persisting changes, mainly in DNA methylation, have been reported to associate with programmed phenotypes. These appear to be stable through mitosis, at least for some time. A key target is the HPA axis. Genes such as those encoding GR, vasopressin, CRH and POMC appear to be susceptible to epigenetic changes associated with the early life environment. Analogous findings are reported in humans, despite the challenges of accessing relevant tissues for what is, at least in part, a tissue-specific process. Interestingly, recent evidence suggests that epigenetic mechanisms such as DNA methylation, may play a role in modulating placental 11β-HSD2 expression (Alikhani-Koopaei et al. 2004; Friso et al. 2008). Indeed, such effects may even be discernible in leucocytes (Drake et al. 2012), implying a potential biomarker of prior enzyme expression. However, caution is required as parallels in methylation in different tissues are not consistently reported (Provençal et al. 2012) and environmental influences in later life may also have an impact (Lam et al. 2012). Perhaps the most that can be claimed, as yet, is that methylation of some key developmental gene promoters may be echoed in later life in adult cells, whether or not there is any clear function of the target gene. How any such changes might be robust enough to become biomarkers of disease risk is unexplored.

Intergenerational Programming

This topic is reviewed in detail elsewhere so here I merely comment on an issue that is currently contentious. In current understanding, most epigenetic modifications of DNA are established during early development and maintained throughout life, which is why they are of potential importance in programming. Then there is reprogramming in the germline and during early embryogenesis to ensure totipotency in the zygote.

However, programming effects are not limited simply to the directly exposed first generation but may be transmitted to one or more subsequent generations

(Drake et al. 2005; Burdge et al. 2007; Jimenez-Chillaron et al. 2009) Non-genetic transmission of physiology and disease risk across generations may be due to persisting adverse environmental conditions or fetal exposure to programmed maternal physiology and structure (the "uterine effect"), and therefore explicable as re-exposure of subsequent generations to programming influences. However, some reports show programming from exposure of the initial generation may follow the paternal line. Such transmission through the male line (Drake et al. 2005; Jimenez-Chillaron et al. 2009) cannot represent any uterine effect. Careful control of the environment rules out re-exposure to challenge postnatally. Such data have spawned interest in putative epigenetic inheritance.

In many mammals, including rodents and humans, germ cells are laid down in early gestation. Such early germ cells undergo extensive epigenetic reprogramming in utero. Mid-late gestation environmental insults might interfere with the pattern of germ cell reprogramming, potentially producing effects in the second generation. Thus the first generation is impacted via direct environmental effects on its developing tissues and organs, whilst the second generation is affected because its early or precursor germ cells are changed, perhaps structurally and/or epigenetically, leading to the "programmed phenotype" in the next generation. This scheme does not invoke epigenetic inheritance and any quasi-Lamarckian inheritance.

In support, whilst glucocorticoid (and nutritionally) programmed rodents in first and second generations have similar gross phenotypes (Drake et al. 2005; Jimenez-Chillaron et al. 2009), the changes in gene expression that underpin these phenotypes are strikingly different. Moreover, even on the same promoter, changes in methylation differ between the directly exposed first generation offspring and the indirectly exposed second generation offspring (Drake et al. 2011). These data suggest differing mechanisms in each generation and not intergenerational inheritance of acquired characteristics, which would be revolutionary indeed. The latter has been occasionally reported following strong early life chemical challenge (Stouder and Paoloni-Giacobino 2011; Anway et al. 2005) but possible genotoxic effects or mutagenesis from such chemicals cannot be excluded with utter confidence. Indeed, such mutagenesis might be expected to change the epigenotype as part of the cellular response to mitigate deleterious mutation, perhaps especially in transposable elements in the male germline (Bao and Yan 2012).

Thus changes in DNA methylation appear to occur in response to early life challenges and persist in specific offspring tissues. Programming phenotypes may recur in subsequent generations, but it remains unlikely that epigenetic changes are transmitted to subsequent generations. Rather there are distinct effects on developing germ cells or there is re-exposure, including via the maternal effect. It is not appropriate yet to propose neo-Lamarckian principles.

Conclusions

There is strong epidemiological evidence that early life influences, notably as denoted by somewhat lower (or much higher) birth weight, associate with a substantially increased risk of cardiometabolic and neuropsychiatric disorders. This pattern is seen not just in modest samples but also in entire population surveys involving millions of individual births (Abel et al. 2010). The magnitude of the associated risk is quite substantial (Whincup et al. 2008).

Overexposure to glucocorticoids in placenta and fetus is a cause of this risk. There are complex mechanisms in placenta and fetal tissues to limit exposure to endogenous glucocorticoids. Bypass or inhibition of these mechanisms, notably of 11β-HSD2, exerts persisting deleterious effects. This may simply be the endogenous fetal system of signalling cells to enter terminal maturation prior to birth. As such it affords an opportunity for the developing organs to prepare for extrauterine life. Getting the timing wrong, or at least changing the timing, may afford improvements in short-term survival at the expense of lesser flexibility of responses, physiological and behavioural, to subsequent environmental challenges. The interesting role of epigenetics in maintaining such alterations in physiological set points is just emerging. However, the different epigenetic processes and distinct patterns in tissue gene expression, in association with the similar phenotypes in a second, unexposed generation, implied strong selectional pressure for phenotype over the challenges of producing this in subsequent generations. The implications of such a biology of the powerful secular trends in human disease are a fertile avenue for exploration.

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Developmental Programming and the Placenta: Focusing in on Glucocorticoids

Caitlin S. Wyrwoll

Abstract Fetal glucocorticoid exposure is a key mechanism involved in adverse programming outcomes in the adult. Impairment of fetal growth has predominantly been attributed to direct effects of glucocorticoids on the fetus, prematurely shifting tissue development from a proliferative to a more functionally mature state. However, fetal growth is dependent on a complex interplay of maternal, placental, and fetal endocrine signals, and glucocorticoid-mediated fetal growth retardation is likely also to relate to disturbances in placental growth and function. Regulation of fetal glucocorticoid exposure is achieved by the placental glucocorticoid barrier, which involves glucocorticoid inactivation within the labyrinth zone of the murine placenta by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). Overexposure to glucocorticoids or depletion of 11β-HSD2 has a dramatic effect on placental development and function, with a reduction in capillary networks and alterations in nutrient transport. This work highlights the finding that adverse programming effects of glucocorticoids are not exclusively due to direct actions on the fetus but are also a consequence of changes in placental development and function.

Developmental Programming

Low birth-weight and other indicators of reduced fetal growth are associated with adult cardio-metabolic and psychiatric disease. This association is the result of "developmental programming," whereby a stimulus during a sensitive period of early development exerts permanent effects on structure, physiology or metabolism (Cottrell and Seckl 2009). The environmental mechanisms of developmental

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programming identified so far can be simplified into two major groups: fetal stress exposure and maternal nutrition, although changes in glucocorticoids appear to underpin the programming effects of both (Langley-Evans et al. 1996; Gardner et al. 2007; Harris and Seckl 2010). In many animals, including mice and humans, there is an increased exposure of the developing fetus to glucocorticoids late in pregnancy, as they have a crucial role in the structural development and functional maturation of fetal organs. However, glucocorticoid overexposure of the fetus can be detrimental, as glucocorticoids cause a shift from cell proliferation to differentiation. Therefore, exposure to excess glucocorticoids in utero alters fetal organ growth and maturation patterns, which can result in adverse consequences in later life. In humans, the actions of glucocorticoids are exploited for preterm births to advance fetal lung maturation (Roberts and Dalziel 2006), although this may set the stage for adverse effects in later life (Benediktsson et al. 1993; Brown et al. 1996a; Levitt et al. 1996; Lindsay et al. 1996; Dodic et al. 1998, 1999, 2002a, b; Gatford et al. 2000; Langdown and Sugden 2001; Jensen et al. 2002).

The Feto-Placental Glucocorticoid Barrier: 11β-HSD2

As glucocorticoids are highly lipophilic, they readily diffuse across biological membranes and, therefore, control of intracellular levels of bioactive glucocorticoid is critical. This control arises from the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), which interconverts the active glucocorticoids cortisol and corticosterone with their biologically inactive forms, cortisone and 11-dehydrocorticosterone (DHC), respectively. There are two distinct forms of 11β-HSD: 11β-HSD1, which is a low affinity, NADP(H)-dependent bidirectional enzyme, although in vivo it appears to act predominantly as an 11β-oxoreductase to enhance glucocorticoid activity; and 11β-HSD2, which is a high affinity NAD-dependent enzyme that exhibits exclusive 11β-dehydrogenase activity (conversion of corticosterone to DHC) to reduce glucocorticoid potency. 11β-HSD2 is highly expressed in aldosterone-selective target tissues such as the distal nephron (Roland et al. 1995), colon (Whorwood et al. 1994), salivary glands (Roland and Funder 1996) and skin (Kenouch et al. 1994), thus serving to confer aldosterone specificity on the mineralocorticoid receptor (MR) to which both corticosterone and aldosterone can bind. Importantly, 11β-HSD2 does not always colocalize with MR, such as within placental and fetal tissues, and so its function has expanded beyond involvement in electrolyte transport to include regulation of corticosteroid action.

During much of normal pregnancy, circulating levels of glucocorticoids in the fetus are substantially lower than in the mother. This difference arises in part from the high expression of 11β-HSD2 in both the placenta and fetus, and this 11β-HSD2 expression serves as a "glucocorticoid barrier," enabling tight regulation of materno-fetal glucocorticoid transfer. Within the placenta, 11β-HSD2 is highly expressed at the interface between maternal and fetal circulations, in the syncytiotrophoblast in humans (Brown et al. 1996a,b) and the labyrinthine zone in rodents

(Waddell et al. 1998). In the rodent, 11β-HSD2 expression within the labyrinthine zone of the placenta falls during late gestation, which may facilitate glucocorticoid passage to the fetus and thus lung maturation (Brown et al. 1996a, b; Burton et al. 1996).

The high expression of 11β-HSD2 in placenta and fetal tissues and the growth-retarding and maturational effects of glucocorticoids upon the fetus (Meyer 1983) have lead to the proposal that variations in feto-placental 11β-HSD2 may underlie developmental programming. Thus, placental 11β-HSD2 activity correlates with birth parameters in rodents and, less consistently, in humans (Benediktsson et al. 1993; Stewart et al. 1995; Murphy et al. 2002), suggesting that normal variation in fetal exposure to maternal glucocorticoids has an impact on fetal growth. Numerous studies have shown that inhibition, deficiency or by-pass (poor substrate steroids such as dexamethasone or betamethasone) of 11β-HSD2 in gestation in rodents and humans associates with alterations in pregnancy duration, birth weight and programmed outcomes in the offspring (Benediktsson et al. 1993; Burton and Waddell 1994; Mune et al. 1995; Lindsay et al. 1996; Dave-Sharma et al. 1998; Nyirenda et al. 1998; Smith and Waddell 2000; Welberg et al. 2000, 2001; O'Regan et al. 2004; Holmes et al. 2006; Wyrwoll et al. 2006, 2007; Newnham and Jobe 2009). Furthermore, maternal stress in rodents during pregnancy has been associated with decreased expression of placental 11β-HSD2 (Mairesse et al. 2007; Lucassen et al. 2009; Pankevich et al. 2009). Interestingly, programming models involving maternal low-protein diet show an increase in maternal and fetal glucocorticoid levels (Lesage et al. 2001; Guzmán et al. 2006) in addition to a decrease in placental 11β-HSD2 activity and/or expression (Langley-Evans et al. 1996; Lesage et al. 2001; Stocker et al. 2004). Moreover, dexamethasone administration during pregnancy decreases food intake (Woods and Weeks 2005). Consequently, there seems to be considerable overlap in mechanisms by which maternal undernutrition and fetal glucocorticoid overexposure elicit developmental programming.

Placental 11β-HSD2 Is More than Just a Glucocorticoid Barrier

As described above, placental 11β-HSD2 may underpin aspects of developmental programming by allowing excess glucocorticoid passage from the "high" glucocorticoid maternal circulation to the "low" glucocorticoid fetal environment (Edwards et al. 1993), thus impairing fetal growth by direct effects of glucocorticoids on the fetus. Fetal growth is, however, dependent on a complex array of maternal, placental and fetal endocrine signals, and glucocorticoid-mediated fetal growth retardation must also relate, at least in part, to disturbances in placental growth and function. Indeed, treatment of rats with glucocorticoids such as dexamethasone, which are poor substrates for 11β-HSD2, restricts placental vascular

development, via inhibition of the endothelial cell-specific mitogen, vascular endothelial growth factor-A (VEGF-A), and peroxisome proliferators-activated receptor gamma (PPARγ), which regulates VEGF-A expression (Hewitt et al. 2006a,b). Impaired vascular arborization within key areas of the placenta that are involved in nutrient exchange between the maternal and fetal circulations is likely to have effects on placental function. However, glucocorticoid effects on placental function have been discordant. Thus, chronic restraint stress during late gestation in rats reduces placental 11ß-HSD2 expression and expression of GLUT1, with an associated reduction in fetal plasma glucose (Mairesse et al. 2007), whereas late gestation dexamethasone increases placental GLUT1 and 3 expression (Langdown and Sugden 2001), and another synthetic glucocorticoid, triamcinolone, downregulates placental GLUT1 and 3 protein and mRNA (Hahn et al. 1999). Any physiological relevance of these manipulations is unresolved. Furthermore, while system A amino acid transporter (SNAT) activity and expression are upregulated by cortisol exposure in BeWo cells (Jones et al. 2006), they are unaltered in human placental villous fragments exposed to cortisol (Jansson et al. 2003; Ericsson et al. 2005). Importantly, it is unknown whether the above observations of altered placental function in whole animal experiments are a direct effect of glucocorticoids on the placenta or occur via indirect effects on the dam. Thus, recent work utilizing 11β-HSD2$^{+/-}$ mice sought to demonstrate a direct effect of increased glucocorticoid exposure on placental function. This model of 11β-HSD2 heterozygous matings, whereby 11β-HSD2$^{+/+}$, $^{+/-}$ and $^{-/-}$ fetuses are generated by the same mother, clearly demonstrates a direct effect of increased glucocorticoid exposure on placental development and function.

Absence of Placental 11β-HSD2 Alters Placental Function and Development

Depletion of 11β-HSD2 in mice (generated by 11β-HSD2$^{+/-}$ matings) has been recently shown to compromise not only fetal but also placental growth (Wyrwoll et al. 2009) and to increase placental and fetal exposure to glucocorticoids (Cottrell et al. 2012). In this model, as 11β-HSD2 is expressed in the labyrinth zone of the placenta (which originates from fetal tissue), the genotype of the fetus is also the genotype of the placenta with regard to 11β-HSD2. At E15, despite a reduction in placental size, fetal weight is maintained, generating an increase in fetal/placental ratio that is indicative of enhanced placental function (Wyrwoll et al. 2009). Indeed, placental amino acid transport of 11β -HSD2$^{-/-}$ fetuses was upregulated at E15 alongside increased expression of the amino acid transporters *Slc38a2* and *Slc38a4* (Wyrwoll et al. 2009). Later in pregnancy, at E18, the smaller placenta of the 11β-HSD2$^{-/-}$ fetus appears unable to maintain normal fetal growth, and fetal weight falls behind control littermates (Wyrwoll et al. 2009). At this time, the transplacental transfer of glucose and plasma glucose levels was reduced in

11β-HSD2$^{-/-}$ fetuses (Wyrwoll et al. 2009). Glucose is a primary nutrient required for fetal development and is transported across the placenta by facilitated diffusion, primarily via GLUT1 and GLUT3 (Uldry and Thorens 2004). The reduced *Slc2a3* expression we observed in the labyrinth zone of placentas from 11β-HSD2$^{-/-}$ fetuses most likely accounts for the reduction in the transplacental transfer of glucose.

This altered placental function is further associated with reduced capillary networks (Wyrwoll et al. 2009). Thus, 11β-HSD2$^{-/-}$ placentas have significantly reduced fetal capillary development within the labyrinth zone, the zone regulating nutrient exchange, accompanied by a decline in VEGF-A and PPARγ mRNA expression, which are factors known to regulate angiogenesis (Wyrwoll et al. 2009). The decrease in fetal capillary vascularity in the 11β-HSD2$^{-/-}$ placentas may have implications for blood flow within the placenta and the umbilical cord. Indeed, ultrasound measures of blood flow have revealed decreased blood flow in the 11β-HSD2$^{-/-}$ placenta towards the end of gestation (Wyrwoll, unpublished data). Furthermore, umbilical vein flow in 11β-HSD2$^{-/-}$ fetuses does not undergo the normal gestational increase that occurs in wild-type littermates (Wyrwoll, unpublished data).

Ultrasound measures have also revealed altered cardiac function in 11β-HSD2$^{-/-}$ fetuses. Thus, the normal increase in E/A wave ratio [the E wave represents passive filling of the left ventricle (LV) and the A wave represents LV filling due to contraction of the atria] over gestation as the fetal heart becomes more compliant is not apparent in 11β-HSD2$^{-/-}$ hearts (Wyrwoll, unpublished data). Furthermore, the resistance index [RI = systole/(systole + diastole); systole being blood flow during maximal contraction of the heart and diastole being maximal relaxation] in the umbilical artery does not undergo the normal gestational decline as blood flow from the fetus to placenta increases (Wyrwoll, unpublished data).

These novel ultrasound data have lead to the proposal that impaired placental hemodynamics in the 11β-HSD2$^{-/-}$ fetus may have direct implications for fetal cardiac function. As the site of gaseous exchange in the fetus is the placenta, fetal circulation is distinct from the postnatal period. Thus oxygenated blood from the placenta travels through the umbilical vein and enters the fetal circulation either through the ductus venosus (a fetal shunt that bypasses the hepatic circulation such that blood is directly delivered into the inferior vena cava) or, after perfusing the liver, enters the inferior vena cava via the hepatic veins. Once circulated, blood returns to the placenta via the umbilical artery. Therefore, given that both the fetal heart and liver are the immediate organs exposed to blood leaving the placenta, the placenta is uniquely placed to have a direct influence on these particular organs. Indeed, epidemiological studies have revealed associations between placental size and the shape and incidence of cardiovascular disease in later life (Barker et al. 2010).

However, the results discussed above provide only indirect evidence of changes in placental blood flow and vascularity impacting on fetal heart development and function. What is required to provide a conclusive link in this relationship is to produce an amelioration of the compromised fetal capillary development in

11β-HSD2$^{-/-}$ placentas and to then investigate how this alters placental and fetal hemodynamics. Indeed, restoration of angiogenic balance in mouse models of preeclampsia has striking effects. Thus, administration of pravastatin (one of a class of lipid-lowering compounds, the HMG-CoA reductase inhibitors, that reduce cholesterol biosynthesis) in various mouse models of preeclampsia appears to ameliorate preeclamptic pathology (Ahmed et al. 2010 , Kumasawa et al. 2011), The precise mechanisms by which this improvement transpires is unclear but there was marked restoration of vasculogenesis in the preeclamptic placentas which has been variously attributed to stimulation of placental VEGF release (Ahmed et al. 2010) or placental growth factor (Kumasawa et al. 2011). The previous finding that VEGF is decreased in the placentas of 11β-HSD2$^{-/-}$ fetuses and thus presumably accounts for the observed decline in normal fetal capillary development is notable (Wyrwoll et al. 2009).

Therefore, recent work has sought to establish the effects of pravastatin on placental blood flow and fetal heart function of 11β-HSD2$^{-/-}$ fetuses. Administration of pravastatin to 11β-HSD2$^{+/-}$ dams from E6 of gestation onwards had marked effects on placental blood flow and fetal heart measures. Thus, both placental blood flow and the fetal cardiac E/A ratio of 11β-HSD2$^{-/-}$ fetuses remained comparable to those in wild-type fetuses in the pravastatin-treated pregnancies (Wyrwoll, unpublished data). Strikingly, gene expression of placental VEGF-A is upregulated in the pravastatin-treated pregnancies, which would presumably enhance angiogenesis within the placenta, with consequent ramifications for blood flow (Wyrwoll, unpublished data). Characterization of vascularity within these placentas is currently being undertaken to establish if this is indeed the case.

Optimal Placental Function, Optimal Health in Later Life?

The work conducted over the last few years on placental function and development of 11β-HSD2$^{-/-}$ fetuses provides a convincing argument that, while maternal glucocorticoids could play a direct role in programming the fetus, placental development and function also play key roles. Thus, the observations of altered placental transport of nutrients may have important ramifications for "setting" fetal metabolism and, thus, adult health in later life. Furthermore, the possibility that placental hemodynamics have the potential to alter fetal cardiac development and function opens up a novel research avenue. Additionally, placental function may shape health outcomes beyond cardio-metabolic disease. Indeed, there is growing recognition that the placenta generates hormones that are critical for neural function prior to the time such hormones are produced by the fetal brain itself, raising the possibility that the placenta may have a significant role in fetal neurodevelopment (Bonnin et al. 2011). Therefore, the placenta is key in influencing fetal development and shaping health outcomes in later life.

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Glucocorticoids, Programming and the Transmission of Effects Across Generations

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Abstract Substantial epidemiological evidence suggests that exposure to an adverse environment in early life is associated with an increased risk for cardiometabolic and neuroendocrine disorders in adulthood, a phenomenon termed "early life programming." One of the major hypotheses advanced to explain early life programming is fetal glucocorticoid overexposure occurring as a consequence of maternal stress, exogenous administration or dysfunction of the placental glucocorticoid barrier. There is evidence from both human and animal studies for an association between prenatal glucocorticoid overexposure and programming effects on cardiovascular and metabolic systems and on the brain. There is much interest in the potential for programmed effects to be transmitted across generations, including the effects of glucocorticoid programming. This review discusses the evidence for glucocorticoid programming and intergenerational effects in animal models and in humans.

Introduction: Glucocorticoids and the Developmental Origins of Health and Disease

The term "developmental origins of health and disease" (DOHaD) is used to describe the concept that the action of a stimulus or insult during a specific critical period in utero or in early postnatal development can lead to "programmed" alterations in tissue structure and function that may predispose an individual to disease later in life (Barker 1998; Gluckman and Hanson 2004). Research in this

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field has yielded extensive epidemiological and experimental evidence to show that the early life environment influences susceptibility to cardiovascular, metabolic and neuroendocrine disorders in later life (Barker and Osmond 1986; Barker 1998; Ellison 1981; Forsdahl 1977). One of the major hypotheses advanced to explain this link has been exposure to excess glucocorticoid in utero (Benediktsson et al. 1993; Edwards et al. 1993; Khulan and Drake 2012). Glucocorticoids are essential for normal physiological function and regulate and/or modulate many pathways, including those involved in metabolism, response to infection, stress responses, blood pressure maintenance and fluid and electrolyte homeostasis (Reynolds 2010). Glucocorticoid receptors (GRs) are expressed in most fetal tissues, including placenta, from early embryonic stages (Cole et al. 1995; Speirs et al. 2004); in humans, GR expression is detectable in many tissues from 8 to 10 weeks of gestation (Condon et al. 1998; Costa et al. 1996). During prenatal development, glucocorticoids promote the maturation of organ systems, and exogenous glucocorticoid administration induces precocious maturation (Bian et al. 1992; Fowden 1995); however, prenatal glucocorticoid overexposure is associated with a reduction in birth weight in both animals and humans (Bloom et al. 2001; French et al. 1999; Nyirenda et al. 1998; Reinisch et al. 1978).

The Role of the Placenta in Glucocorticoid Programming

Although glucocorticoids should freely cross the placenta, circulating glucocorticoid levels are much higher in the mother than in the fetus, and this gradient is maintained by the action of placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which catalyzes the conversion of active glucocorticoids to their inactive 11-keto metabolites (Seckl 1997). The activity of 11β-HSD2 may therefore impact on fetal glucocorticoid overexposure and, indeed, placental 11β-HSD2 activity correlates with fetal size in rodents (Benediktsson et al. 1995; Holmes et al. 2006) and in some (Stewart et al. 1995) although not all (Rogerson et al. 1997) human studies.

The importance of the placental glucocorticoid barrier in mediating a protective effect has been demonstrated in a number of animal studies. Placental 11β-HSD2 is not a complete barrier to glucocorticoids, so that maternal stress, resulting in increased maternal glucocorticoid concentrations, may result in increased fetal glucocorticoid exposure. Consistent with this finding, restraint stress in pregnancy results in lower birth weight and glucose intolerance in rats (Lesage et al. 2004), and this effect may be further augmented since placental expression of 11β-HSD2 is reduced by chronic maternal stress in rats (Mairesse et al. 2007). The administration of synthetic glucocorticoids that are not metabolized by 11β-HSD2 (e.g., dexamethasone and betamethasone) reduces birth weight and leads to effects on blood pressure and glucose-insulin homeostasis in rats (Benediktsson et al. 1993; Nyirenda et al. 1998), sheep (Dodic et al. 2002; Gatford et al. 2000; Jensen et al. 2002) and non-human primates (de Vries et al. 2007; Nyirenda et al. 2009).

Similar programmed effects are reported as a consequence of administration of liquorice and its derivatives, such as carbenoxolone, which inhibit 11β-HSD2, resulting in reduced birth weight and increased offspring blood pressure (Lindsay et al. 1996a, b; Seckl 1997).

Glucocorticoid Programming: Evidence from Animal Models

Programming of Blood Pressure and Metabolism

Prenatal glucocorticoid overexposure is associated with increased blood pressure in rats (Benediktsson et al. 1993; Sugden et al. 2001), sheep (Berry et al. 1997; Tangalakis et al. 1992) and non-human primates (de Vries et al. 2007; Koenen et al. 2002); potential mechanisms include alterations in kidney and cardiovascular system structure and function. For example, prenatal glucocorticoid excess reduces nephron number (Ortiz et al. 2001), increases renal glucocorticoid sensitivity, decreases renal 11β-HSD2 expression and alters renin-angiotensin-aldosterone system activity (O'Regan et al. 2004). Additionally, studies have described effects on vascular responsiveness (Hadoke et al. 2006; Molnar et al. 2003; Roghair et al. 2005, 2007), and changes in cardiac noradrenergic innervation and sympathetic activity have been reported in offspring exposed to excess glucocorticoid prenatally (Bian et al. 1993).

In rats, sheep and non-human primates, prenatal glucocorticoid overexposure is also associated with offspring hyperglycemia and hyperinsulinemia (de Vries et al. 2007; Lesage et al. 2004; Lindsay et al. 1996b; Nyirenda et al. 1998; Sloboda et al. 2002), which may occur as a consequence of programmed effects on several target organs, including the pancreas and insulin-sensitive target tissues including liver, muscle and adipose tissue. Glucocorticoid overexposure impairs β-cell development in rats (Blondeau et al. 2001) and non-human primates (de Vries et al. 2007), and in vitro studies demonstrate that dexamethasone alters the expression of transcription factors that play important roles in pancreatic development (Gesina et al. 2004). Prenatal glucocorticoid overexposure in rats has lasting effects on hepatic function, resulting in a permanent increase in the expression and activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK; Nyirenda et al. 1998), perhaps as a consequence of the increased expression of key transcription factors including GR (Cleasby et al. 2003; Nyirenda et al. 1998) and hepatocyte nuclear factor 4α (HNF4α; Nyirenda et al. 2006). In marmosets, prenatal dexamethasone programs increased hepatic expression of 11β-HSD1, which catalyzes the regeneration of active glucocorticoids from their inactive metabolites, and this stage precedes the development of obesity or the metabolic syndrome in this model (Nyirenda et al. 2009). In rats, prenatal dexamethasone overexposure also programs increased susceptibility to the development of fatty liver when animals are maintained on a high fat diet following weaning, in

association with altered expression of genes that are important in lipid metabolism (Drake et al. 2010). Adult offspring of rat dams exposed to dexamethasone during gestation have adipose depot-specific alterations in gene expression (Drake et al. 2010) in addition to increased GR expression and attenuated fatty acid uptake in visceral adipose tissue (Cleasby et al. 2003); in the marmoset, prenatal glucocorticoid overexposure programs increased 11β-HSD1 expression in subcutaneous fat (Nyirenda et al. 2009). Thus, glucocorticoid programming of insulin resistance and hyperglycemia may also occur as a consequence of effects on the expression of genes regulating glucose uptake and fuel metabolism in tissues involved in insulin-sensitive glucose disposal and fat storage and metabolism (Cleasby et al. 2003).

Programming of the Brain

Glucocorticoids are involved in normal brain development (Meyer 1983), and exposure of the fetal brain to excess glucocorticoids at critical stages of development may affect neuroendocrine function, memory and behavior (Uno et al. 1990; Wyrwoll and Holmes 2012). In rodents, prenatal glucocorticoid overexposure affects hippocampal growth, impairs motor development and leads to programming of adult behavior and affective function (Alonso et al. 2000; Mandyam et al. 2008; Welberg et al. 2001; Wyrwoll and Holmes 2012); in sheep, betamethasone exposure in utero is associated with delayed maturation of neurons, myelination and glia (Huang et al. 2001) and, in the rhesus macaque, prenatal stress increases basal cortisol levels and reduces neurogenesis (Pryce et al. 2011). Potential targets for prenatal glucocorticoids include molecules involved in the regulation of neuronal survival, HPA axis function, other higher center functions and behavior (Avishai-Eliner et al. 2002). In rats, prenatal glucocorticoid overexposure increases corticotrophin releasing hormone (CRH) mRNA levels in the central nucleus of the amygdala and reduces mineralocorticoid receptor (MR) and GR levels in the hippocampus, with permanent effects on HPA axis activity (Welberg et al. 2000, 2001). Prenatal glucocorticoid exposure also affects the developing serotonergic and dopaminergic systems (Wyrwoll and Holmes 2012), with implications for the development of psychiatric disorders including schizo-affective, attention-deficit hyperactivity and extrapyramidal disorders.

Glucocorticoid Programming: Evidence from Human Studies

Exogenous Steroids

In humans, glucocorticoids are frequently used in obstetric practice to accelerate fetal lung maturation in threatened preterm labor (Kattner et al. 1992) and, in the

past, repeated courses of glucocorticoids have been used in this context (Brocklehurst et al. 1999). While there is no doubt that the use of glucocorticoids in this way reduces both morbidity and mortality in preterm infants, whether there are adverse consequences of repeated exposure are unknown. A recent meta-analysis of randomized controlled trials reported that weekly or bi-weekly betamethasone treatment reduced fetal growth but had no effect on neurodevelopment or growth at 2 years of age (Peltoniemi et al. 2011); however, further, longer-term follow up of these children is essential. Studies reporting effects of prenatal glucocorticoid administration on blood pressure in adolescence or adulthood have included small numbers of individuals, and while one randomized controlled trial involving 81 individuals aged 20 years reported lower mean systolic blood pressure in exposed individuals (Dessens et al. 2000), a second reported higher mean systolic and diastolic blood pressure in a non-randomized cohort of 177 adolescents (Doyle et al. 2000). Additionally, prenatal administration of glucocorticoids to women at risk of preterm delivery has been associated with a reduced glomerular filtration rate in the offspring aged 19 years (Finken et al. 2008). In terms of glucose-insulin homeostasis, although one long-term follow-up study of individuals exposed to antenatal betamethasone did not find a significant change in lipid profile or in the prevalence of diabetes or cardiovascular disease at age 30, individuals had higher insulin levels, suggesting that the risk for diabetes could increase with aging (Dalziel et al. 2005). Prenatal exposure to synthetic glucocorticoids may also impact on the developing HPA axis: children born at term to mothers who had received glucocorticoid treatment antenatally for threatened preterm labor have higher cortisol responses to a painful stimulus on the first day of life (Davis et al. 2011) and increased responses to acute psychosocial stress in childhood (Alexander et al. 2012).

The administration of low dose dexamethasone during the first trimester has also been advocated in the management of women at risk of bearing fetuses with congenital adrenal hyperplasia (CAH), and it is effective in reducing virilization in affected girls. In this regime, glucocorticoids are commenced early in pregnancy, before prenatal testing or sex determination is possible, so that seven of eight fetuses (i.e., all boys and CAH-unaffected girls) are exposed to exogenous glucocorticoids until prenatal testing at the end of the first trimester, when fetal sex and disease status can be determined. At this time, treatment is stopped in all except CAH-affected girls. Many of the children exposed in this way have not been enrolled in clinical trials, so follow up data are not available; however, there is growing evidence for deleterious effects in exposed individuals. A number of studies suggest that exposure to glucocorticoids may impact on offspring behavior and working memory, and one study reported that verbal working memory capacity correlated with the children's self-perception of difficulties in scholastic ability (Hirvikoski et al. 2007, 2008; Trautman et al. 1995). CAH-unaffected, glucocorticoid exposed children reported increased social anxiety and, in studies of gender role behavior, there were more neutral behaviors in glucocorticoid-exposed boys (Hirvikoski et al. 2011). Indeed, such is the concern about the potential sequelae of such glucocorticoid use, particularly since most exposed fetuses do *not* have CAH,

that the use of antenatal glucocorticoids for this purpose has recently been suspended in Sweden (Hirvikoski et al. 2012).

Endogenous Steroids and Offspring Neurodevelopment

Maternal prenatal stress and anxiety have been associated with lower scores on neonatal assessment (suggesting adverse effects on neurodevelopment; Rieger et al. 2004), with behavioral and emotional problems in offspring at the age of four (O'Connor et al. 2002) and with decreased gray matter density at the age of 6–9 years (Buss et al. 2010). Severe maternal stress in pregnancy has been associated with lower cognitive and language abilities in childhood (Laplante et al. 2008). Further data suggesting glucocorticoid excess impacts on neurodevelopment came from Finland, where the consumption of large amounts of liquorice (an 11β-HSD inhibitor) is common. Maternal liquorice consumption during pregnancy is associated with altered HPA axis activity in children, with those whose mothers consumed the most liquorice during pregnancy having the highest circulating cortisol levels (Raikkonen et al. 2010). Additionally, higher maternal liquorice consumption is associated with detrimental effects on offspring cognitive and psychiatric outcomes (Raikkonen et al. 2009).

Transmission of Glucocorticoid-Induced Programming Effects Across Generations

Evidence from Animal Models

There is much evidence from both human and animal studies showing that programmed effects may not be limited to the first (directly exposed) generation but may be transmitted to subsequent generations. Although there has been much recent interest in this phenomenon of intergenerational effects, it is not a new concept. Using rat models of prenatal stress, Pollard (1986) reported effects on birth weight and postnatal growth in a second generation, and Pinto and Shetty (1995) reported that the effects of maternal exercise stress persisted into a second generation. We showed that prenatal exposure to dexamethasone is associated with effects on birth weight and glucose-insulin homeostasis in a second generation in rats (Drake et al. 2005), with marked parent-of-origin effects showing that programmed effects can be transmitted through both maternal and paternal lines. Effects on birth weight and glucose tolerance have been demonstrated in the grand-offspring of sheep treated with "clinically relevant" doses of dexamethasone during pregnancy (Long et al. 2012). The transmission of glucocorticoid-induced programming effects on behavior and HPA axis activity across generations has also been

reported in a number of animal models, including in guinea pigs, in which prenatal betamethasone is associated with sex-specific effects on HPA axis activity and behavior in second generation offspring following transmission through the maternal line (Iqbal et al. 2012). Importantly, stress acting in the early postnatal period can program altered behavior in the offspring, and these effects can also be transmitted to subsequent generations through the paternal lineage (Franklin et al. 2010). Finally, such effects can influence sexual behavior; Morgan and Bale (2011) have reported programming of "dysmasculinization" in first and second generation male offspring following a period of stress during the first week of gestation, with altered behavior and gene expression in the brain evident in both first and second generation males.

Transmission of Glucocorticoid-Induced Programmed Effects: Evidence from Human Studies

While a number of well-known studies in humans have demonstrated the transmission of "programmed" effects across several generations (Kaati et al. 2002; Painter et al. 2008; Stein and Lumey 2000), most of these have related to nutritional influences. There are, however, a few intriguing studies suggesting that stress or abuse in early life may lead to effects that are transmissible across generations, although interpretation of these studies is complex given the likelihood that ongoing mental health issues in the parents may impact on the upbringing of the child. It is well known that maternal experience of childhood maltreatment and maternal antenatal depression are both associated with offspring childhood maltreatment and offspring adjustment problems. A prospective longitudinal study of 125 families in London identified that mothers who had themselves experienced childhood maltreatment were more likely to suffer depression during pregnancy. Importantly, the offspring of mothers who experienced both maternal childhood maltreatment *and* antenatal depression were themselves exposed to significantly greater levels of childhood maltreatment and exhibited significantly higher levels of adolescent antisocial behavior, suggesting an intergenerational cycle of maltreatment and psychopathology (Plant et al. 2013). Early life trauma is associated with altered HPA axis activity in adulthood; in a further study, maternal child abuse was associated with steeper declines in cortisol in a group of mothers on exposure to a stress paradigm and, additionally, with lower baseline cortisol in their infants. Among this group of infants, a history of early maternal abuse and comorbid maternal post-traumatic stress disorder (PTSD) was associated with the greatest increases in cortisol levels (Brand et al. 2010). In a study in pre-school children, those who showed the highest levels of cortisol in response to stressful tasks were those whose parents had a past history of depression and who demonstrated hostility toward their child (Dougherty et al. 2011). Importantly, this effect was specific to offspring who had also been exposed to maternal depression during the

first few years of life, suggesting that early intervention in these families might be effective in decreasing the risk of transgenerational effects (Dougherty et al. 2011). Evidence also suggests that the risk of PTSD may be transmitted transgenerationally (Roberts et al. 2012).

Mechanisms

The transmission of programmed effects across generations suggests that there are mechanisms by which environmentally induced changes in parental phenotype can be transmitted to the offspring (Drake and Walker 2004; Gluckman et al. 2007; Jablonka and Lamb 2005). There are a number of potential explanations for the transmission of programmed effects across generations. Firstly, the persistence of similar environmental conditions that impact on development and/or behavior is known to re-create the same phenotype in a number of generations in animals (reviewed in Jablonka and Lamb 2005). A second potential mechanism for the transmission of programming effects is that programmed changes in the mother, e.g., changes in physiology, size or behavior, may influence the development of her own offspring, so that the phenotype is established de novo in each new generation. Programming of maternal physiology, including altered stress hormone levels, has been proposed as one mechanism for transgenerational effects on health in African-Americans in the US (Kuzawa and Sweet 2009).

Finally, there is increasing interest in the role of epigenetic effects that may be transmissible through the germline. Although the precise mechanisms by which epigenetic modifications escape reprogramming in the germline or after fertilization remain unclear (Youngson and Whitelaw 2008), emerging evidence suggests that epigenetic information, including DNA methylation, histone marks and small RNAs, may be transmissible in sperm (Hammoud et al. 2009; Lalancette et al. 2008; Rassoulzadegan et al. 2006). In the glucocorticoid-programmed rat, we showed that prenatal dexamethasone overexposure is associated with altered DNA methylation at the imprinted gene insulin-like growth factor 2 (Igf2) in both first and second generation offspring, although this occurs at different differentially methylated regions (DMR) in the two generations and there were no changes in DNA methylation at either DMR in sperm, so that the mechanism for the transmission of effects remains to be determined. Changes in DNA methylation have also been described as a consequence of prenatal betamethasone exposure in two generations of guinea pigs (Crudo et al. 2012). In mice, chronic, unpredictable stress in the postnatal period leads to altered behavior in the first, directly exposed generation and effects on behavior were also present in a second generation (Franklin et al. 2010). Intriguingly, alterations in DNA methylation were identified in sperm from first generation males and in the brain of second generation offspring, suggesting a mechanism for the transmission of effects across generations in this model (Franklin et al. 2010).

Conclusions

Studies in both animal models and in humans suggest that exposure to excess glucocorticoids in early life, whether exogenous or endogenous, has important effects on offspring development, increasing the risk of cardiometabolic and neuroendocrine disease. Evidence also suggests that these effects can be transmissible across generations. Since these conditions have a marked impact on the health and well-being of society, I suggest that understanding the mechanisms underlying glucocorticoid-induced programmed effects is of great importance for public health.

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Maternal Stress and in Utero Programming

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Abstract The idea that a mother's psychological state can negatively influence the outcome of pregnancy and the health of her unborn child has been deeply ingrained into many societies since the beginning of modern civilisation. This concept was revived in the twentieth century when a series of large-scale, manmade and natural disasters, such as war and famine, coupled to population level data, provided an opportunity to examine the effect of stress during pregnancy on the subsequent mental and physical health of the unborn infant. The establishment of the Scandinavian registers, such as those in Sweden and Denmark, has facilitated a closer examination of the effect of antenatal maternal stress on subsequent offspring outcomes at an individual level. There is now a considerable body of work to support the hypothesis that maternal psychological stress in the peri-conception and antenatal period is associated with a diverse range of adverse outcomes for the offspring, including immediate obstetric complications and later psychiatric disorders and chronic physical ill-health. Here we discuss the most recent and robust data emanating from Scandinavian Register-based research programmes and contemporary birth cohorts. We provide evidence that psychological stress is associated with a range of adverse outcomes, which in some cases are gender specific. Moreover, we provide evidence that the timing of exposure is critical and provides tantalising clues into the potential underlying causal pathways.

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Introduction

The idea that a mother's psychological state can negatively influence the outcome of pregnancy and the health of her unborn child has been deeply ingrained into many societies since the beginning of modern civilisation. One of the first references to this in literature can be found in the major Sanskrit epic of ancient India from The Mahabharata (-500BC).

> The daughter of Virata. . . (was) exceedingly afflicted by grief on account of the death of her husband. . .they all feared that the embryo in her womb might be destroyed.
> (Narasimhan 1997)

In modern times, manmade and natural disasters such as war, atomic bombing, earthquakes and famine have offered the opportunity to examine the effect of stress on the subsequent health of the unborn infants of mothers exposed to these events. A large body of literature has reported associations between events such as famine and war and both immediate obstetric and longer-term psychiatric adverse outcomes (Susser and Lin 1992; Susser et al. 1996; Selten et al. 2003; St Clair et al. 2005; Smits et al. 2006).

However, the use of such occurrences as a measure of stress is complicated for three reasons. Firstly, these events may combine several risk factors other than stress, such as nutritional deficiency, ionizing radiation and infectious diseases, all of which are associated with adverse health effects in their own right.

Secondly, correctly identifying the subjects as exposed or unexposed may be difficult. For example, in the famine studies (Susser and Lin 1992; Susser et al. 1996; St Clair et al. 2005), the measurement of exposure was based on official food rations distributed to the public. However, there were no records or control on unofficial food that may have been available to some people. In the war studies (van Os and Selten 1998; Selten et al. 2003), cohorts of people who were born in the previous and subsequent 2 years were used as the unexposed/reference group. However, it is well known that the years that preceded and followed those wars were not peaceful. The May 1940 invasion of the Netherlands was followed by the Nazi blockade and famine a few months later. The years that preceded and followed the 1967 and 1973 wars in the Middle East are likely to have been full of fear and tension for the same population considered as unexposed to the stress of the intervening wars.

Finally, the exposures to war and famine are measured at the population level rather than the individual level, and thus the intensity of exposure may be different depending on geographical and personal circumstances.

There are also several other methodological concerns with studies examining maternal stress on subsequent offspring outcomes. There is now substantial evidence that adverse outcomes associated with or caused by in utero exposure to maternal stress are critically dependent upon the window of exposure. For example, several authors have reported that stress in the first but not subsequent trimesters is associated with schizophrenia in the offspring (van Os and Selten 1998), whereas a more recent study by Smits et al. (2006) reported a significant reduction in mean

birth weight of babies who were exposed in utero, through media coverage, to the 9/11 attacks, during the second and third trimesters, but not the first trimester. Ignoring the timing of the exposure may lead to a dilution in the relationship between maternal stress and outcomes if that relationship is specific to only one trimester. Furthermore, precise knowledge of the timing of the exposure is essential to identify possible mechanisms for the stress effect.

Additionally, many studies that have reported both positive and negative associations between maternal stress and adverse offspring outcome have failed to control for potential confounders such as family history of illness, social class and place of birth.

Finally, there is a growing body of evidence to suggest that the effects of in utero exposure to stress are gender specific (Susser and Lin 1992; Susser et al. 1996; van Os and Selten 1998); thus it is important to accurately ascertain infant sex.

The limitations of previous studies reporting population-level exposures and the requirements for more detailed and precise information regarding exposures and outcomes have prompted researchers to utilise the Scandinavian registers more often. In recent years, these registers have proven to be invaluable tools in the progress of research into in utero exposures and later health outcomes.

Registery-Based Research

> The founders had faith that the seeds they planted would contribute to the health of the Norwegian people. What the founders could not have foreseen was the benefits that the Registry would provide to the world at large.
>
> Allen Wilcox on the Norwegian Medical Birth Register (Wilcox 2007)

The establishment of the Scandinavian registers, such as those in Norway, Sweden and Denmark, has been extremely valuable to international epidemiology research. The major advantage of the registers is that they cover the entire population but, as all information in the registers is connected to each citizen's unique personal identification number, which enables linkage between various registers and biobanks, it is possible to look at health effects across an entire population on an individual level.

The registration of births, deaths and marriages in Denmark has for centuries taken place in the parish registers (Malig 1996; Pedersen et al. 2006). This process was formalised in 1924 with the national registration of Danish residents. This information was recorded manually until 1968 when the electronic Danish Civil Registration System (CRS) was established.

Persons who live legally in Denmark for a certain period of time must register with the national registration offices. Registered persons are assigned an identification number called the "Civil Person Registration" number (CPR-number); the number is unique and used uniformly across all public authorities as well as by major banks and insurance companies.

The CRS contains vital demographic information, including name, address, gender, marital status, place of birth, and date of birth for all individuals who live or have lived, legally, for a certain period of time in Denmark (since 1968) and in Greenland (since 1972). The CRS also includes the legal parents' CPR-numbers and the spouse's CPR-number as well as any those of previous spouses. Persons who are registered in the CRS can be linked to their siblings using the maternal CPR-number.

The Medical Birth Registry (MBR) was established in 1968 to monitor the health of newborns and the quality of antenatal and delivery care services (Knudsen and Olsen 1998). The MBR, which covers more than 99 % of all births in Denmark (Kristensen et al. 1996), contains information about live and stillbirths and infant deaths since the January 1, 1973. This registry contains several variables, such as birth weight, gestational age, infant sex and parity and information about the obstetric complications that the mother may have had during pregnancy or during delivery. Information about the mother's marital status, date of marriage and place of residence is also available through the MBR, and there is a high degree of concordance between the MBR data and data within medical records (Kristensen et al. 1996).

The Psychiatric Register is one of the oldest registers in Denmark. Manual systematic psychiatric data collection started in 1938, and this was replaced by an electronic register in 1969 (Munk-Jorgensen and Mortensen 1997). The register includes all admissions and outpatient attendances for psychiatric reasons at hospitals in Denmark, Greenland and the Faroe Islands.

The Hospital Discharge Register was established in 1975. Data have been collected nationally on all somatic hospital admissions since 1977 and on all outpatients and emergency patients since 1995. Like the other registers, the International Classification of Diseases, 8th Revision (ICD-8) was used from its establishment until 1993, and the International Statistical Classification of Diseases, 10th Revision (ICD-10), from 1994 onwards (Anderson 1999). The Hospital Discharge Register is an important tool of register-based research. By linking it to other registers, it is possible to investigate the relationship between a certain disease and demographic variables such as age and sex. It is also possible to investigate the mortality rate in people diagnosed with a certain disease.

It is thus apparent that the comprehensive, contemporaneous and integrated nature of the linked registers represents a powerful tool for epidemiological research. In a decade-long collaboration with colleagues at the Centre for Integrated Register Based Research at Aarhus University in Denmark, we have used the Danish registers to assess the effect of in utero stress on a wide range of subsequent outcomes (Khashan et al. 2008a, b, 2009, 2011, 2012). Here we describe a summary of our key findings.

Maternal Stress and Obstetric Outcomes

We used the Danish national registers to investigate the association between maternal exposure to severe life events and pregnancy outcome. We defined severe life events as the experience of death or serious illness in a close relative, as we believe that bereavement—the permanent loss of the deceased person and the relationship they enjoyed with them—is a severe and objective stress (Zisook et al. 1994). Life stressors, which involve actual or threatened death or serious injury to a family member, are considered to be traumatic stressful events according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition criterion (Lindberg and Wellisch 2004). We would expect these life events to affect any person, regardless of their personality or strength of social network (Hansen et al. 2000).

Preterm Birth

Preterm birth is a pregnancy complication with significant healthcare implications; in 2004, 12.8 % of all births in the USA were preterm (National Centre for Health Statistics 2006), and recent figures have shown that 1.3 % of all deliveries in the UK (approximately 10,000 births/year) occurred before 32 weeks gestation (NHS Maternity Statistics 2007), when mortality and morbidity are most severe. In the UK, 75 % of neonatal deaths, and the majority of neonatal intensive care admissions, are in preterm babies (Slattery and Morrison 2002). In some industrialised countries, particularly the USA, the preterm birth rate has remained high over the past 20–30 years; indeed, there has been a small upward trend (Goldenberg and Rouse 1998; Tucker and McGuire 2004). However, this increase in preterm births rate has been in part attributed to obstetric interventions, such as induction of labour (Thompson et al. 2006).

Several psychosocial stressors, such as pregnancy-related anxiety (Orr et al. 2007), stressful life events (Wadhwa et al. 1993), distress (Rondó et al. 2003), death of husband (Cepicky and Mandys 1989) and low self-esteem (Copper et al. 1996) or optimism (Rini et al. 1999), have been investigated as potential risk factors for preterm birth. Although the majority of researchers have reported a significant association between preterm delivery and maternal stress (Hedegaard et al. 1993; Wadhwa et al. 1993; Copper et al. 1996; Nordentoft et al. 1996; Lobel et al. 2000; Dole et al. 2003; Rondó et al. 2003), other studies have questioned the association (Pagel et al. 1990; Perkin et al. 1993; Peacock et al. 1995). There are several potential explanations for such conflicting conclusions, including poor definitions of stressors and inadequate sample size, which may have influenced the results of previous studies.

Table 1 Association between maternal exposure to severe life events and risk of preterm birth

Variable	Number of pre-term births	RR (95 % CI)
Death of any relative		
Unexposed	41,523	1.00
Exposed at any time	1,611	1.09 (1.04, 1.14)
Before pregnancy	884	1.16 (1.08, 1.23)
Trimester 1	364	1.03 (0.93, 1.13)
Trimester 2	363	1.01 (0.91, 1.11)

Adapted from (Khashan et al. 2009)
Adjusted for year of birth, parity, maternal age, maternal history of diabetes, and maternal history of hypertension, acute myocardial infarction, and renal disease

Using the Danish National Registers, we investigated the association between maternal exposure to severe life events and risk of preterm birth. Mothers of all singleton live births ($n = 1.35$ million births) in Denmark between January 1, 1979 and December 31, 2002 were linked to data about their children, parents, siblings and partners. As previously discussed, we defined exposure as the death or serious illness in close relatives in the first or second trimesters or in the 6 months before conception. Log-linear binomial regression was used to estimate the effect of exposure on preterm birth, very preterm birth and extremely preterm birth.

There were 58,626 (4.34 %) preterm births (<37 weeks), 11,732 (0.87 %) very preterm births and 3,288 (0.24 %) extremely preterm births in the study cohort. Severe life events in close relatives in the 6 months before conception increased the risk of preterm birth by 16 % [relative risk (RR) = 1.16,(95 % confidence interval (CI): 1.08–1.23); Table 1)]. Severe life events in older children in the 6 months before conception increased the risk of preterm birth even more, by 23 % [RR = 1.23, (95 % CI: 1.02–1.49)] and the risk of very preterm birth by 59 % [RR = 1.59 (95 % CI: 1.08–2.35; Khashan et al. 2009)].

This study suggests that maternal stress, as mediated by severe life events, may play a role in increasing the risk of preterm birth. It also suggests the timing of the exposure is crucial to the provocation of preterm birth, as the effect is only present with pre-pregnancy and first trimester exposure. It is possible that the effects are mediated by an alteration in the uterine environment early in fetal/placental development.

Maternal Stress and Fetal Growth

Intrauterine growth restriction (IUGR), in which a baby fails to reach its growth potential, is a serious complication of pregnancy. Perinatal mortality rates in IUGR fetuses are four to ten times higher than those in normally grown infants

(Chiswick 1985), and approximately 5–10 % of all pregnancies complicated by IUGR will result in either stillbirth or neonatal death (McIntire et al. 1999; Thornton et al. 2004). Suboptimal fetal growth is responsible for at least one quarter of all stillbirths (Morrison and Olsen 1985), and recent evidence suggests that this figure is probably higher (Gardosi et al. 2005). Analysis of over 23,000 fetal deaths in California on population-based percentile curves showed a strong link between low fetal weight for gestational age and fetal demise (Williams et al. 1982).

Previous studies have suggested an association between stress and low birth weight (<2,500 g; Rondó et al. 2003), anxiety (Rini et al. 1999), depression (MacKey et al. 2000), distress (Rondó et al. 2003), 9/11 attacks (Smits et al. 2006), life events (Sable and Wilkinson 2000), lifestyle (Grjibovski et al. 2004), and work-related stress (Oths et al. 2001). The authors of most of these studies reported that maternal exposure to psychosocial stress decreased birth weight significantly. Smits et al. (2006) defined stress as exposure to the 9/11 attacks via media reporting in a study of Dutch neonates. The authors reported around a 50-g reduction in mean birth weight of the offspring of exposed women compared with those who were unexposed. Pritchard and Teo (1994) found a significant association (odds ratio = 4.08) between high levels of household strain at 20 and 30 weeks gestation and the odds ratio of low birth weight. Dejin-Karlsson et al. (2000) reported a more than threefold increased odds ratio of small for gestational age (SGA) infants in relation to a poor social network and an almost threefold increased odds ratio of SGA in relation to poor social support. In contrast, Homer et al. (1990) found no significant association between maternal work-related stress and risk of low birth weight. In another study, there was no significant association between maternal exposure to life events and mean birth weight (Stein et al. 1987). Several reasons may have contributed to the inconsistent results reported from these studies, including different definitions of psychosocial stress, small sample size, and retrospective designs (Istvan 1986).

In a large study, Smith et al. (2002) reported that first-trimester plasma concentrations of a protein associated with the transfer of nutrient flow from mother to fetus in the placenta was significantly correlated with final fetal weight. Using the Danish National Registers, we investigated the hypothesis that the greatest effect of stress on final fetal weight would occur after exposure in the first trimester. Furthermore, fetal weight is sex dependent (on average, males are heavier at birth than females); therefore, we also hypothesized that any stress effects on fetal weight would be sex dependent.

Mothers of 1.38 million singleton live births in Denmark between January 1, 1979 and December 31, 2002 were linked to information on their spouses, parents, siblings, and older children. As before, exposure to stress was defined as death or serious illness in a relative during pregnancy or in the 6 months before conception. Linear regression was used to examine the effect of exposure on birth weight. Log-linear binomial regression was used to assess the effect of exposure on SGA.

Table 2 Association between maternal exposure to death of a relative and birth of a baby weighing below the 10th percentile

Variable	Number of births	SGA no.	RR (95 % CI)
Death of any relative			
Unexposed	1,024,275	81,101	
Exposed at any time	25,404	2,227	1.17 (1.13, 1.22)
Before pregnancy	11,235	923	1.13 (1.06, 1.20)
Trimester 1	4,240	373	1.17 (1.07, 1.29)
Trimester 2	5,657	406	1.24 (1.13, 1.36)
Trimester 3	333,273	523	1.22 (1.12, 1.32)

Adjusted for year of birth, parity, maternal age, maternal history of diabetes, and maternal history of hypertension, acute myocardial infarction, and renal disease (adapted from Khashan et al. 2008b)

We found that the death of a relative during pregnancy or in the 6 months before conception reduced birth weight by 27 g (adjusted estimate −27 g, 95 % CI = −33, −22). There was a significant association between maternal exposure to death of a relative and risk of a baby weighing below the 10th percentile (adjusted RR = 1.17, 95 % CI = 1.13, 1.22) and 5th percentile (adjusted RR = 1.22, 95 % CI = 1.15, 1.29; Khashan et al. 2008b; Table 2).

Mothers exposed to severe life events before conception or at any point during pregnancy have babies with significantly lower birth weights. Contrary to our hypothesis, the effect of maternal stress on fetal growth was maximal in the second trimester; at this crucial time, fetal growth and organ development are associated with an increase in the maternal blood supply to the fetus and placenta, consequent on invasion of the maternal spiral arteries by placental trophoblast cells (Goldman-Wohl and Yagel 2002). Thus, if this association is causal, the potential mechanisms of stress-related effects on birth weight include changes in lifestyle due to the exposure and stress-related dysregulation of the hypothalamic-pituitary-adrenal axis during pregnancy.

Maternal Stress and Psychiatric Outcomes

Schizophrenia

Schizophrenia is a serious, potentially lifelong and disabling condition that is associated with abnormalities of brain structure and function. A large body of evidence from “natural experiments” such as twin studies suggests that it is highly heritable (up to 85 %; Cardno et al. 1999). Increasingly, schizophrenia is believed to be a disorder of early brain development influenced by environmental risk factors

that interact with the combined effects of multiple (small-effect) susceptibility genes (Tsuang et al. 1993; Murray et al. 2004).

There is an extensive literature linking exposures such as war (van Os and Selten 1998), famine (Susser and Lin 1992; St Clair et al. 2005), A-bomb radiation (Imamura et al. 1999) and unwanted pregnancy (Myhrman et al. 1996) to offspring schizophrenia risk. Most of these studies reported a positive association between exposure of a population to a severe life event and schizophrenia risk in subsequent offspring cohorts. However, apart from Myhrman et al. (1996), they have been unable to examine exposure of individual subjects to risk factors/stressors. Furthermore, exposures like famine, the A-bomb, and war are complex and may combine several risk factors other than emotional stress, including malnutrition, ionizing radiation, and infectious disease. In addition, most of these studies failed to control for potential confounders such as family history of mental illness and urbanicity.

We used the Danish population registers to examine rates of schizophrenia in a cohort of offspring whose mothers had been exposed antenatally to an objective and incontrovertible measure of stress that would be independent of the psychological characteristics of the mother: death or illness in a first-degree relative (mother, father, spouse/partner, sibling, or older child). Based on findings reported by Hansen et al. (2000), we hypothesized that the strongest association would be to stressors affecting the mother during the first trimester.

In a cohort of 1.38 million Danish births from 1973 to 1995, mothers were considered exposed if one (or more) of their close relatives died or was diagnosed with cancer, acute myocardial infarction, or stroke syndrome up to 6 months before conception or during pregnancy. Offspring were followed up from their 10th birthday until their death, migration, onset of schizophrenia, or June 30, 2005; admissions were identified by linkage to the Central Psychiatric Register.

The risk of schizophrenia and related disorders was raised in offspring whose mothers were exposed to the death of a relative during the first trimester [adjusted RR, 1.67 (95 % CI, 1.02–2.73)]. The death of a relative during other trimesters or up to 6 months before pregnancy was not linked with a higher risk of schizophrenia (Khashan et al. 2008a) (Table 3).

Our population-based study suggests that severe stress to a mother during the first trimester may alter the risk of schizophrenia in offspring. This finding is consistent with ecological evidence from whole populations exposed to severe stressors and suggests that environment may influence neurodevelopment at the feto-placental-maternal interface.

Affective Disorders

Affective disorders are mental disorders characterized by dramatic changes or extremes of mood. Although the affective disorders are not a clearly delineated group of illnesses, they include unipolar and bipolar depression, generalised anxiety disorder, and more specific anxiety disorders such as agoraphobia, panic disorder

Table 3 Estimates for offspring schizophrenia risk according to timing of exposure during the antenatal period (adapted from Khashan et al. 2008a)

Death of any relative	No. of cases	Partially adjusted RR (95 % CI)[a]	Adjusted RR (95 % CI)[b]
Unexposed	1,813	1 (REF)	1 (REF)
Exposed before pregnancy	46	1.01 (0.75–1.36)	1.01 (0.75–1.35)
Exposed 1st trimester	16	1.74 (1.06–2.85)	1.67 (1.02–2.73)
Exposed 2nd trimester	9	1.04 (0.54–1.99)	0.97 (0.50–1.87)
Exposed 3rd trimester	9	0.76 (0.39–1.46)	0.74 (0.38–1.43)

[a]Partially adjusted for calendar year and statistical interaction between offspring age and sex
[b]Adjusted for offspring age, sex, place of birth, family history of mental illness, maternal age, calendar year, unknown father and statistical interaction between offspring age and sex

and social phobia, obsessive-compulsive disorder and post traumatic stress disorder (PTSD). They aggregate strongly in families and may therefore share common heritable anomalies. There is evidence to suggest an association between prenatal exposure to major life events and a risk of affective disorders in offspring; furthermore, this association may be gender specific (Brown et al. 1995, 2000; Watson et al. 1999). Prenatal exposure to the Dutch famine was associated with a higher risk of affective disorders (Brown et al. 1995, 2000). Brown et al. (1995) performed two studies to investigate the effects of prenatal exposure to the Dutch famine (1944–1945) on the risk of affective disorders in offspring. The authors used inpatient data from the Dutch Psychiatric Registry to identify affective disorder diagnoses between 1978 and 1991 in the offspring who were born between 1944 and 1946 and survived to at least 18 years. They found more than a twofold increased risk of affective disorder in male offspring who were prenatally exposed to the famine in the second trimester, but not in females. A few years later, the authors repeated the investigation by including all affective disorder cases between 1970 and 1996. They reported a 70 % increase in risk of affective disorder in male offspring and a 30 % increase in female offspring in the second and third trimesters but not the first (Brown et al. 2000). Watson et al. (1999) compared the risk of affective disorder in 18-year-old high school students who were exposed in utero to the Tangshan earthquake in China in 1976 with 18-year-old students who were not exposed. The authors reported an increased risk of severe depression in all trimesters in male offspring but not in females.

Based on our previous work (Khashan et al. 2008a) and that of others (Brown et al. 2000), we hypothesised that prenatal exposure to severe life events in the first, second or third trimesters would increase the risk of affective disorders. We also aimed to explore whether there would be gender differences in the effects of prenatal exposure to severe life events on affective disorders.

In a cohort of 1.1 million Danish births from May 1978 until December 1997, mothers were considered exposed if one (or more) of their close relatives died or was diagnosed with a serious illness up to 6 months before conception or during pregnancy. Offspring were followed up from their 10th birthday until their death,

Table 4 Estimates for offspring affective disorders risk according to offspring gender and timing of exposure during the antenatal period (adapted from Khashan et al. 2011)

Death or illness of any relative	No. of cases (males)	Adjusted RR (95 % CI)[a]	Adjusted RR in males (95 % CI)[b]	Adjusted RR in females (95 % CI)[b]
Unexposed	7,144 (2,179)	1 (REF)	1 (REF)	1 (REF)
Exposed during any period	347 (123)	1.03 (0.93, 1.15)	1.20 (1.00, 1.44)	0.96 (0.84, 1.10)
Exposed before pregnancy	151 (45)	1.01 (0.86, 1.19)	1.00 (0.75, 1.35)	1.02 (0.84, 1.23)
Exposed 1st trimester	60 (26)	1.00 (0.78, 1.29)	1.26 (0.85, 1.89)	0.88 (0.64, 1.23)
Exposed 2nd trimester	64 (26)	1.18 (0.93, 1.15)	1.55 (1.05, 2.28)	1.02 (0.74, 1.40)
Exposed 3rd trimester	72 (25)	0.99 (0.79, 1.25)	1.27 (0.88, 1.85)	0.87 (0.65, 1.17)

[a]Variables included in the model were calendar year and statistical interaction between offspring age and sex

[b]Variables included in the model were calendar year, statistical interaction between exposure and offspring sex, and statistical interaction between offspring age and sex. *GA* gestational age

migration, onset of affective disorder or 31 December 2007; hospital admissions were identified by linkage to the Central Psychiatric Register. Log-linear Poisson regression was used for data analysis.

The risk of affective disorders was increased in male offspring whose mothers were exposed to severe life events during the second trimester [adjusted RR 1.55 (95 % CI 1.05–2.28)] (Table 4). In addition there was an increased risk of affective disorders in male offspring in relation to maternal exposure to death of a relative in the second trimester [adjusted RR 1.74 (95 % CI 1.06–2.84)] or a serious illness in a relative before pregnancy [adjusted RR 1.44 (95 % CI 1.02–2.05)]. There was no evidence of an association between prenatal exposure to severe life events and risk of affective disorders in female offspring (data not shown).

Our population-based study suggests that prenatal maternal exposure to severe life events may increase the risk of affective disorders in male offspring. These findings are consistent with studies of populations exposed to famine and earthquake disasters that indicate that prenatal environment may influence the neurodevelopment of the unborn child.

Summary

There is a growing body of research to support an association between antenatal maternal stress and offspring outcomes. These findings have implications in the search for the effect of antenatal maternal stress on the offspring. The effect of the stressor on the development of the fetus depends on the timing of that particular stressor during the exposure period. Understanding the effect of the timing of stress may play an important role in exploring the mechanism through which the stress

affects the obstetric and psychiatric outcomes. One of the main advantages of utilising Scandinavian registers such as the Danish registers is that short-term (obstetric outcomes) and long-term (mental ill-health) consequences of maternal stress can be investigated. Moreover, the registers facilitate the investigation of potential pathways between maternal stress and offspring outcomes. Identifying these pathways could have a great impact on the design and conduct of future intervention studies. Increased understanding of such mechanisms may result in approaches to reduce the incidence of obstetric complications and psychiatric morbidity.

The demonstration of direct hormonal mediation between antenatal maternal stress and schizophrenia would necessitate drug therapies; alternatively, if mediation is via pregnancy complications, antenatal surveillance/preventative strategies after exposure to stress will be of paramount importance. Drug therapies would be necessary also if the association between maternal stress and obstetric outcomes is mediated by hormonal changes. However, mediation via smoking, alcoholism and/or drug addiction would demand special support to the stressed pregnant women in order to encourage avoidance of using such products.

Finally, policymakers need to design and conduct intervention studies (since prevention studies are almost impossible with this type of exposure) to reduce the effect of maternal stress on offspring outcomes. The target of such interventions should be improving obstetric outcomes in stressed women because these outcomes would not need long follow-up periods compared to schizophrenia. Improvements in obstetric outcomes may have important consequences on the cost of neonatal health care by, most importantly, reducing the number of infant deaths.

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The Role of the Placenta in Fetal Programming

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Abstract The placenta occupies a critical place in fetal programming by modulating fetal responses to alterations in the maternal environment, by regulating transfer of nutrients between mother and fetus and by responding to changes in environmental stimuli that affect maternal and/or fetal physiology. Placental size and function are exquisitely sensitive to alterations in maternal nutrition, oxygen tension and exposure to glucocorticoids. These factors affect the activity of proteins such as

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the System A transporter that shuttles neutral amino acids to the fetus, the activity of cells such as those producing placental lactogen, a key metabolic hormone of pregnancy, and the generation of peptides such as the urocortins, members of the corticotrophin releasing hormone family with pro-and anti-inflammatory activities, and placental 11βhydroxysteroid dehydrogenase-2, which regulates placental metabolism of maternal cortisol. Maternal administration of exogenous synthetic glucocorticoid bypasses this metabolic barrier, programming reductions in fetal body weight and composition, and changing development of the fetal neurologic, pancreatic and hypothalamic-pituitary-adrenal (HPA) axes, leading to altered stress and inflammatory responses, cardiovascular activity and predisposition to insulin resistance in later life. Interestingly, many of these placental functions appear to be regulated in a manner that is dependent upon the sex of the fetus.

Introduction

It is now well accepted that the propensity to adult disease is influenced by aspects of postnatal lifestyle, including poor nutrition and inadequate exercise, superimposed upon a particular genetic background. However, that genetic background will have been modified and determined by in utero exposure to different environments and adversity (Barker 1994a, b). The fetus might respond to adversity in the environment in a manner that is appropriate for intrauterine physiologic adaptation, or the fetus may recognize maternally derived cues that signal the anticipated post-natal environment. If the prediction is wrong, then mismatch occurs (Gluckman and Hanson 2006). In many circumstances, the responses of the fetus to adversity may result in epigenetic changes to the fetal genome. The interaction between the modified prenatal epigenome and postnatal environment dictates conditions of health and disease and the later life development of noncommunicable disorders (Gluckman et al. 2011). The placenta plays a critical role in transmission of maternal cues to the fetus. The placenta transfers nutrients from the mother to the fetus, produces hormones that influence fetal development either directly or indirectly by affecting maternal metabolism, and provides a metabolic barrier that maintains an appropriate glucocorticoid gradient between mother and fetus (Burton et al. 2011). Many aspects of fetal growth and development are influenced by glucocorticoids. In general, cortisol promotes maturation of fetal organ systems while diminishing the rate of proliferation. It is apparent that inappropriate exposure to high levels of glucocorticoid at an inappropriate stage of development may interfere with the normal sequences of maturation (Benediktsson et al. 1993; Matthews et al. 2002; Murphy et al. 2006).

In this chapter we shall conclude the following: the programming of later life health and development begins in utero and occurs in response to the environment within the womb; prenatal responses may predispose to altered postnatal

development and patterns of disease through mismatch between pre- and postnatal environments; definition of the first 1,000 days of development begins at conception, or before, but not just at birth; and finally, the placenta has a critical role in influencing these events.

The Fetal Supply Line

The nutritional supply line between mother and fetus is complex and subject to multiple points of physiologic regulation and pathologic interruption (Barker 1994b; Rumball et al. 2008). Maternal body composition and maternal nutritional intake are starting points for fetal nutrition. The uptake of maternal nutrients is influenced by the maternal gastrointestinal microbiome and the efficiency of nutrient transfer from the maternal gut to the maternal circulation, the transfer across the placenta, blood flow within the umbilical and fetal circulations and the efficiency of nutrient uptake into fetal tissues. Placental transfer for glucose and amino acids is regulated by different transporter systems, as well as by the placental blood flow (Jansson and Powell 2006). In our studies, below, we have examined the developmental regulation of one of these transporter systems, the System A neutral amino acid transporter, and have shown that regulation exists in response to change in the adrenal glucocorticoid system.

Fowden and colleagues (1998) have summarized interactions between nutrition and alterations in maternal and/or fetal glucocorticoids. Briefly, maternal undernutrition may provoke a stress stimulus to the mother, resulting in increases in circulating glucocorticoids that can be transmitted across the placenta to the fetus. In the human or sheep, where the active glucocorticoid is cortisol, maternal undernutrition reduces activity of the placental 11β hydroxysteroid dehydrogenase-2 (11β HSD-2), resulting in less placental inactivation of maternal cortisol and greater transfer of cortisol to the fetal compartment (Challis and Connor 2009). Fetal under-nutrition and/or hypoxemia may result from reductions of utero-placental blood flow or from other placental pathologies and may also occur in response to environmental change such as high altitude. Hypoxemia and hypoglycemia in the fetus provoke acute or chronic activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis and stimulate rises in fetal adrenal cortisol output and elevated concentrations of cortisol in the fetal compartment (Alfaidy et al. 2002).

Maternal nutrition and transplacental transfer of nutrients may affect fetal growth directly and may program later responses to different postnatal diets. Early work had shown that reduction of maternal dietary protein fed to pregnant rats reduced fetal weights at birth, and those offspring developed hypertension and became glucose intolerant by 16 weeks of postnatal age (Langley-Evans et al. 1996). Interestingly, placental weights were significantly increased in these animals, potentially reflecting an adaptive hyperplastic response. Subsequently, the interaction between nutritional state in utero and responses to altered postnatal nutrition was demonstrated more clearly in an elegant series of studies from Mark

Vickers and colleagues. Vickers et al. (2000) used more discreetly balanced diets to reproduce the reduction in fetal weight that follows a reduction in maternal protein intake. They found that the offspring again developed hypertension but, in addition, had elevated levels of leptin, insulin, and percentage fat accompanied by a predisposition to diabetes (Vickers et al. 2007). The offspring showed reduced anxiety behaviors, reflecting neurological adaptation, and had increased food intake, suggesting altered appetite control. Thus, it appeared that maternal under-nutrition during pregnancy generated an altered metabolic state in the offspring and influenced appetite control pathways, a topic that we shall return to later in this review. In addition, these offspring had elevated levels of C-peptide, perhaps reflecting altered inflammatory responsiveness. Thompson et al. (2007) later showed that offspring of animals undernourished during pregnancy responded after birth with catch-up growth when fed normal rat chow but had accelerated growth and gross obesity after exposure to a high fat diet. These studies illustrate the important interactions that take place between the intrauterine and postnatal environments.

Our own studies examined regulation of the System A neutral amino acid transporter in the placenta as one factor affecting the fetal supply line. This system transports small, unbranched neutral amino acids. It is located in the syncytiotrophoblast and consists of three isoforms: SNAT1, SNAT2, SNAT4. The expression of these different isoforms changes in the human placenta during pregnancy (Desforges et al. 2009). In vitro studies with human placental explants or trophoblast cells showed that SNAT proteins are upregulated by insulin, leptin and IGF-I and down-regulated by hypoxemia, nitric oxide and free oxygen radicals (Jones et al. 2007). The activity of System A is reduced in placentas of growth-restricted fetuses (Mahendran et al. 2003; Glazier et al. 1997), and inhibition of System A across gestation in the rats resulted in reductions in fetal weight. We found that glucocorticoid exposure of the human placenta in vitro was associated with increased System A activity, but this increase was likely a product of accelerated syncytiotrophoblast proliferation in culture (Audette et al. 2010). In the mouse, we found that placental System A activity rose progressively during gestation, and this increase was associated with modest increases in levels of mRNA encoding each of the SNAT isoforms. Animals treated with synthetic glucocorticoids on days 13.5 and 14.5 of gestation showed no immediate change in System A activity but a 50 % reduction in activity by day 18.5, at term (Audette et al. 2011). Responses were similar in placentas of both male and female fetuses. Surprisingly perhaps, there was no corresponding reduction in SNAT mRNA expression, and it was argued that the change in activity might reflect a post-translational modification of the transporter protein or altered incorporation of protein into the trophoblast cell membrane. Offspring from these pregnancies were not growth restricted, which may reflect altered compensatory activity in other nutrient transport pathways. However, these studies clearly demonstrate an aspect of the interaction between glucocorticoids and fetal nutrition.

Placental Influences on Development of the Fetal HPA Axis

Early studies in rats had shown that inhibition of placental 11β HSD2 with licorice resulted in fetal growth restriction and postnatal hypertension, similar to the responses to maternal under-nutrition (Seckl 1997). These elegant experiments led to an understanding of the importance of placental 11β HSD2 in maintaining the gradient of bioactive glucocorticoid between mother and fetus and the potential adverse consequences of maternal glucocorticoid on the fetus when that barrier was reduced. Because synthetic glucocorticoids are less well metabolized by placental 11β HSD2 than naturally occurring glucocorticoids, we used maternal synthetic glucocorticoid (sGC) administration as a means of examining these responses (Sloboda et al. 2000; Moss et al. 2001). In sheep, administration of sGC in early gestation produced elevations of systolic blood pressure in the offspring after birth. sGC given in early pregnancy also enhanced the activity of the fetal HPA axis in late gestation (Braun et al. 2009). This response appeared to be exerted directly at the level of the fetal adrenal. Immediately following sGC administration on days 39 and 40 (term 145 days), fetal cortisol levels were reduced but without any change in fetal circulating ACTH. In late gestation, the fetal cortisol concentrations of sGC-treated fetuses were significantly higher than controls, but the ACTH concentrations were similar, implying enhanced responsiveness of the fetal adrenal. Adrenal tissue from female fetuses had increased expression of 3β hydroxysteroid dehydrogenase and 17α hydroxylase, potentially accounting for the increase in cortisol output, but these enzymes were not elevated in adrenal tissue from male fetuses. The explanation for the rise in cortisol in males is unclear; it does not appear to be due to changes in corticosteroid-binding globulin (CBG) or in expression of key hypothalamic or pituitary neuropeptides, and it is a subject of ongoing investigation.

sGCs are administered to women at risk of early preterm birth with the goal of enhancing maturation of the lungs and other organs (Liggins and Howie 1972; Newnham and Jobe 2009). Other beneficial effects of sGC for the baby born preterm include reduced ventricular hemorrhage and necrotizing enterocolitis. There is a suggestion that the offspring may be at risk of increased insulin resistance, but further follow up of different cohorts is necessary to resolve the long-term sequelae of these treatments, which remains an area of active debate. When sGCs were administered to late pregnant sheep in clinically relevant amounts and weekly intervals, fetal and placental weights were reduced and the lambs were growth restricted at birth (Ikegami et al. 1997). The administration of sGC reduced the numbers of binucleate cells in the placenta and of the outputs of placental lactogen (PL) into both maternal and fetal circulations (Braun et al. 2007). In the human, PL is an important metabolic hormone that increases transplacental uptake of substrate by the fetus, but it is not clear whether PL has the same importance in ovine pregnancy. Levels of IGF-I and IGF-II in the fetal circulation were also reduced in sGC-treated animals and were associated with reductions in fetal organ

development and body weight, consistent with earlier reports that cortisol administration to fetal sheep reduced tissue IGF expression (Gatford et al. 2008).

We have reported extensively on the effects of administering sGC in late gestation on other aspects of fetal growth and development in sheep (Newnham et al. 2009; Sloboda et al. 2005, 2006; Challis 2012). These include decreases in placental weight, decreases in body weights and in individual organ and brain weights; delayed skeletal maturation predicting altered pre- and postnatal growth patterns; delayed myelination of the optic nerve and reduced weights of specific brain regions, predicting later life neuropathology; altered cardiac function, predicting later life hypertension; altered HPA axis responses, increased at 6 months but decreased at 3 years, predicting altered stress responsiveness and potentially immunologic function in later life; and glucose intolerance, predicting later life type 2 diabetes and obesity. More recent studies have shown that the adverse effects of sGC given in late pregnancy appear more marked than those of early pregnancy treatment (Li et al. 2013). This finding may be of increasing relevance given current recommendations around the use of sGC for women undergoing elective Cesarean section at late preterm and early term gestations. Indeed it has been shown recently that sGCs in sheep at term result in increased adiposity at 12 months of age.

Effects of Maternal Under-Nutrition on Appetite Control Pathways

The relationship between under-nutrition, placental function and the developing HPA axis has been explored in great detail in sheep exposed to periconceptional under-nutrition between days −60 to +30 of gestation. The level of under-nutrition was designed to produce a modest (10 %) decrease in maternal body weight that recovered following conclusion of the nutritional challenge. A high proportion of fetuses in these pregnancies had advanced HPA axis maturation at day 110 of gestation, almost 100 days after completion of the periconceptional insult. These animals exhibited an increased prevalence of preterm birth, although the lamb weights were not inappropriate for gestational age (Bloomfield et al. 2003, 2004). The mechanism of these responses is of great interest. Maternal circulating concentrations of cortisol were reduced during the period of under-nutrition. However, we have also reported that placental 11β HSD2 activity was reduced modestly at day 50 and significantly in tissue collected at day 85 of gestation, and at these two times the ratio of cortisol:cortisone in the fetal circulation was significantly raised (Connor et al. 2009). Thus, the effects of periconceptional under-nutrition on development might reflect altered levels of cortisol in the early and mid-gestation fetuses, as suggested above, or might result directly from epigenetic responses of critical genes to the adversity of under-nutrition.

Periconceptional under-nutrition is known to result in obesity in the offspring (Vickers et al. 2007). Therefore, we predicted that the maternal insult might be

impacting on appetite-regulating genes such as the glucocorticoid receptor (GR) in the hypothalamus of the offspring. We found that, at term, methylation of the GR promoter was reduced and levels of GR mRNA increased in hypothalami of fetuses from undernourished mothers (Stevens et al. 2010). This response was specific to the ventral hypothalamus and was not seen in other brain areas such as the hippocampus or pituitary. Furthermore, twinning had a similar effect to under-nutrition in that there was hypomethylation of the GR promoter in twins from normally nourished mothers but no further effect in twin fetuses from mothers who were undernourished (Begum et al. 2012). The levels of methylation correlated with respective levels of DNA methyltransferase activity. Effects on histone acetylation and trimethylation were found in control twins as well as singleton and twin undernourished animals (Begum et al. 2012). These data clearly imply that periconceptional under-nutrition produces epigenetic changes that could be associated with changes in gene expression in specific brain regions that can be detected more than 100 days after withdrawal of the stimulus. They further suggest that these changes are more pronounced in pregnancies with a singleton fetus and that the control twin fetus may already be functioning at a level of compromise similar to that of the undernourished singleton. Additional studies will be required to determine whether these responses might occur in response to changes in placental function that could include the reductions in 11βHSD2 described above.

Increased glucocorticoids are known to predispose animals to obesity and it is thought that this is in large part due to regulating appetite and energy balance via neuronal networks in the ventral hypothalamus involving both pro-opiomelanocortin (POMC) and neuropeptide Y (NPY). As the neural circuitry develops towards the end of gestation in the sheep, at a similar time to the human, changes in the neuropeptides may not be detected until the offspring are feeding independently. Therefore, increased expression of the GR in this region as a result of maternal under-nutrition would be predicted to cause changes in these neuropeptides, resulting in obesity in the offspring. Studies are currently underway to determine if the epigenetic changes in the GR in the fetal sheep persist in the adult offspring and predispose the adults to obesity. Such a finding would be consistent with the match-mismatch thesis of developmental programming and consistent with the increased appetite of offspring from nutritionally deprived rats. Although appetite was not measured in our studies, the male offspring were obese (Jaquiery et al. 2012).

Placental Neuropeptides

Collectively, these studies have implied that there is interaction between maternal nutrition and the maternal HPA axis, between transplacental nutrient transfer and the regulation of bioactive glucocorticoid levels, and changes in delivery of nutrient with altered HPA axis function in the fetus. Our recent studies have demonstrated that other hormones produced locally within the placenta may have important

activities in relation to placental function and hence fetal growth and development (Petraglia et al. 1996, 2010). It has been established for many years that the human placenta produces a range of neuropeptide hormones that are analagous to those produced in the pituitary and brain. For example, the placenta produces corticotrophin releasing hormone (CRH). CRH output increases in late pregnancy and is higher in women at risk of preterm delivery. The output of placental CRH is regulated by glucocorticoid hormones, and cortisol increases placental CRH gene expression and peptide secretion. Placental CRH is also regulated by cytokines, nitric oxide, and oxytocin and down regulated by progesterone (see Petraglia et al. 2010). Placental CRH is thought to have direct actions on the myometrium and, importantly, increases placental blood flow (Clifton et al. 1995). Hence, one can envisage that, in response to adversity such as hypoxemia or under-nutrition, the fetal HPA axis produces increasing amounts of cortisol that, in turn, up-regulates expression of CRH, a placental vasodilator, in an attempt to increase blood flow and nutrient transfer.

Recently, we became interested in the production of other peptide hormones, the urocortins, which are members of the corticotrophin family and may impact placental function and hence fetal development. Urocortins are present in placental tissue from very early in gestation (Imperatore et al. 2006). Trophoblast cells in vitro produce increasing amounts of urocortin 2 and urocortin 3 in response to lowered oxygen tension (Imperatore et al. 2010). Furthermore, urocortins and CRH increase expression of the placental aromatase enzyme, thereby increasing the production of estrogen in the placenta using C_{19} steroids derived from the fetal or maternal adrenal as precursor (Imperatore et al. 2009). Potentially this provides another regulatory pathway to increasing utero placental blood flow. Hence hypoxemia would tend to increase placental output of the CRH family of peptides that increase blood flow, directly and indirectly, thereby promoting oxygen delivery to the placenta and fetus. Placental CRH preferentially stimulates the output of dehydroepiandrosterone sulphate (DHEAS) by the fetal zone of the fetal adrenal (Sirianni et al. 2005), thereby increasing the availability of estrogen precursor reaching the placenta. Hypoxemia provokes fetal adrenal cortisol output (as well as placental urocortins), which then further stimulates placental CRH, accounting for the elevated levels of maternal and umbilical arterial CRH in the presence of a growth-restricted fetus (see Petraglia et al. 2010).

In all these activities, the level of cortisol acting on the placenta or crossing the placenta into the fetus is regulated by the enzyme 11βhydroxysteroid dehydrogenase-2 in the placental syncytiotrophoblast layer. Regulation of the placental 11β HSD2 enzyme may be critical in different circumstances (Wyrwoll et al. 2009). The enzyme is reduced in response to under-nutrition in sheep, rats and human placental tissue. It is also reduced in response to hypoxemia and infection, circumstances of threat to the fetus. Clifton (2010) has demonstrated that, in asthmatic pregnant women, 11β HSD2 in the placenta increases with maternal glucocorticoid treatment, but only with a female fetus. In this situation, where the placental barrier to cortisol transfer is maintained, autonomous HPA maturation may occur more readily with a female than with a male fetus, contributing to an

enhanced postnatal performance, including lung maturation, following preterm delivery.

Members of the CRH/urocortin family also affect the inflammatory pathway within the placenta. Urocortin synergizes with lipopolysaccharide (LPS) to stimulate output of the proinflammatory cytokine, tumor necrosis factor α, TNFα, by placental cells in vitro. In contrast, urocortin 2 decreased output of proinflammatory cytokines produced in response to the LPS stimulus (Torricelli et al. 2011). We found that placental tissue collected from women in preterm labor with chorioamnionitis expressed increased amounts of CRH, urocortin 2 and CRH-receptor 1 compared to tissue from women delivered preterm without infection (Torricelli et al. 2011). The same pattern of response was produced with LPS stimulation of the tissue. Hence, it is becoming clear that this family of peptides may exert local regulation of inflammatory responses in placental tissue and that these responses in turn may be influenced by the local oxygen tension. We have speculated that a low-grade placental inflammatory reaction may predispose towards fetal growth restriction and preterm labor and that an altered inflammatory pathway could present a common mechanism between these pathologies. Further study of these peptides will be of considerable interest.

Glucocorticoids and Human Pregnancy

Synthetic glucocorticoids, which largely by-pass the placental 11βHSD2 metabolic barrier, are administered to women presenting at risk of early preterm birth to promote fetal lung maturation (McKinlay et al. 2012). As discussed above, this obvious benefit may be tempered by potential adverse effects on fetal growth, development, and postnatal responses (Murphy et al. 2008; Davis et al. 2009). However, many women receiving glucocorticoids in pregnancy do not deliver preterm, since it remains difficult to predict this condition with accuracy. Hence the human fetus may be exposed unnecessarily to single or repeated doses of glucocorticoid in late gestation (Challis 2012). Follow-up of these infants is of great importance. Recently, Alexander et al. (2012) have shown that offspring from mothers who had received glucocorticoid during pregnancy had slightly lower weights at term. At 6–11 years of age, however, these children responded to a social stress test with far greater elevations in salivary cortisol than children from mothers who had delivered at term in the absence of glucocorticoid treatment or children from mothers exposed to an endogenous "stress" during pregnancy but who did not receive synthetic glucocorticoid. The response was greater in girls than in boys, but the reason for this sex-related difference is unknown. Continued observation of this cohort of children will be of great interest to determine childhood effects on school performance and potential neurological differences between the groups. Future studies will need to examine whether glucocorticoids have similar effects when administered at different developmental stages of pregnancy.

Conclusions

Studies described in this brief report have shown the complexity of regulation of fetal growth and have intimated at the relationship between changing activities of the stress axes, in mother, fetus and placenta, with alterations in nutritional state on fetal development. Studies have demonstrated that reduced nutrition very early in pregnancy can program regulation of appetite-control pathways and that undernutrition in pregnancy interacts with the nutritional state postpartum to generate different adolescent and adult phenotypes. It is very clear that, as the gateway between the mother and the fetus, the placenta plays a critical role in the integration of these different responses.

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Male and Female Placentas Have Divergent Transcriptomic and Epigenomic Responses to Maternal Diets: Not Just Hormones

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Abstract There is mounting evidence that the placenta can be considered as a programming agent of adult health and diseases. Placental weight and shape at term are correlated with the development of metabolic diseases in adulthood in humans. Maternal obesity and malnutrition predispose the offspring to developing metabolic syndrome, a vicious cycle leading to transmission to subsequent generation(s), with differences in response and susceptibility according to the sex of the individual. Adaptations in placental phenotype in response to maternal diet and body composition alter fetal nutrient provision. This finding implies important epigenetic changes. However, the epigenetics of placental development in studies of developmental origins of health and disease (DOHaD) is still poorly documented, particularly concerning overnutrition. We used histology, microarray analyses and epigenetic techniques to investigate the effects of a high fat diet (HFD) or low protein diet on mouse placental development, respectively. We showed for the first time that not only the gene sets but also their biological functions affected by the HFD differed markedly between the two sexes. Remarkably, genes of the epigenetic machinery as well as global DNA methylation level showed sexual dimorphism. Imprinted gene expression was altered, with locus-specific changes in DNA methylation. Thus, these findings demonstrate a striking sexual dimorphism of programming trajectories in response to the same environmental challenge,

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implicating sex chromosome genes, not just hormones. Explaining the sex-specific causal variables and how males versus females respond and adapt, and to what extent, to environmental perturbations should help physicians and patients anticipate disease susceptibility.

Introduction to the Sexually Dimorphic Effects of Gestational Obesity and Suboptimal Nutrition

According to the Developmental Origins of Adult Health and Disease (DOHaD) concept, environmental conditions during specific windows of mammalian development can have lasting effects on cell fate, organogenesis, metabolic pathways and physiology, thereby influencing life-long physical health and the susceptibility to lifestyle-induced diseases in adulthood (Barker and Osmond 1988; McMillen and Robinson 2005). There is evidence to suggest that maternal overnutrition, gestational diabetes and obesity are deleterious to the health of offspring, inducing the same range of defects as maternal mal- or undernutrition and leading to the development of metabolic syndrome (Armitage et al. 2005; Boloker et al. 2002; Dabelea et al. 2000; Levin and Govek 1998; Nathanielsz et al. 2007) in offspring, with a striking sex specificity (Dunn et al. 2010; Gabory et al. 2009, 2012, 2013; Gallou-Kabani et al. 2007). The number of overweight or obese women of childbearing age is growing, reaching 25 % in Europe and 50 % in the US (WHO), and potentially triggering a vicious cycle, with transmission to subsequent generations and increasing prevalence of these lifestyle diet-induced disorders. Interestingly, the adverse metabolic consequences of dietary manipulations can be improved or prevented by applying mild food restriction or a normal control diet to the mother (Giraudo et al. 2010; Srinivasan et al. 2006), by reducing maternal obesity by bariatric surgery (Kral et al. 2006; Smith et al. 2009) or by the addition to the maternal diet of specific nutrients involved at different levels of carbon metabolism essential for DNA methylation (Boujendar et al. 2003; Burdge and Lillycrop 2010; Dolinoy et al. 2006; Lillycrop et al. 2005; Torrens et al. 2006; Waterland et al. 2007).

The principal modifiers of disease risk, presentation and treatment include sex and age. Differences between the sexes have been reported for most non-communicable diseases (NCDs), including metabolic diseases, hypertension, cardiovascular disease, cancer and psychiatric and neurological disorders. Sex differences in responses to drugs and diets have also been described, and many DOHaD reports have indicated that exposure to an adverse environment during specific windows of developmental programming may affect the long-term health and susceptibility to NCDs of the offspring in a sex-specific manner (Bale 2011; Barker 1992; Gabory et al. 2009; van Abeelen et al. 2011; Waddell and McCarthy 2012). Large sex differences in the incidence and progression of diseases suggest

that sex-biased factors are an untapped source of factors providing protection against disease (Arnold and Lusis 2012).

Sociocultural considerations have largely obscured divergences in the biology of the sexes, which have been studied essentially only in terms of the organizational and activational effects of sex hormones after sexual differentiation. However, unequal gene expression by the sex chromosomes has an impact much earlier, beginning at conception, and may set the backdrop for events in later life (reviewed in Al-Khan et al. 2011; Clifton 2010; Gabory et al. 2009). The sex chromosomes have a disproportionately large impact on human health and disease. The unique evolutionary pathway of the X and Y chromosomes has resulted in these chromosomes having highly atypical gene contents and activities (Graves 2010), resulting in differences between the sexes even before the initiation of adrenal and gonad development (Penaloza et al. 2009).

In all adult tissues examined to date, including the gonads and brain, the expression of different genes with different functions from different networks is modulated in a sex-specific manner. Gene expression analyses, either for candidate genes or at the genome-wide level, show that both the trajectories under basal conditions and those modulating responses differ between the sexes (Gabory et al. 2009). The mechanisms regulating the differences between the sexes in adults have been investigated intensively in the liver, but dimorphic gene expression has also been reported in several mouse tissues, including the kidneys, lacrimal gland and brain (reviewed in Gabory et al. 2009). A microarray analysis revealed that 14 % (brain) to 70 % (liver) of active genes display sexual dimorphism. Interestingly, these genes cluster together not only on the sex chromosomes but also on several autosomes (Yang et al. 2006). van Nas et al. (2009) identified sexually dimorphic modules of networks of genes with highly correlated patterns of expression implicated in genetic and metabolic traits and genes affected by gonadal hormones in the adipose tissue, brain, liver and muscle. In adult liver, the genes mapping to the sex chromosomes play a lesser role in the modulation of sex-specific gene expression than gonadal hormones (Wauthier et al. 2010). Statistically significant sex differences have been reported for 78 % of the metabolites in the human plasma metabolome (Mittelstrass et al. 2011). For this reason, molecular investigations of the similarities and differences between the responses of male and female conceptuses to various maternal exposures are of considerable interest.

Sex differences in the rate of fetal growth have long been recognized (Lubchenco et al. 1963). The sex of the embryo affects the size of both the fetus and the placenta, together with the ability of the placenta to respond to adverse stimuli (Clifton 2010; Clifton et al. 2010; Mao et al. 2010). In mice and cattle, accelerated development is already evident in XY blastocysts; cell division among male embryos occurs more rapidly than in female embryos (Mittwoch 1993) and, in humans, boys grow more rapidly than girls from the earliest stages of gestation (Eriksson et al. 2009). These differences may start as early as the blastocyst stage in bovines: one third of the genes actively expressed in bovine blastocysts display sexually dimorphic expression (Bermejo-Alvarez et al. 2008, 2010). Analysis of genes involved in amino acid transport and metabolism identified sex differences

both in average placental gene expression between male and female and in the relationships between placental gene expression and maternal factors (Sturmey et al. 2010). Ontological analysis of such data suggests a higher global transcriptional level in females and greater protein metabolism levels in males. Specifically, global glucose metabolism and pentose-phosphate pathway activity are twice and four times greater in bovine male vs. female blastocysts, respectively, with similar metabolic differences being seen for human embryos at the same stages (for review Bermejo-Alvarez et al. 2011). At birth, placental weights and FPI (fetus-to-placenta weight ratio index, reflecting placental efficiency) tend to be greater in boys than girls (Lampl et al. 2010). These observations suggest that males may be both more responsive to growth-promoting influences and more susceptible to supply disturbances (Lampl et al. 2010; Wallace et al. 2012).

As a critical messenger between the maternal environment and the fetus, the placenta may play a key role not only in buffering environmental effects transmitted by the mother but also in expressing and modulating effects due to preconceptional exposure of both the mother and the father to stressful conditions. Sexually dimorphic patterns of expression have recently been reported for individual genes or pathways in placentas from humans and rodents, potentially accounting for differences in the sensitivity of male and female fetuses to maternal diet (reviewed in Gabory et al. 2009). However, few groups have studied global sexual dimorphism in the placenta with microarrays, focusing in particular on the impact of maternal diet, asthma or stress on placental gene expression through systematic investigations of the relationship between diet and the expression of sexually dimorphic genes (Mao et al. 2010; Sood et al. 2006). Even fewer studies have investigated the associated epigenetic changes (Gabory et al. 2012; Gallou-Kabani et al. 2010).

Figure 1 shows how such influences may operate on the transmission of environmental influences to subsequent generation(s) and illustrates the central role of the placenta in the sex specificity of these parent-of-origin effects. Support for the possibility of inter- and transgenerational effects is also emerging, making it important to know the role played by the placenta and the possible maternal and or paternal epigenetic imprints carried by the gametes forming the zygote. Indeed, maternally or paternally transmitted, non-erased epigenetic alterations of key developmental genes may perturb early trophoblast development in a sex-specific manner (Fig. 1).

Placenta Is a Sexually Dimorphic Organ

During pregnancy, the placenta maintains fetal homeostasis by regulating nutrient transfer from the mother to the fetus. This ‘gateway’ to the fetus is affected by numerous environmental factors, each of which may modifies epigenetic marks and gene expression within the placenta (Novakovic and Saffery 2012). Various environmental factors, including nutrient status and tissue oxygenation, may modify

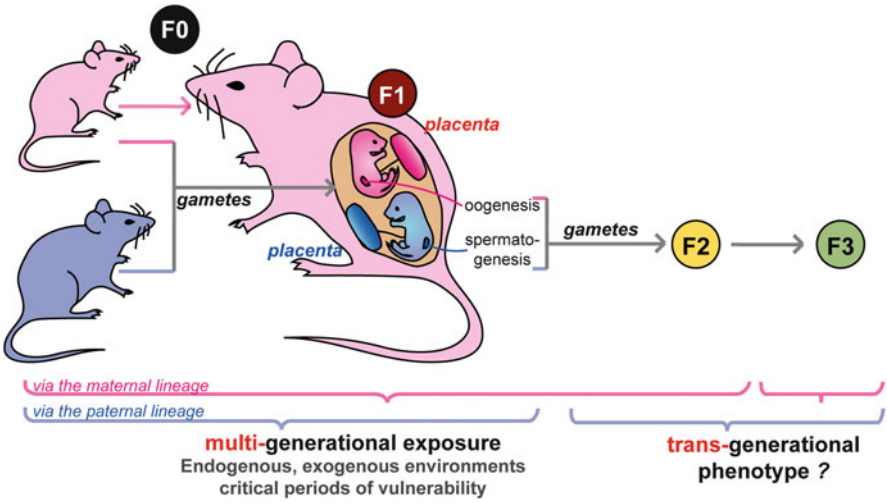

Fig. 1 Sex-specific transmission of exposure to environment to subsequent generations. Environmental factors—including nutrition, psychosocial stress, toxins, endocrine disruptors, tobacco, alcohol, and microbiota—impact individual (F0) epigenetic landscapes, and hence gene pathways and networks, in ways that differ between the sexes. For example, maternal and paternal preconceptional exposures can alter gamete epigenetic marks, some of which can be transmitted to the subsequent (F1) generation. Additionally consequences of maternal F0 exposure during pregnancy (stress, metabolism, diet, hormonal changes...) can be transmitted from the maternal to the fetal compartment via the placenta in a sex-specific manner and can affect F1 tissue development. Programming of somatic tissues can lead to changes in long-term health outcomes in the first generation. Moreover, primordial germ cells, which develop and undergo reprogramming during fetal development, can also be affected by F0 maternal environment and contribute genetic and epigenetic information to the F2 generation. Maternal and paternal lineages affect the transmission of such influences differently. In particular, intergenerational effects of F0 exposure on the maternal lineage can be seen in the F1 and F2 generations, and transgenerational to F3, whereas the paternal lineage can transmit intergenerational effects to the F1 and transgenerational effects to the F2 and F3 generations

placental development and function (Cross and Mickelson 2006; Liang et al. 2010). Studies of rodents and large animals have shown placental development to be highly adaptable, with many means of compensating for poor nutritional conditions (Coan et al. 2010; Constância et al. 2005; Gheorghe et al. 2010; Mao et al. 2010; Reynolds et al. 2010).

The placenta has traditionally been considered an asexual organ, and many studies focusing on the placenta have not taken the sex of the embryo into account (Clifton 2010). Predisposition to various diseases in adulthood, including type 2 diabetes (T2D), hypertension and cardiovascular disease (CVD), is affected by the food consumed by the mother during pregnancy. It thus appears likely that sex-specific placental functions contribute to the differences in frequency of these diseases between the sexes. The sex of the embryo affects the size of both the fetus and the placenta, together with morbidity and the ability of the placenta to respond

to adverse stimuli (Clifton 2010; Clifton et al. 2010; Mao et al. 2010). Moreover, Wang et al. (2011) showed that the maternal decidual renin-angiotensin system (RAS) in humans is regulated in a sex-specific manner at term, both before and after labor. Thus, the sex of the fetus affects gene expression, indicating possible functional differences as a function of fetal sex.

The fact that boys tend to be longer at birth than girls suggests that the placenta may be more efficient for male than for female fetuses, although it may have a smaller reserve capacity. From the earliest stages of gestation, boys grow more rapidly than girls and are therefore at greater risk of undernutrition (Eriksson et al. 2009). Unfavorable programming, whether immediately before conception or during gestation, may result in various defects potentially translated into differences in susceptibility to disease between boys and girls (Clifton 2010; Dunn et al. 2010; Eriksson et al. 2009; Gabory et al. 2009; Gallou-Kabani et al. 2010; Ng et al. 2010). Thus, the placenta constitutes an ideal organ in which to study the sensing, by the fetus, of stresses, starvation, endocrine disruptors and obesity-prone diets or lifestyles, in a sex-specific manner.

Neither Sex Nor Maternal HFD Triggers Gross Morphological Changes in Placental Layers

We investigated the impact of a high-fat diet (HFD) during gestation on fetoplacental development in a mouse model. We collected placentas in the middle of the fetal period, when the morphological development of the placenta is complete and fetal growth is maximal (Gallou-Kabani et al. 2010). We observed a major effect of sex on placental weight, with male placentas being larger than female placentas. Under the HFD, the placental weight was increased in both males and females. There was no effect of sex or diet on fetal weight. Therefore the FPI was greater in females than in males and was reduced under a HFD.

Experimental and epidemiological studies in humans and animal models have demonstrated that predisposition to impaired glucose tolerance, blood pressure and coronary heart disease is associated with either a low or high fetus-to-placenta weight ratio and a decrease in FPI under maternal HFD, for both male and female placentas (Gallou-Kabani et al. 2010). This finding indicates a change in placental efficiency, which could be due to either a change in placental development or to gross morphological changes in the different layers. Using in situ hybridization with several probes, we could not detect any changes in the respective proportion of the two layers (Gallou-Kabani et al. 2010). To assess whether these weight changes were associated with morphological changes, we analyzed the placenta with classical histological approaches. No obvious changes were observed in the structure of the labyrinth or spongiotrophoblast. The proportion of the placenta occupied by the labyrinth was not affected by diet or sex of the offspring. However, as shown by the dysregulation of important genes involved in placental development, we cannot

exclude subtle changes (Cross et al. 2003; Rawn and Cross 2008). Altogether this finding suggests that the observed increase in placental weight was distributed evenly between the layers. Moreover, overall placental and labyrinth shapes, estimated from minor and major lengths, did not differ between the four groups. Thus, neither the sex of the offspring nor HFD in the mother triggered gross morphological changes in the respective structure and size of the placental layers (Gabory et al. 2012).

Sexual Dimorphism and Sensitivity to Diet for 9/20 Genes from Four Clusters of Imprinted Genes

It has been suggested that changes in imprinted gene dosage in the placentas may compromise the prenatal control of nutritional resources (Charalambous et al. 2007). Indeed, monoallelic behavior and sensitivity to changes in regional epigenetic state render imprinted genes both vulnerable and adaptable. However, the underlying mechanisms remain unclear.

We investigated whether a HFD during pregnancy modified the expression of imprinted genes in the placenta. We compared gene expression patterns in total placenta homogenates, for male and female offspring, by the RT-qPCR analysis of 20 imprinted genes. Sexual dimorphism and/or sensitivity to diet were observed for nine genes from four clusters on chromosomes 6, 7, 12 and 17. Six genes (*Slc22a1*, *Slc22a2*, *Slc22a3*, *Rtl1*, *Dlk1* and *Dio3*) displayed changes in expression pattern when the mother was fed the HFD. We observed sex-specific sensitivity to the HFD, with effects limited to or more pronounced in the female placenta for *Dlk1*, *Dio3*, *Slc22a1* or in the male placenta for *Slc22a2* only. Our results are therefore consistent with previous findings that female placentas display more striking changes in gene expression in response to maternal diet than do male placentas. As suggested by Penaloza et al. (2009), this difference in cell behavior and sensitivity appears to be driven by the genetic sex of the cells, with the effects of factors such as hormones subsequently being superimposed on this difference.

Concerning the chromosome 17 cluster (*Slc22a2*, *Slc22a1* and *Slc22a3*), the sex steroid hormone estrogen down-regulates renal organic cation transport in animals and may contribute to sex-related differences in xenobiotic accumulation and excretion (Asaka et al. 2006; Pelis et al. 2007; Urakami et al. 2000). However, caution is required when extrapolating transport-related sex differences between species and organs. These data on sexual dimorphism in organic cation transport are nonetheless potentially interesting when trying to understand the differences between the sexes in terms of the response in the placenta. For the chromosome 12 cluster, an effect of diet was observed for the paternally expressed *Dlk1*, *Rtl1* and *Dio3* genes but not for the maternally expressed *Gtl2/meg3* genes, with female placentas again being more sensitive than male placentas to the effects of the HFD.

This study was the first to demonstrate that the placentas of male and female fetuses from mothers fed a HFD displayed changes in the expression of selected imprinted genes from different clusters, with these changes differing between the sexes.

Differential CpG Methylation of the Igf2r DMR

The transporter genes *Slc22a2* and *Slc22a3* are imprinted specifically in mouse placenta (Wagschal and Feil 2006). The promoters of the repressed paternal alleles of these genes do not display DNA methylation (Sleutels et al. 2002). The *Igf2r* imprint control element (ICE), which is a differentially methylated région (DMR) containing 30 CpG, plays a crucial role in regulating many imprinted genes in this cluster. We therefore investigated whether adaptation of the nutrient supply to fetal demand in pregnant mice fed a HFD involved the ICE/DMR regulating these important placental transporter systems (Gallou-Kabani et al. 2010). In a bisulphite-sequencing analysis of all 30 CpG within the DMR of the chromosome 17 cluster, we found no statistically significant difference between the sexes or the two diets. However, a CpG by CpG analysis revealed sex- and diet-specific differential methylation of individual CpGs in two conspicuous subregions of the DMR. Significantly different levels of methylation between the sexes were found for the first four CpGs in fetuses from mothers fed the HFD. Similarly, different levels of methylation between the sexes were found for the next five CpG and for CpG 20 in fetuses from mothers fed the control diet (CD). CpG 2 was the only CpG displaying both diet response and sexual dimorphism (Gallou-Kabani et al. 2010).

Bioinformatic analysis suggested that the CpGs displaying sex- and/or diet-specific differential methylation in the DMR might lie within recognition elements or binding sites for transcription factors or factors involved in chromatin remodelling, or within a higher-order chromatin architecture including Pax4, Smarca3, Nrf2/Arp, Ppar/Rxr, Egr3, Rxr, Stat6 (Gallou-Kabani et al. 2010).

Placental Gene Expression Is Sexually Dimorphic Under CD and Under HFD

Using Affymetrix exon microarrays, we analyzed gene expression in our placenta samples. We obtained expression data for four groups: female (F) or male (M) placentas from mothers under the control (CD) or high-fat (HFD) diet (F CD, F HFD, M CD and M HFD). First, we analyzed the data to detect sexual dimorphism in gene expression by comparing M CD vs F CD and M HFD vs F HFD. Second, to describe the placental response to a maternal HFD challenge, we compared F HFD vs F CD and M HFD vs M CD. Altogether, for these four

Category (function annotations)
Cellular Development
spermatogenesis
Nervous System Development and Function
Free Radical Scavenging
Cell-To-Cell Signalling and Interaction
adhesion of immune cells
Cellular Movement
chemotaxis of mononuclear leukocytes
chemotaxix of cells
chemotaxis of myeloid cells
chemotaxix of phagocytes
Hematological System Development and Function
chemotaxis of mononuclear leukocytes
chemotaxis of myeloid cells
chemotaxix of phagocytes
hematopoiesis
adhesion of immune cells
Immune Cell Trafficking
chemotaxis of mononuclear leukocytes
chemotaxis of myeloid cells
chemotaxix of phagocytes
adhesion of immune cells
Amino Acid Metabolism
Carbohydrate Metabolism

Category (function annotations)
Cell Cycle
Embryonic Development
development of organ
Organ development
development of organ
Organismal Development
development of organ
Tissue Development
development of organ
tissue development
aggregation of cells
Cellular Compromise
Cellular Assembly and Organisation
outgrowth of neurites
Cellular Development
growth of tumor cell lines
Nervous System Development and Function
outgrowth of neurites
Cell Signaling
quantity of Ca2+
activation of blood cells
activation of cells

Fig. 2 Sexual dimorphism in placental gene expression under control diet (CD) or high fat diet (HFD). The Venn diagram illustrates that, among the genes mapped by Ingenuity Pathway Analysis (IPA), 97 were sexually dimorphic in CD conditions and 93 in HFD conditions. Eighty-six genes were dimorphic in basal conditions (CD) but not in HFD placentas, 81 were not dimorphic in basal conditions but became dimorphic under HFD conditions and only 11 genes displayed sex-dimorphic expression in both CD and HFD placentas. The tables corresponds to the list of biological functions and their annotations associated with the gene sets, as analyzed by IPA

comparisons, a bioinformatic analysis was carried out using the Ingenuity Pathway Analysis (IPA) software.

We first investigated whether there was a basal sexual dimorphism between female and male placentas from mothers fed a CD during pregnancy. We detected 104 genes displaying sexual dimorphism, of which 97 were mapped in IPA: 54 with higher expression in female than in male placentas and 43 with higher expression in male than in female placentas (Fig. 2). Among the genes mapped in IPA, 97 genes were sexually dimorphic in CD conditions and 93 genes in HFD conditions. Interestingly, only 11 genes displayed sex-dimorphic expression in both CD and HFD placentas. Among these 11 genes, *Adra1a*, *Eif2s3x*, *Kdm5c* (*Jarid1c*) and *Ogt* were more strongly expressed in female placentas (the last three are located on the X-chromosome) and *Cst6*, *Ppp2r2c*, *Zfp36l2*, *Ddx3y*, *Eif2s3y* and *Kdm5d* (*Jarid1d*)

were more strongly expressed in male placentas. Interestingly, four Y-linked genes were represented on the microarray: *Ddx3y*, *Eif2s3y*, *Kdm5d* and *Sry*. The expression of this last gene is undetectable in placenta. Therefore, we can observe male-specific expression for three of four Y-linked genes in our microarray data. Notably, the amplitude of the differences in gene expression levels observed between males and females was very close under a CD and under a HFD. Interestingly, concerning the known classical sex hormone receptor genes, only the expressions of the Estrogen Receptor Related beta (*Esrrb*) or *Nr3b2* and the hormone nuclear receptor Retinoid X receptor gamma (*Rxrg*) or *Nr2b3* genes were sexually dimorphic under a CD (more expressed in male and more expressed in female, respectively).

The Transcriptional Response of Placenta to Maternal HFD Is Female- and Male-Specific

To characterize the effect of the maternal HFD on gene expression and whether a HFD triggers different changes in males and females, we analyzed this effect in the two sexes separately. We compared the female HFD with the female CD microarrays and the male HFD with the male CD microarrays. We found that 168 genes were affected by maternal HFD in female placentas, with 164 genes mapped in IPA, whereas 190 genes were affected in male placentas, with 187 mapped in IPA. About half of these genes were upregulated and half downregulated in both female and male placentas (Fig. 3). Interestingly, only 16 genes were dysregulated in both sexes, with the same amplitude, namely *CcdC56*, *Sh3bgrl3* and *Sumo1*, which were upregulated, and *Cxcl2*, *GcGr*, *Klk8*, *Mpg*, *Pcca*, *Pdgfß*, *Pla2g15*, *Ppp2r3c*, *Rhbdf2*, *Slc7a2*, *Tmem62* and *Zfp37*, which were downregulated. Among dysregulated genes and gene families, some have been reported to be important for placental development: *Adra2a*, *Ceacam10*, *Crabp2*, *Gata1*, *Gcm1*, *Mmp1a*, *Mmp 16*, *Pdgfß* and *Rarb* (Cross et al. 2003; Rawn and Cross 2008; Watson and Cross 2005).

Several studies have reported differences in gene expression between male and female placentas in humans and mice (Clifton et al. 2009; Mao et al. 2010; Sood et al. 2006; Suter et al. 2010). In humans, Clifton et al. showed that the placenta adapts in a sexually dimorphic manner to chronic maternal asthma. The growth of female fetuses is reduced, increasing chances of survival, whereas normal growth of male fetuses is associated with a poor outcome in the case of acute asthma exacerbation (Clifton 2010; Clifton et al. 2009). In mice, Mao et al. (2010) reported that, in E12.5 mice fed a low-fat diet (10 %) or a very HFD (60 %), similar to the CD and HFD used in this study, respectively, or an intermediate chow diet (25 %), there were more changes in gene expression with both diets in female than male placentas. In the present report, but at a later stage, E15.5, we confirmed that the affected gene sets differed between females and males. However our transcriptomic analysis provides no evidence for a greater reactivity to maternal HFD in either

Category (function annotations)
Cell Death
cell death of neuroblastoma cell lines
Cell Signaling
stimulation of lymphocytes
binding of phagocytes
quantity of monoamines
stimulation of cells
Cellular Development
growth of tumor cells
Digestive System Development and Function
Organ Development
Organ Morphology
Small Molecule Biochemistry
uptake of amino acids
quantity of glutathione
phosphorylation of L-tyrosine
quantity of monoamines
Tissue Development
mass of connective tissue
Amino Acid Metabolism
uptake of amino acids
quantity of glutathione
quantity of monoamines

Category (function annotations)
Cardiovascular System Development and Function
blood pressure
development of blood vessel
systolic pressure
Embryonic Development
development of organ
Organismal Development
development of blood vessel
development of organ
Lipid Metabolism
oxidation of fatty acid
quantity of triacylglycerol
Small Molecule Biochemistry
uptake of 2-deoxyglucose
oxidation of fatty acid
quantity of D-glucose
quantity of triacylglycerol
Cell Morphology
extension of cellular protrusions
Nervous System Development and Function
memory
spatial memory
proliferation of neuroglia

Fig. 3 Effect of maternal HFD on gene expression in female or in male placentas. The Venn diagram illustrates that, among the genes mapped by Ingenuity Pathway Analysis (IPA), 164 were affected by maternal HFD in female placentas, whereas 187 were affected in male placentas. A total of 148 genes are specific to the female response and 171 are specific to the male response. Only 16 genes are affected by maternal HFD in both sexes. The tables corresponds to the list of biological functions and their annotations associated with the gene sets, as analyzed by IPA

male or female mouse placentas in terms of placental and fetal growth or the numbers of dysregulated genes. Moreover, using the Ingenuity Pathway Analysis, we show for the first time that the gene sets change not only from a quantitative point of view but also, more strikingly, from a qualitative point of view. Indeed, the associated biological functions affected by the HFD differed markedly between the two sexes. In the absence of a greater reactivity but with clearly different trajectories, it is difficult to say whether one sex copes better than the other under a HFD, as previously suggested in other contexts (Clifton 2010; Eriksson et al. 2009). Differences in adaptation between males and females may therefore be context-, species- and stage-specific.

Diet Effect and Sexual Dimorphism in Global DNA Methylation in Mouse Tissues and Placenta

Global DNA methylation was assessed by the LUMA technique, in which the ratio of genomic DNA digested by the methylation-sensitive enzyme HpaII to that digested with the methylation-insensitive enzyme MspI indicates the level of cytosine demethylation (Karimi et al. 2006). An effect of sex was observed under the CD. Male placentas displayed lower levels (3.3 %) of methylation than female placentas. Diet had an effect on global % methylation but was statistically significant only in females (2.4 %). Female placentas from mothers fed the HFD displayed lower levels of methylation. Such a global hypomethylation was also observed in brains of offspring of HFD-fed mouse mothers (Vucetic et al. 2010). However, it remains difficult to speculate about the potential role of the high fat/low carbohydrate composition of the diet on the one carbon metabolism, in the absence of relevant mechanisms to account for a potential link.

This dimorphism may be due to the presence of an inactive X (Xi) chromosome in the female. However, Weber et al. (2005) overturned previous views by showing that Xi was hypermethylated at only a subset of gene-rich regions and, unexpectedly, displayed overall hypomethylation with respect to its active counterpart. Hellman and Chess (2007) have shown that the active X (Xa) chromosome in females has levels of allele-specific methylation that are twice those of Xi. A bipartite methylation-demethylation program results in Xa-specific hypomethylation at gene promoters and hypermethylation at gene bodies in both male and female active Xa chromosomes (Hellman and Chess 2007).

We investigated this difference in methylation further by assessing the methylation levels of the two major repetitive elements containing most of the genomic 5-methylcytosine bases: LINE-1 (long interspersed nucleotide element-1) and SINE-1 (short interspersed nucleotide element-1), represented by human Alu elements and the homologous mouse B1 elements. The methylation levels of both LINE-1 and SINE-1 have been reported to be good indicators of cellular 5-methylcytosine level (i.e., global DNA methylation level; Fryer et al. 2009; Jeong and Lee 2005). In our placenta model, no difference in the level of LINE-1 or B1 repetitive element methylation was observed between the sexes or between the diets (CD and HFD). These differences are therefore probably located in non-genic regions, gene bodies and centromeric heterochromatin.

Effect of Maternal Diet and Sexual Dimorphism on the Epigenetic Machinery Enzymes

The mouse extraembryonic membranes, yolk sac and placenta are characterized by global undermethylation of DNA with respect to embryonic somatic lineages. The major differences in methylation between the two lineages probably affect

non-gene regions such as, for example, centromeric heterochromatin (Ng et al. 2008). Indeed, in both chorionic villi and placental fibroblasts, large differences have been observed both globally and between various chromosome structures within individual metaphases (Kokalj-Vokac et al. 1998). As previously mentioned, female placentas displayed significantly higher levels of methylation than male placentas under control conditions (Gallou-Kabani et al. 2010). We also showed an effect of maternal HFD, with hypomethylation in female placentas only (Gallou-Kabani et al. 2010). However, the levels of expression of neither *Dnmt3a*, *Dnmt3b* nor *Dnmt1* differed between the sexes or between diets. In contrast, this hypomethylation was consistent with the observation of Dnmt3l downregulation only in female HFD placentas. *Dnmt3l* has no DNA methylase activity but is a cofactor of the de novo DNA methyltransferases (Kobayashi et al. 2012; Smallwood et al. 2011). Our data do not exclude a role for *Dnmt3a*, *Dnmt3b* and *Dnmt1* in global methylation differences. Indeed the expression of these genes and the levels of the corresponding proteins may have varied before the E15.5 stage. Time course studies and studies of the cell type distribution of DNA methylation during development are therefore of interest.

Six other epigenetic machinery genes, *Kmt1a*, *Kmt1b*, *Kmt2f*, *Kdm5c*, *Kdm5d*, and *Prmt7*, were dysregulated under maternal HFD as an effect of diet, sex of the fetus, or both. *Kmt1a* and *Kmt1b* encode methyltransferases involved in trimethylation of the lysine 9 residue of histone H3 (H3K9me3 is mostly associated with a repressed chromatin state). The two paralogues, *Kdm5c* and *Kdm5d*, demethylases of lysine 4 of histone H3 (H3K4me3 is mostly associated with an active chromatin state), map to the X and Y chromosomes, respectively. *Kdm5c* escapes X-inactivation and is more strongly expressed in females than in males (Li and Carrel 2008). *Kdm5c* and *Kdm5d* are highly similar in nucleotide and amino acid sequence, but whether the role and targets of these two enzymes are identical, divergent or with partial compensation in their functions is still unclear (Xu et al. 2008). *Prmt7* is an arginine methyl transferase (H3R2) and *Setd1a* (*Kmt2f*) is a H3K4 methyltransferase. Little is known about these two enzymes and their role is not described in placenta.

The proteins encoded by Y-linked genes may or may not have the same functions, the same target sequences or the same patterns of expression, according to age or tissue, as their X paralogues. In our study, in placentas of HFD-fed mouse mothers, the Y- and X-linked histone demethylase paralogue genes *Kdm5c* and *Kdm5d* were sexually dimorphic. In another report, in mouse brain, expression of the Y version of the gene in male mice did not compensate for the dosage imbalance between the two sexes in the expression of their X homologs escaping X-inactivation. Figure 4 shows that, in placentas from mothers on a CD or HFD, the Y-linked *Kdm5d* gene expression in males was not able to compensate for the expression of *Kdm5c*, its X-linked paralogue escaping XIC, in females (Gabory et al. 2012). Thus the epigenetic enzymes produced by these two genes could mark the epigenome in a sex-specific manner, both at the quantitative and qualitative levels (Xu et al. 2002).

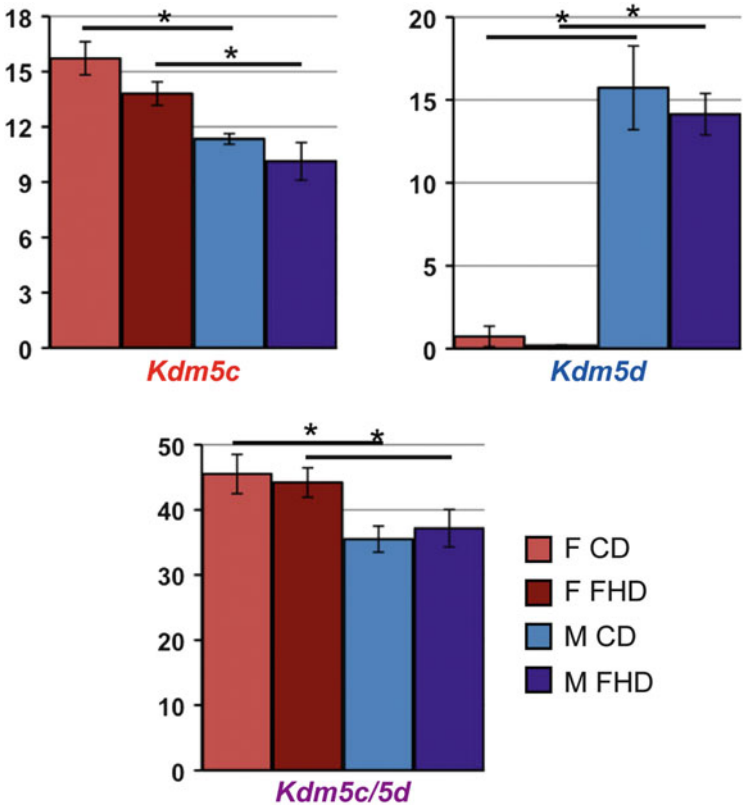

Fig. 4 Analysis of the mRNA expression of the *Kdm5c* and *Kdm5d* paralogues. Three PCR primer pairs have been designed for recognizing specifically either *Kdm5c* or *Kdm5d* cDNA and for recognizing both *Kdm5c/5d* cDNA. Their expression was studied in male and female placentas in pregnant female mice fed either a control diet (CD) or a high-fat diet (HFD) from E0.5 to sacrifice at E15.5 stage. *Kdm5c* expression is higher in females (*pink bars*) than males (*blue bars*), and *Kdm5d* is expressed only in males, regardless of maternal diet. The *Kdm5c/5d* PCR shows that the combined expression of *Kdm5d* and *Kdm5c* expression in males is not of equivalent magnitude as the expression of *Kdm5c* from both alleles in females

The involvement of all these enzymes in an important network is consistent with (1) documented crosstalk between the H3K4 and H3K9 methylation marks, with H3K9 methylation partly controlled by the Kmt1a and Kmt1b enzymes, and (2) crosstalks between histone methylation and acetylation and DNA methylation. Dnmt3l has been shown to recruit histone deacetylases (Deplus et al. 2002) and to interact with H3K4me3 (Ooi et al. 2007). Moreover, the H3K4 demethylase Kdm5c interacts with H3K9me3, which can be methylated by Kmt1a and 1b (Iwase et al. 2007). It will therefore be important to determine how the crosstalk between key repressive or activating marks and their modifying enzymes can be disturbed by environmental challenges, leading to developmental and metabolic malprogramming.

Effects of Maternal Diet and Sexual Dimorphism on Relevant Histone Marks

Since Kdm5c, Kdm5d, Kmt1a and Kmt1b enzymes are responsible for histone modifications of H3K4 and H3K9, respectively, we investigated whether the differences in gene expression led to differences in the global levels of pertinent histone methylation marks. In line with the changes in expression of histone modifying enzymes, we analyzed global histone modifications. The H3K4me3 signal was not sexually dimorphic, as might have been expected given the dimorphic expression of *Kdm5c* and *Kdm5d*, and was equivalent in both diets despite the dysregulation of *Kmt2f*. Similarly, H3K9me3 was not affected by diet despite the downregulation of *Kmt1a* and *Kmt1b*. However, Western blotting provides a global evaluation of histone marks and cannot exclude subtle changes in particular target sequences of these enzymes. Thus, diet itself or the mechanisms used by cells to compensate for dietary imbalances for adaptation must have a major impact on the epigenetic machinery. As previously highlighted for DNA methylation, time course studies and studies of the cell type distribution of histone marks during development are required.

Not Just Hormones: Role of X and Y Chromosome-Linked Genes

Increasing numbers of reports are challenging the traditional view of the influences of gonadal hormones and highlighting other roles for sex chromosomes (reviewed in Davies and Wilkinson 2006; Dunn et al. 2010; Gabory et al. 2009, 2013; Howerton and Bale 2012). In bovine blastocysts, sex determines the expression levels of one third of all actively expressed genes (Bermejo-Alvarez et al. 2010). Sexual dimorphism has also been observed in total embryonic cells isolated from mice at E10.5, before the occurrence of sexual differentiation. These cells responded differently to the dietary stressors applied, even before the production of fetal sex hormones (Penaloza et al. 2009). The genes present on the sex chromosomes (which are asymmetrically inherited between males and females) may influence sexually dimorphic gene expression (Davies and Wilkinson 2006). In most placental mammals, X inactivation is random. However, extraembryonic X inactivation is thought to be paternally imprinted in rodents. At least 150 loci are known to escape inactivation and may therefore be expressed from both X chromosomes (Carrel and Willard 2005). Studies in mice and rats demonstrating sex differences in placental responses to changes in the maternal environment may thus indicate a role for these escaped genes, as the placentas of female fetuses may produce twice as much of the corresponding proteins as those of male fetuses (Howerton and Bale 2012). Alternatively, the small number of expressed genes present on the Y chromosome may be involved. Many X-homologous regions are

found on the Y chromosome, but most do not recombine and are referred to as male-specific regions. These regions contain 78 single and multicopy genes encoding about 27 different proteins in humans. In total, 29 genes are conserved in the pseudo-autosomal regions (PARs) of the X and Y chromosomes. In human term placentas, Sood et al. (2006) showed that many of the sex-correlated genes are located on the sex chromosomes, but that some are autosomal. Thus X- and Y-linked genes may modulate the expression of different sets of autosomal genes, leading to physiological differences between males and females (Gabory et al. 2013).

Conclusions: Why Sex Matters

These findings highlight the importance of studying both sexes in epidemiological protocols or dietary interventions in both humans and experimental animal models. Our results pave the way for explorations concerning the possible targeting, by fatty acids and other nutrients, of conspicuous regions in the genome harboring binding sites for the recruitment of diet- and tissue-specific chromatin remodeling complexes. Elucidation of the ways in which epigenetic modifications fix the effects of early environmental events, in a sex-specific manner, ensuring sustained responses to transient stimuli resulting in modified gene expression patterns and phenotypes later in life, remains a key challenge (Attig et al. 2010).

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Early Growth and Later Health: Findings from the Helsinki Birth Cohort Study

Johan G. Eriksson

Abstract It is well established that factors active during prenatal life and early childhood largely influence later health outcomes. Slow rates of growth during prenatal and postnatal life increase the later risk for coronary heart disease, type 2 diabetes and hypertension. Lifestyle is closely associated with these non-communicable disease outcomes. The programming of food choices and exercise habits seems to take place early in life and could be one factor explaining the association between early growth and later health outcomes.

Introduction

Large-scale epidemiological studies, as well as experimental animal work, have introduced the concept of Developmental Origins of Health and Disease (DOHaD): environmental factors present in early life, often reflected by specific patterns of early growth, cause life-long changes in organ structure and function that have substantial effects on health outcomes later in life (Hanson and Gluckman 2011; Barker 2012).

Over the past decades it has become increasingly clear that conditions during sensitive early phases of growth and development have a substantial impact on an individual's susceptibility to several chronic diseases. The evidence is strongest for an association between prenatal growth and cardiovascular disease and type 2 diabetes (Barker et al. 2005, 2011; Eriksson et al. 2006; Whincup et al. 2008).

The underlying mechanisms that explain the associations between growth during critical phases and later health outcomes are largely unknown. Most common

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non-communicable diseases develop as a consequence of unfavorable environmental conditions in combination with a genetic predisposition for the disease. Typically, these disorders are heterogeneous and complex and are likely influenced by multiple genetic variants in addition to multiple environmental factors and epigenetic factors.

Helsinki Birth Cohort Study

The Helsinki Birth Cohort Study (HBCS) includes 13,345 subjects born at Helsinki University Central Hospital or at the Midwives' Hospital in Helsinki, Finland, between 1934 and 1944. Data on prenatal and childhood growth have been abstracted from hospital birth records and child welfare clinic and school health care records. These records include information on health and growth during childhood but also information on socio-economic factors. The hospital birth records include data on birth weight, length at birth, gestational age and maternal characteristics. Serial measurements of body size throughout childhood are available and the subjects have, on average, 17 measurements of height and weight from birth to 11 years of age. The cohort has been followed up by register linkage to national Finnish registers providing information on both morbidity and mortality.

Clinical examinations including over 2,000 individuals have provided more detailed information on metabolic, cognitive and psychological outcomes. These examinations have included oral glucose tolerance testing (OGTT), measurements of height, weight, waist circumference, blood pressure and body composition. Blood has been drawn for measurements of blood lipids, inflammatory markers and adipocytokines. Dietary and exercise habits have been assessed by validated questionnaires several times. Psychological and personality factors have also been assessed repeatedly.

Growth During Prenatal Life and Infancy

There is a large body of evidence showing that people whose body size at birth is towards the lower end of the normal range are at increased risk of cardiovascular disease, type 2 diabetes, depression and cognitive decline (Eriksson et al. 2001, 2003; Räikkönen et al. 2007, 2012). Size at birth is a result of the intrauterine environment, which can have permanent effects on the structure and function of the body, a phenomenon referred to as prenatal programming. HBCS has added significantly to previous knowledge by showing that not only small body size at birth but also slow growth during infancy is associated with increased risk of many common age-related disorders, including cardiovascular disease and type 2 diabetes. The risks for coronary heart disease and type 2 diabetes are further aggravated in individuals who are small during infancy and who put on weight rapidly

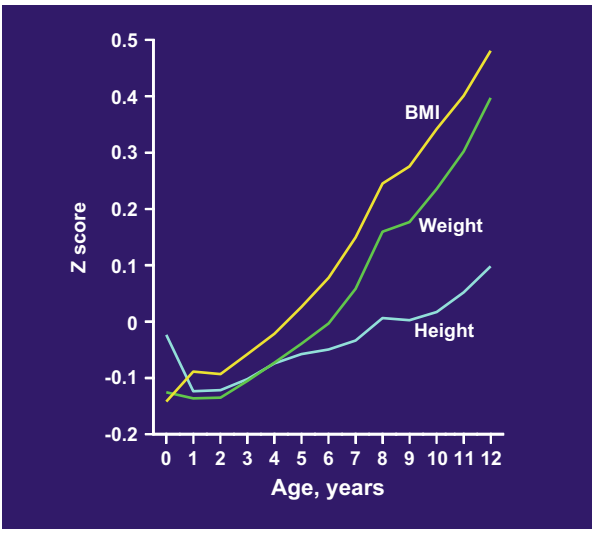

Fig. 1 Growth in body mass index (BMI), height and weight of 311 people who developed type 2 diabetes in relation to the growth of the whole cohort (n = 2,003) expressed as z scores

thereafter (Eriksson et al. 1999, 2001). The term "mismatch" has been used to describe this pattern of growth (Godfrey et al. 2007). Type 2 diabetes and coronary heart disease (CHD) share several risk factors, and the prenatal and early postnatal growth patterns that associate with these disorders are similar. Figure 1 shows the growth pattern during early life that is associated with an increased risk for type 2 diabetes. Slow prenatal growth and slow growth in infancy increased the risk for type 2 diabetes. A rapid increase in body mass index between 2 and 11 years of life was another characteristic growth feature (Eriksson et al. 2006). The underlying mechanisms explaining these associations could include impairment in insulin secretion as well as insulin resistance; both are known to be associated with a small body size at birth. Furthermore, we have shown that the growth patterns associated with type 2 diabetes are also associated with a body composition with an unfavorable fat-lean body mass distribution that could further increase the risk for type 2 diabetes in later life (Eriksson et al. 2006; Ylihärsilä et al. 2007, 2008; Pulizzi et al. 2009).

Programming of Dietary Habits and Food Choices

Lifestyle is closely associated with both cardiovascular disease and type 2 diabetes. Lifestyle factors including unhealthy dietary habits and physical inactivity are well-known, modifiable risk factors for cardiovascular disease and type 2 diabetes. One plausible mechanism explaining the association between prenatal growth and an increased risk for non-communicable diseases could be early programming of lifestyle and lifestyle-associated factors. However, the association between adult lifestyle and prenatal growth has been little studied, and only a few studies have

focused on the association between prenatal growth and food preferences in later life. Within the HBCS we have been examining the association between birth size and food intake in adult life. Diet has been assessed with a validated, self-administered, 128-item, food-frequency questionnaire (FFQ) designed to assess the usual diet over the previous 12 months (Perälä et al. 2012).

We observed several associations between body size at birth and food intake in adulthood. A small body size at birth was associated with lower consumption of fruits and berries and of rye and rye products in men and women at the age of 64 (Perälä et al. 2012). Those who were small at birth had a higher intake of fat and lower intake of carbohydrates and fiber. A 1-kg higher birth weight was associated with about 83 g higher daily intake of fruits and berries. This number perhaps is not that impressive but equals a weekly consumption of fruits and berries of 580 g. It is well known that lower consumption of fruits and berries reflects an unhealthy diet, which may increase the risk for cardiovascular disease. In other words, our results strongly suggest that intrauterine growth modifies food intake in adult life, and this may largely affect health outcomes in later life.

Body size at birth was also associated with macronutrient intake in later life in such a way that a 1-kg increase in birth weight was associated with a lower intake of total fat (Perälä et al. 2012). Animal studies support our findings; rats whose mothers were fed a low-protein diet during gestation had a preference for a high-fat diet (Bellinger and Langley-Evans 2005). There are also human findings showing that a low birth weight is associated with an unhealthy diet; in children, fat intake increased with decreasing birth weight (Shultis et al. 2005). Findings from the Dutch hunger winter have shown that prenatal exposure to famine was associated with a preference for greater fat intake in later life (Lussana et al. 2008; Stein et al. 2009). However, no relationships between birth size and macronutrient intake were found. There are some potential underlying mechanisms explaining these findings; for example, it has been suggested that a low-protein diet during gestation may alter the expression of neuropeptides regulating eating behavior (Plagemann et al. 2000).

Programming of Physical Activity and Cardiorespiratory Fitness

Physical activity is a major factor influencing risk for chronic diseases. Findings mainly based upon animal experiments have suggested that exercise habits might have a prenatal origin. A weak association between birth weight and physical activity in later life has been reported among people born with normal birth weight. However, both low birth weight and high birth weight were associated with less physical activity during leisure time (Andersen et al. 2009). Based upon findings in the HBCS, we have reported that body size at birth is positively associated with intensity of total leisure time physical activity. Height during childhood was

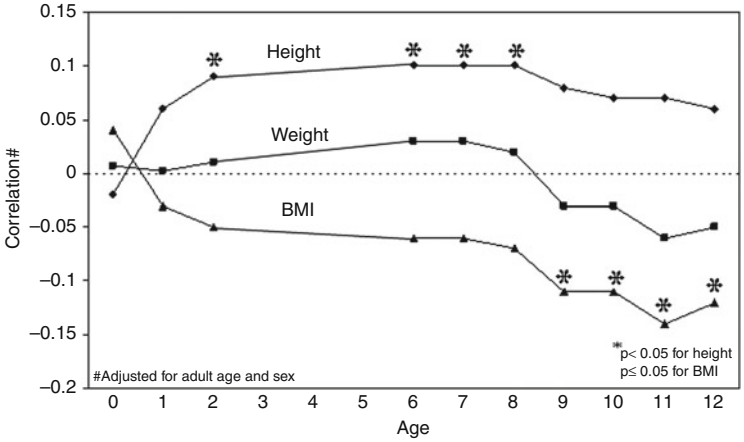

Fig. 2 Age- and sex-adjusted correlations between VO2max and height, weight and BMI annually from birth to 12 years (from Salonen et al. 2011b)

positively associated with the intensity of conditioning leisure time physical activity. In other words, children who were heavier and taller at birth and heavier during infancy reported higher intensity levels of leisure time physical activity in adult life. This higher physical activity was also associated with more favorable anthropometric profiles. We believe that the higher weight and body mass index (BMI) in childhood of these people are reflections of a higher lean body mass rather than obesity; childhood obesity was not common in the 1930s and 1940s in Finland (Salonen et al. 2011a).

Besides physical activity, cardiorespiratory fitness (CRF) is another factor influencing health and disease. We did not observe any significant associations between CRF and body size at birth. Interestingly, childhood growth was associated with CRF. Figure 2 shows that height in childhood was positively associated with CRF in adult life. The underlying mechanisms are still poorly understood but muscle fitness may play a major role (Salonen et al. 2011b).

Early Life Stress

Severe early life stress (ELS) is known to be associated with unfavorable psychological outcomes (Pesonen et al. 2007). However, much less is known about its potential long-term consequences on physiological functions in adult life.

We have been studying the long-term effects of childhood separation during World War II on cardiovascular outcomes and type 2 diabetes. During World War II, approximately 70,000 Finnish children experienced ELS through temporary separation from their parents. To be protected from the strains of war, Finnish

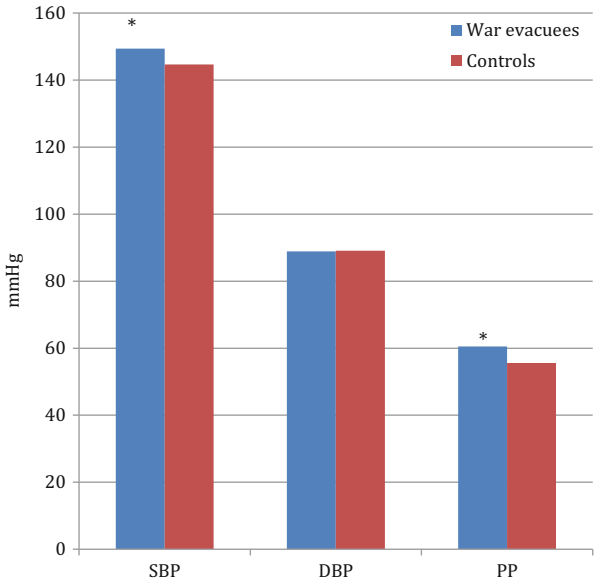

Fig. 3 Systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP) among former war evacuees and non-evacuated controls. *p < 0.001 (unadjusted)

children were evacuated, without their parents, mainly to Sweden and Denmark. Being sent away alone to a foreign country represented an extreme form of ELS. After the war, the children were separated again from their foster families when returning to Finland. Some children experienced these separations several times. Roughly 15 % of the participants in the HBCS had experienced childhood separations as war evacuees.

Former war evacuees were two times more likely to have cardiovascular disease, their risk for type 2 diabetes was 1.4-fold and for hypertension 1.3-fold higher than for non-evacuees (Alastalo et al. 2009). Figure 3 shows systolic, diastolic and pulse pressure among former war evacuees and non-evacuated controls. Not only does the separation experience seem to be important but also the age and duration of evacuation were important factors influencing the outcome (Alastalo et al. 2012, 2013). We did not observe any differences in total cholesterol, LDL-cholesterol, HDL-cholesterol or triglyceride concentrations. Interestingly lipoprotein (a) was significantly higher among former war evacuees. High plasma lipoprotein (a) levels have been associated with an increased risk for CHD.

Psychological stress is known to modulate the hypothalamic-pituitary-adrenal (HPA) axis activity. Interestingly, HPA axis dysfunction is a known risk factor for CHD (Rosmond and Björntorp 2000). The separation experience was associated with higher salivary cortisol and ACTH concentrations compared to a non-separated group (Pesonen et al. 2010). The former evacuees also showed higher reactivity to a standardized stress test, with stronger findings in men.

Conclusion

Findings in the HBCS support the idea that lifestyle, including dietary and exercise habits, seems to be programmed early in life. Furthermore, the importance of early life stress on later health outcomes through programming of the HPA axis is demonstrated.

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Human Fetal Growth Disorders and Imprinting Anomalies

C. Gicquel, S. Azzi, S. Rossignol, J. Demars, F. Brioude, I. Netchine, and Y. Le Bouc

Abstract Epigenetic mechanisms play a key role in regulating gene expression. One of the best-studied epigenetic modifications is DNA methylation at cytosine residues of CpG dinucleotides in gene promoters, transposons and imprinting control regions (ICR). Genomic imprinting refers to the epigenetic marking of genes that results in monoallelic expression, depending on their parental origin. Several hormone genes involved in embryonic and fetal growth are imprinted. There are two critical time periods in epigenetic reprogramming: gametogenesis and early preimplantation development. Major reprogramming takes place in primordial germ cells in which parental imprints are erased and totipotency is restored. Imprint marks are then re-established during spermatogenesis or oogenesis, depending on sex. Upon fertilization, there is genome-wide demethylation followed by a wave of de novo methylation, both of which are resisted by imprinted loci. Disruption of imprinting causes disorders involving growth defects, such as the Beckwith-Wiedemann overgrowth syndrome (BWS) and Silver-Russell syndrome (SRS) with the opposite phenotype, involving intrauterine and postnatal growth retardation. These growth disorders are caused, in most cases, by abnormal DNA methylation at the 11p15 imprinted region that contains many imprinted genes, including Insulin-like Growth Factor 2 (*IGF2*). Loss of methylation (LOM) on the maternal allele at the centromeric ICR2/*KCNQ1OT1* region or gain of methylation (GOM) on the maternal allele at the telomeric ICR1/*IGF2*/*H19* region has been shown in BWS. This latter defect is associated with a higher risk of

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pediatric tumors, such as nephroblastoma. By contrast, LOM on the paternal allele at the telomeric ICR1 is observed in SRS. There is an abnormally high prevalence of conceptions by assisted reproductive technology (ART) among patients with BWS and SRS, suggesting that ART may favor imprinting alterations at the imprinted centromeric 11p15 locus (LOM at the maternally methylated ICR2 or LOM at the paternally methylated ICR1, respectively). The underlying cause of these imprinting defects (following ART or occurring spontaneously) remains unclear. However, recent data indicate that, in patients with BWS or SRS, including those born following ART for BWS, the methylation defect involves imprinted loci other than 11p15. Moreover, some patients exhibit LOM at both maternally and paternally methylated ICR, which suggests that unfaithful maintenance of DNA methylation marks following fertilization involves the dysregulation of a *trans*-acting regulatory factor.

Introduction

The genetic code has been known for decades; in contrast, epigenetic mechanisms controlling gene expression were discovered within the last 30 years. Epigenetic mechanisms are involved in many physiological processes, including during development. Genomic imprinting (also called parental imprinting) refers to the epigenetic marking of genes, resulting in monoallelic expression depending on the parental origin. Several human syndromes are associated with the failure of maintenance and/or establishment of genomic imprinting (Azzi et al. 2013). The fetal overgrowth syndrome, Beckwith-Wiedemann syndrome (BWS), and the intrauterine and postnatal growth retardation syndrome, Silver-Russell syndrome (SRS), are both due to imprinting defects. Here, we review clinical aspects of BWS and SRS, including the relationships between phenotype and (epi)/genotype. We also describe epigenetic and genetic anomalies leading to the imprinting defects (isolated locus or multilocus) involved in these developmental diseases, and we consider the potential role of environmental factors such as assisted reproductive technology (ART) in the occurrence of imprinting defects.

Epigenetics and Genomic Imprinting

Epigenetic modifications of the genome play important roles in the regulation of gene expression in diverse cell lineages. Epigenetic marks are various changes to the chromatin but do not include changes in the nucleotide sequence of the DNA (genetic code). Epigenetic marks are dynamically reprogrammed but, once established, they are stably transmitted to daughter cells during mitosis. Epigenetic

modifications regulate the expression of genes and confer cell lineage specificity. The nucleosome is the basic unit of chromatin organization and consists of an octamer of histone proteins around which wraps the strand of DNA (Fig. 1). The best-known epigenetic marks are DNA methylation at CpG islands (DNA domains rich in CG dinucleotides) and various post-translational modifications (notably acetylation and methylation) of histones H3 and H4. In any particular region of chromatin, a combination of post-translational modifications of histones (histone code) and the DNA status (methylated/unmethylated) induces either compaction of the chromatin (repressive form) or decondensation of the chromatin (active form; Reik et al. 2001).

In general, DNA methylation is associated with histone deacetylation in regions where chromatin is compacted and thus gene expression is prevented. However, when the DNA is demethylated and histones acetylated, chromatin is in an open conformation. Thus, two loci can be identical in nucleotide sequence (genetic code) but, due to genomic imprinting, they can be functionally different. Expression is monoallelic: one of the two parental alleles is expressed and the other is silent. The two alleles have different epigenetic modifications (DNA methylation and post-translational histone modifications: including methylation and/or acetylation) according to their parental origin, resulting in the expression or non-expression of a gene (Fig. 1). Parental imprinting was identified in mammals in the 1980s through nuclear transfer experiments that demonstrated the non-equivalence of the two parental genomes: zygotes generated with two maternal genomes (gynogenotes) led to embryo development but no development of the embryonic annexes, whereas the zygotes generated with two paternal genomes (androgenotes) developed embryonic annexes but failed to develop an embryo. These experiments showed the importance of the contribution of both parental genomes to achieve normal development and suggested that some genes are expressed from only the paternal or the maternal allele (McGrath and Solter 1984).

These genes were called imprinted genes. One of the first imprinted genes to be identified by gene inactivation ("knockout") in mice was *Igf-2* (DeChiara et al. 1991).

Mice heterozygous for a deletion of the *Igf2* gene exhibited growth retardation at birth only if the deletion had been paternally transmitted; growth was normal if the deleted gene was inherited from the mother. The phenotype of mice homozygous for the mutation was not more severe than that of heterozygous mice carrying the mutation on the paternal allele.

The vast majority of genes are expressed by the two parental alleles, and less than 1 % of genes are subject to parental imprinting. There is dynamic reprogramming of genomic imprinting during development: imprinting marks are first erased in primordial germ cells and thereafter re-established differently depending on the sex of the individual during gamete maturation (Lucifero et al. 2002). After fertilization during the preimplantation period, these epigenetic marks are protected against a wave of demethylation and then a wave of global genome de novo remethylation (Santos and Dean 2004).

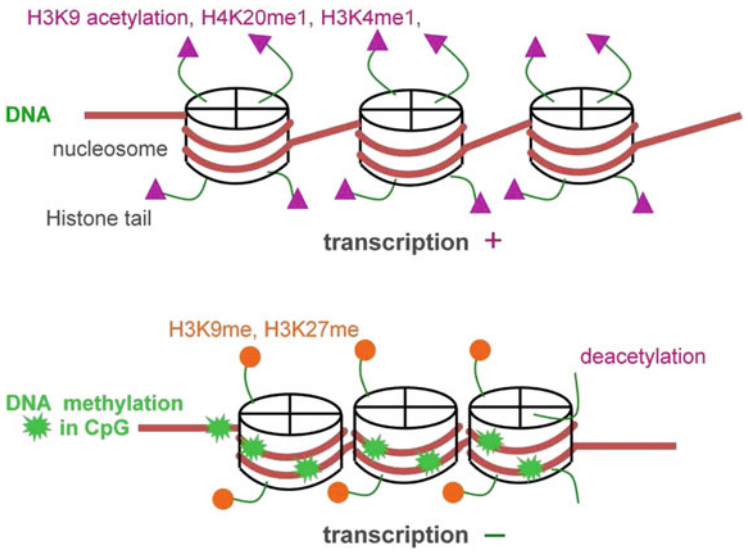

Fig. 1 Parental imprinting. These two alleles have different epigenetic modifications (methylation, acetylation) according to their parental origin, resulting in expression or non-expression of a gene. DNA methylation and repressive histone marks (such as H3K9me1 or me3, H3K27me1 and me3, and H4K20me3) are associated with the absence of transcription. Conversely, unmethylated DNA and permissive histone marks (H3K9 ac, H4K20me1, H3K4me1 or me2) are associated with transcription activity

Several chromosomal regions have now been identified as being imprinted in several mammalian species. Imprinted genes are organized into clusters throughout the genome and many are regulated by a regulatory element called the imprinting center region (ICR).

Several transacting factors involved in the regulation of DNA methylation and imprinting have been identified. DNA methyltransferases (DNMT; Cheng and Blumenthal 2008) and Methyl CpG Binding Domain proteins (MBDs; Klose and Bird 2006) are well-known regulatory factors of DNA methylation. There are also many other regulatory proteins involved in the regulation of DNA methylation and imprinting and in the regulation of the modifications of histone tails by histone acetyltransferases or deacetylases and by histone methylases or demethylases. All these epigenetic changes affect chromatin structure and are thus determinant for the control of gene expression of the cluster.

There are two main regulatory pathways that govern the monoallelic expression of imprinted genes in a cluster: the “chromatin insulator or boundary” mechanism and the long non-coding RNA mechanism (Ideraabdullah et al. 2008; Wan and Bartolomei 2008).

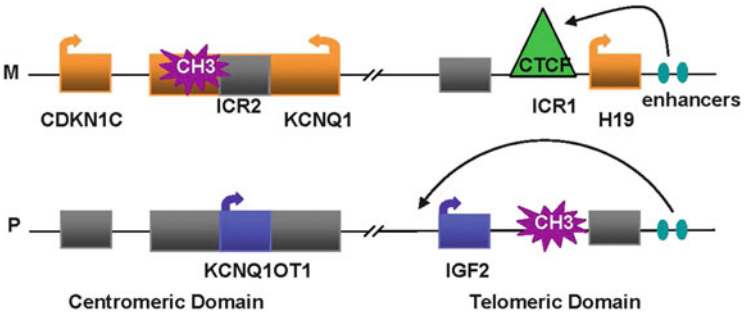

Fig. 2 The two imprinted domains of the 11p15 region. The reciprocal imprinting of the maternally (*M*) expressed *H19* and the paternally (*P*) expressed *IGF-2* genes depends on the differentially methylated ICR1 upstream from the *H19* gene, which acts as an insulator. CTCF binds to the maternal unmethylated ICR1 and prevents the *IGF-2* gene promoter from interacting with enhancers downstream from the *H19* gene, resulting in transcriptional silencing of the maternal *IGF-2* allele (Bell and Felsenfeld 2000; Hark et al. 2000). On the paternal allele, ICR1 is methylated, preventing CTCF binding and thereby leading to *IGF-2* transcription on the paternal allele. The centromeric ICR2 domain functions as a silencer by producing a non-coding RNA (antisense *KCNQ1OT1* RNA), which results in paternal silencing of the genes in this domain. The *KCNQ1OT1* RNA is probably involved in targeting repressive histone modifications to the flanking genes (Lewis et al. 2004; Umlauf et al. 2004; Monk et al. 2006). Paternally expressed genes are represented as *blue boxes*, maternally expressed genes as *orange boxes*, and non-expressed genes as *gray boxes*

Fetal Development, Imprinting and the 11p15 Region

The imprinted 11p15 region is organized into two domains: a telomeric domain including the *IGF2* and *H19* genes and a centromeric domain including the *CDKN1C* (cyclin-dependent kinase inhibitor 1C), *KCNQ1* (potassium voltage-gated channel, subfamily Q, member 1) and *KCNQ1OT1* (KCNQ1-overlapping transcript 1) genes. Each domain is controlled by its own ICR: ICR1, for the telomeric domain, controls the allele-specific expression of *IGF2* and *H19*; and ICR2, for the centromeric domain, controls the allele-specific expression of *KCNQ1OT1*, *KCNQ1* and *CDKN1C* (Fig. 2).

Reciprocal imprinting of the *H19* gene (the gene for a maternally expressed noncoding RNA) expressed by the maternal allele (M) and of the *IGF2* gene expressed by the paternal allele (P) depends on the differentially methylated ICR1 (upstream from the *H19* gene), which acts as an insulator. CTCF binds to the unmethylated maternal ICR1 and prevents *IGF2* promoters from interacting with the shared enhancers downstream from the *H19* gene, thereby abolishing *IGF2* expression. In contrast, on the paternal allele, ICR1 is methylated, which prevents the binding of CTCF, thus leading to the transcription of the paternal *IGF2* gene. The centromeric domain ICR2 acts as a "silencer" by producing a long non-coding RNA (*KCNQ1OT1*) from the unmethylated paternal allele. *KCNQ1OT1* in turn silences the neighboring paternal genes of the centromeric domain, in *cis*, including

the *CDKN1C* gene (Edwards and Ferguson-Smith 2007; Ideraabdullah et al. 2008; Wan and Bartolomei 2008).

This imprinted 11p15 region is extremely important during fetal growth and development.

Indeed, the BWS overgrowth syndrome results from various molecular or chromosomal abnormalities at 11p15 that cause overexpression of the paternally expressed genes or underexpression of the maternally expressed genes (Gaston et al. 2001; Gicquel et al. 2005b; Weksberg et al. 2005). SRS, a fetal growth restriction, is mainly due to an imprinting anomaly affecting the paternal ICR1.

Most known 11p15 defects display a mosaic pattern, where the ratio between cells with defects and normal cells differs between tissues (including kidney, muscle, liver, leucocytes and fibroblasts) and patients; this is probably a main cause of the variability of the BWS and SRS phenotypes.

A particular tissue, the placenta, exhibits some imprinting differences as compared to the tissues of the embryo. The placenta in eutherian mammals is a distinct organ ensuring maternal-fetal nutrient allocation and is, consequently, crucial for fetal growth. Thus, the effectiveness with which the placenta transfers nutrients to the fetus is a determinant of fetal growth. Imprinted genes play an important role in placental development (Coan et al. 2005; Frost and Moore 2010). This was first demonstrated by parthenogenesis experiments that provided evidence for the necessity for both paternal and maternal genomes for correct development of both embryo and annexes (Barton et al. 1984; McGrath and Solter 1984; Surani et al. 1984). A subset of genes is imprinted only in the placenta and some imprinted genes are expressed exclusively in the placenta (Gutierrez-Marcos et al. 2012; Lefebvre 2012). The inactivation of the maternally expressed *Cdkn1c*, in mice, results in a large placenta weighing 140 % of the normal weight (Takahashi et al. 2000). The inactivation of any of the paternally expressed *Igf2*, *Peg3* and *Peg1* genes results in a small placenta (Reik et al. 2003). *Igf2* is particularly interesting because it has a variant, *Igf2 P0*, expressed specifically in the mouse labyrinthine trophoblast (Constância et al. 2002). Inactivation of this variant is associated with reduced expression of *Igf2* in the placenta, whereas its expression in the fetus is normal. The placental weight and passive transport of nutrients in *Igf2 P0*$^{-/-}$ mice were both lower than those in controls. These experimental models have highlighted the important role of imprinted genes in the control of placental development and function (Coan et al. 2005; Angiolini et al. 2006; Frost and Moore 2010; Fowden et al. 2011; Nelissen et al. 2011; Gutierrez-Marcos et al. 2012).

The placenta is the mediator between the mother and the fetus; it is sensitive to its environment and can adapt its capacity in response to environmental variations to ensure an appropriate nutrient supply to the fetus. This characteristic has been extensively documented by caloric restriction studies in animals, and also in humans (Sibley et al. 2010; Sandovici et al. 2012; Wu et al. 2012). Indeed, most of these studies show that placental development and function are compromised by caloric restriction (Heasman et al. 1998; Fowden et al. 2008), which may also affect DNA methylation of imprinted genes (Lumey 1998; Heijmans et al. 2008).

All these various observations and findings highlight the importance of imprinted genes in the regulation of feto-placental development.

Fetal Growth Disorders and Imprinting Defects: Clinical Aspects

In humans, two syndromes in particular, with opposite and severe fetal growth anomalies, have been associated with dysregulation of imprinted gene expression: BWS and SRS.

BWS

BWS is an overgrowth disorder involving developmental abnormalities and an increased risk of childhood tumors. It has an estimated population incidence of 1 in 13,700 (Thorburn et al. 1970), but this is probably an underestimate because of the existence of mild phenotypes that may not be detected or reported (Sotelo-Avila et al. 1950; Schneid et al. 1993).

Its phenotypic expression is indeed variable and diagnosis is still based on clinical signs, although there is no consensus on the clinical definition of the syndrome (Wiedemann 1969; Pettenati et al. 1986; Elliott et al. 1994; DeBaun and Tucker 1998; Weksberg et al. 2001). It is generally accepted that the diagnosis of BWS requires at least three clinical findings, including at least two major findings: the major clinical criteria are macroglossia, macrosomia, abdominal wall defects (exomphalos, umbilical hernia) and selective visceromegaly (involving kidneys, liver or spleen). Less frequent and minor clinical findings are neonatal hypoglycemia, anterior ear lobe creases and/or posterior helical pits, facial nevus flammeus, hemihyperplasia and polyhydramnios. Diverse molecular defects within the 11p15 region are associated with BWS; it can be the result of various molecular or chromosomal alterations that cause overexpression of paternally expressed genes or impair the expression of maternally expressed genes (Gaston et al. 2001; Gicquel et al. 2005b; Weksberg et al. 2005).

More powerful techniques for the molecular diagnosis of BWS are now available and can confirm the diagnosis in patients with incomplete phenotypes, which suggests that BWS should now be defined molecularly. About 7.5–10 % of BWS patients develop a tumor before the age of 5 years. However, it is now clear that this risk differs very substantially depending on the molecular defect involved (Bliek et al. 2004; Cooper et al. 2005; Rump et al. 2005; Brioude et al. 2013a; Eggermann et al. 2013) (see section “Genotype-Phenotype Relationships”).

SRS

SRS is a clinically heterogeneous syndrome involving severe pre- and postnatal growth retardation. It was first described by Silver et al. (1953) and Russell (Russell and Jackson 1954). Their common findings were short stature without catch-up growth, normal head circumference for age, distinctive triangular face morphology with prominent forehead, low-set ears, clinodactyly of the fifth fingers, and skeletal asymmetry. The clinical presentation of SRS covers a spectrum of manifestations such that it is fairly easy to recognize in extreme cases but can be difficult to diagnose in less severely affected individuals, especially if there is no body asymmetry. Based on reviews of the published data, we and others have proposed a clinical scoring system to overcome these difficulties (Price et al. 1999; Netchine et al. 2007; Abu-Amero et al. 2010; Wakeling et al. 2010). For a diagnosis of SRS under these systems, the patient must be born small for gestational age (SGA: birth weight and/or length ≤ -2 SDS for gestational age) and also present at least three of the five following features: postnatal growth retardation (at 2 years of age or at the nearest measure available), relative macrocephaly [arbitrarily defined as a head circumference at birth at least 1.5 SDS above that expected for the birth weight and/or length SDS according to Usher and McLean charts (Usher and McLean 1969)], body asymmetry, prominent forehead and feeding difficulties during early childhood and/or postnatal Body Mass Index (BMI) below -2 SDS (at 2 years of age or at the nearest measure available; Netchine et al. 2007).

Molecular Aspects of BWS and SRS

Imprinting disorders may arise in several ways: through copy-number changes for imprinted domains, uniparental isodisomy, disruption of regulatory sequences, and mutation of the active allele or "primary" imprinting defects such as gain or loss of DNA methylation. In BWS, various cytogenetic, genetic and epigenetic defects in 11p15 result in the down-regulation of maternally expressed genes and/or the up-regulation of paternally expressed genes; conversely, in SRS, genetic or epigenetic defects in the 11p15 region result in the down-regulation of paternally expressed genes and/or the up-regulation of maternally expressed genes.

Genetic Defects

Duplications in BWS and SRS Patients

Approximately ten unbalanced translocations involving both imprinted 11p15 domains have been described. Duplications of the whole 11p15 domain (both

ICR1 and ICR2) resulting from unbalanced translocations cause an SRS or fetal growth retardation phenotype if they involve the maternal allele and a BWS or overgrowth phenotype if they involve the paternal allele (review in Bliek et al. 2009a; Demars et al. 2011a; Azzi et al. 2013; Soejima and Higashimoto 2013; Table 1).

Cis-duplications confined to one of the two domains are rare and until recently the picture was relatively clear. *Cis*-duplications involving the whole ICR1 *IGF2/H19* domain always result in BWS if the paternal chromosome is involved with no phenotype if the maternal chromosome is involved (Russo et al. 2006; Algar et al. 2007; Bliek et al. 2009a; Demars et al. 2011b; Table 1). On the other hand, *cis*-duplications involving the whole ICR2 *KCNQ1/CDKN1C* domain result in SRS if the maternal chromosome is involved with no phenotype if the paternal chromosome is involved (Schönherr et al. 2007; Bonaldi et al. 2011; Table 1). *Cis*-duplications involving only part of one of the two imprinted domains have recently been described in SRS and BWS cases (Chiesa et al. 2011; Demars et al. 2011b) and provide interesting information on how imprinting control mechanisms normally work and how they can be altered in human imprinting disorders. A *cis*-duplication involving only part of the ICR1 *IGF2/H19* domain (the imprinting control region and the *H19* gene) results in a SRS phenotype only if maternally inherited, whereas there is no phenotype upon paternal transmission (Demars et al. 2011b; Table 1). Both the parental transmission pattern and the phenotype in these cases differ from previously reported ICR1 duplications (Russo et al. 2006; Algar et al. 2007; Bliek et al. 2009a; Demars et al. 2011b; Beygo et al. 2013). Hence, a partial maternal *cis*-duplication of the *IGF2/H19* domain results in a SRS phenotype whereas a maternal *cis*-duplication involving the whole *IGF2/H19* domain does not result in any phenotype (Table 1). Two copies of the active maternal *H19* gene are expressed in both cases but, in the partial *cis*-duplication, one maternal *H19* gene is not engaged in a *cis*-effect. We (Demars et al. 2011b) and others (Chiesa et al. 2011; De Crescenzo et al. 2013) have also described partial *cis*-duplications of the ICR2 *KCNQ1/CDKN1C* domain; they result in a BWS phenotype only if maternally inherited whereas there is no phenotype upon paternal transmission (Table 1). One of these partial *cis*-duplications (Demars et al. 2011b) does not involve ICR2 but involves a region displaying CTCF- and cohesin-binding sites, suggesting that *cis*-regulatory elements other than ICR2 contribute to the establishment/maintenance of imprinting.

Uniparental 11p15 Isodisomy

Uniparental isodisomy (UPiD) is the presence of two copies of the same parental chromosome and results in unbalanced expression of imprinted genes. Paternal isodisomy (patUPiD) of the 11p15 region is a common cause of BWS and is present in 20–25 % of BWS cases (reviewed in Demars et al. 2011a; Azzi et al. 2013; Brioude et al. 2013a).

Table 1 Defects in 11p 15 *cis*-regulatory elements and the resulting phenotypes associated with maternal and paternal transmission

	Maternal chromosome/ transmission	Paternal chromosome/ transmission	Reference
Duplication			
ICR1 and ICR2 domains	SRS	BWS	Reviewed in Bliek *et al*. 2009a; Demars *et al*. 2011a
Whole ICR1 domain	No phenotype	BWS	Russo et al. 2006; Algar *et al*. 2007; Bliek *et al*. 2009a; Demars et al. 2011b
Part of the ICR1 domain	SRS	No phenotype	Demars et al. 2011b
Whole ICR2 domain	SRS	No phenotype	Schönherr *et al*. 2007; Bonaldi *et al*. 2011
Part of the ICR2 domain	BWS	No phenotype	Chiesa *et al*. 2011; Demars *et al*. 2011b
Deletion			
ICR1	BWS	No phenotype	Sparago *et al*. 2004; Prawitt *et al*. 2005; Sparago *et al*. 2007; Demars *et al*. 2010; De Crescenzo *et al*. 2011; Demars *et al*. 2011b
ICR2	BWS	No phenotype	Niemitz *et al*. 2004; Zollino *et al*. 2010; Algar *et al*. 2011
Enhancers	No phenotype	SRS	Grønskov et al. 2011
Mutation and small deletion			
ICR1 OCT4/ SOX2binding sites	BWS	No phenotype	Demars et al. 2010; Poole et al. 2011; Berland et al. 2013
Inactivating *CDKN1C mutation*	BWS	No phenotype	Reviewed in Choufani et al. 2010
Activating *CDKN1C mutation*	IMAGe/SRS	No phenotype	Arboleda *et al*. 2012; Brioude et al. 2013b
UPiD	I.	II.	III.
ICR1 and ICR2 domains	SRS	BWS	Cooper et al. 2007; Bullman et al. 2008; Romanelli et al. 2011
ICR1 domain	Not identified	Not identified	Demars et al. 2011b
ICR2 domain	Not identified	Not identified	Demars et al. 2011b

BWS Beckwith-Wiedemann Syndrome, *SRS* Silver-Russell Syndrome, *UPiD* uniparental isodisomy

We recently showed that SNP array analysis is a powerful diagnostic technique for BWS. Such arrays can be used to distinguish patUPiDs from trisomies more precisely than karyotyping and FISH, and they help to determine the size and

mosaicism rate of patUPiDs even in cases of low-rate patUPiD mosaicism (Keren et al. 2013).

Maternal isodisomy has been reported in only one SRS case (Bullman et al. 2008). UPiD is the consequence of a postzygotic event due to mitotic recombination in early embryogenesis and therefore results in mosaicism. Postzygotic mitotic recombination produces a mixed population composed of normal cells and cells with maternal UPiD or paternal UPiD. Maternal UPiD is rare, suggesting that cells with paternal UPiD have a selective growth advantage.

The extent of isodisomy along chromosome 11 is variable. It can extend to the long arm (10 % of cases) and always involves the two imprinted 11p15 domains (Cooper et al. 2007; Romanelli et al. 2011). Segmental UPiDs confined to the *IGF2/H19* or to the *KCNQ1OT1/CDKN1C* domains do not account for DNA methylation defects restricted to one of the two imprinted 11p15 domains (Demars et al. 2011b).

UPD of Chromosome 7

The molecular cause of SRS has long been unknown (Azzi et al. 2013). Several chromosomal abnormalities were reported to be associated with SRS or SRS-like phenotypes (Hitchins et al. 2001b) but the most relevant abnormality found in a significant number of patients (5–10 % of cases) was UPD of chromosome 7 (mUPD7; Preece 2002). This cytogenetic anomaly implicates imprinted genes on chromosome 7 in the SRS phenotype. Indeed, at least two imprinted domains, located at 7p11.1-p14 and 7q31, have been identified within chromosome 7. These regions harbor at least two imprinted genes involved in the control of growth: the maternally expressed growth factor receptor-binding protein 10 (*GRB10* at 7p11.1-p14) and paternally expressed gene 1/mesodermal expressed transcript (*PEG1/MEST* at 7q31). Because a number of SRS patients with duplications (or inversions) or segmental UPD have been reported (Joyce et al. 1999; Monk et al. 2000; Hannula et al. 2001), these two regions have been the focus of research to identify causative mutations or epimutations. However, screening SRS patients without mUPD7 failed to identify either mutations of *GRB10* or *PEG1/MEST* or epimutations in their DMRs (Riesewijk et al. 1998; Yoshihashi et al. 2000; Hannula et al. 2001; Hitchins et al. 2001a; Kobayashi et al. 2001; McCann et al. 2001; Arnaud et al. 2003). Recently, Kagami and coworkers reported hypermethylation of the *PEG1/MEST* DMR in a girl with the SRS phenotype born after in vitro fertilization (Kagami et al. 2007); subsequently, Eggermann et al. reported a SRS boy carrying a de novo deletion of 3.7 Mb of the paternal allele on 7q32 causing the loss of 53 genes, including *PEG1/MEST* (Eggermann et al. 2012). These observations, despite providing evidence of the involvement of imprinted genes in these regions, do not incriminate a particular gene as being causative of SRS.

CDKN1C Mutations

Inactivating mutations in the *CDKN1C* gene (also known as $p57^{KIP2}$), which encodes a maternally expressed cell-cycle inhibitor, are found in approximately 5 % of BWS patients (Choufani et al. 2010) and account for approximately half of familial BWS cases. Mice lacking the imprinted Cdk inhibitor $p57^{(kip2)}$ show altered cell proliferation and differentiation, leading to abdominal muscle defects and many of the phenotypes seen in patients with BWS (Zhang et al. 1997; Tunster et al. 2011).

Although *Cdkn1c* transgenic mice display a SRS phenotype (Andrews et al. 2007), no *CDKN1C* mutations had been found associated with SRS until recently (Obermann et al. 2004). Indeed, maternally transmitted activating mutations of *CDKN1C* were recently described in cases of the IMAGe syndrome, which shares some phenotypes with SRS, such as fetal growth retardation and facial dysmorphia (Arboleda et al. 2012; Table 1). Then, very recently, an activating mutation was found in a familial case of SRS (Brioude et al. 2013b).

Epigenetic Defects

Isolated DNA Methylation Defects

A large subgroup of BWS and SRS patients displays no obvious genetic defects in a *cis*-regulatory element or a transacting factor; these cases are identified as ICR1 or ICR2 "primary" DNA methylation defects. However, the prevalence of secondary DNA methylation defects might be underestimated because there is generally no search for mutations in *cis*-regulatory elements or transacting factors for routine diagnosis of BWS or SRS.

DNA Methylation Defects at ICR2 Result in a BWS Phenotype

Approximately 60 % of BWS patients display ICR2 loss of methylation. In rare cases, the loss of methylation is caused by a deletion involving ICR2 on the maternal allele (Niemitz et al. 2004; Zollino et al. 2010; Algar et al. 2011; Demars et al. 2011a; Azzi et al. 2013; De Crescenzo et al. 2013). At least 25 % of BWS patients with loss of methylation at ICR2 also display loss of methylation at imprinted loci other than 11p15 (see section on "The Multilocus Hypomethylation Disorder"), and this pattern defines the multilocus hypomethylation disorder (MHD). Abnormal methylation of ICR2 DNA (i.e., gain of methylation) has never been identified in SRS patients (Gicquel et al. 2005a; Eggermann et al. 2006; Netchine et al. 2007; Penaherrera et al. 2010).

DNA Methylation Defects at ICR1 Result in Both BWS and SRS Phenotypes

A gain of methylation at ICR1 is found in 10 % of BWS patients and results in biallelic expression of the *IGF2* gene. Conversely, a loss of methylation at ICR1 is observed in 50–60 % of SRS patients and results in loss of expression of the *IGF2* gene (Gicquel et al. 2005a; Netchine et al. 2007; Azzi et al. 2013). The gain of methylation in BWS is strictly localized at ICR1 and does not involve other imprinted loci (Bliek et al. 2006; Azzi et al. 2009). The situation is different in SRS, with at least 10–17 % of SRS patients displaying loss of DNA methylation at imprinted loci other than 11p15 (Azzi et al. 2009, 2013; Turner et al. 2010; Court et al. 2013; Poole et al. 2013; see section on "The Multilocus Hypomethylation Disorder").

Dysregulation of the Histone Code in BWS and SRS

Although histone marks are determinant for the regulation of 11p15 genomic imprinting (Henckel et al. 2009), their role in the pathogenesis of BWS and SRS and their link to DNA methylation defects have not been extensively addressed. Very recently, Nativio et al. (2011) showed that trimethylated Lysine 9 of histone H3 and trimethylated Lysine 20 of histone H4 (H3K9me3 and H4K20me3), both repressive histone marks, are associated with the methylated paternal ICR1 allele. In addition, dimethylated Lysine 4 of histone H3 and acetylated Lysine 9 of histone H3 (H3K4me2, H3K9ac), both permissive histone marks, are associated with the non-methylated maternal ICR1 allele. In BWS and SRS patients, the asymmetric distribution of these epigenetic marks is lost: H3K9me3 and H4K20me3 are biallelic in BWS, and H3K4me2 and H3K9ac are biallelic in SRS.

Mechanisms of Imprinting Dysregulation

Genomic imprinting is a multistep process and some specific stages, such as imprint establishment in germ cells or imprint maintenance after fertilization, are critical in the regulation of genomic imprinting. Deregulation of genomic imprinting during one of those stages will result in imprinting disorders.

Evidence for Secondary Imprinting Defects in BWS and SRS

The Prevalence of Mutations in Cis*-Regulatory Elements is Probably Underestimated*

Investigation of BWS and SRS patients relies mostly on diagnostic techniques, such as MLPA, which identify copy number variants in the kb range but do not recognize small deletions or mutations involving transacting factor-binding sites. The main function of the CTCF protein at ICR1 is to maintain the unmethylated state of the maternal allele. It has therefore been suggested that the loss of some CTCF-binding sites impairs protection by CTCF and results in gain of methylation on the maternal allele. Maternally inherited deletions of between two and six CTCF-binding sites have been identified in several BWS cases (Sparago et al. 2004, 2007; Prawitt et al. 2005; Scott et al. 2008; Demars et al. 2010, 2011b; Beygo et al. 2013) but no such deletions have been found in SRS cases (Bliek et al. 2006; Yamazawa et al. 2008b; Bartholdi et al. 2009; Bruce et al. 2010; Demars et al. 2010). No mutation of CTCF-binding sites has been in BWS (Sparago et al. 2007; Demars et al. 2010) or SRS (Bliek et al. 2006; Yamazawa et al. 2008a; Bruce et al. 2009; Demars et al. 2010).

Novel transacting factors have recently been identified, and there is emerging evidence that CTCF function is modulated by neighboring DNA-binding factors (Weth and Renkawitz 2011). These factors include pluripotency factors that may be involved in the regulation of genomic imprinting. Mutations and small deletions of OCT4- and SOX2-binding sites have been described within ICR1 in BWS patients and are associated with gain of ICR1 methylation (Table 1). In all cases, the BWS phenotype segregated with transmission of the mutation through the female germline, with no phenotype showing paternal transmission (Demars et al. 2010; Poole and Leith 2012; Berland et al. 2013). These observations suggest that loss of binding of pluripotency factors at ICR1 impairs the maintenance of the unmethylated state of the maternal ICR1. Possibly, OCT4 and SOX2 protect the maternal allele from gain of DNA methylation, especially at CTCF-binding sites (Hori et al. 2002). A recent YAC transgenic mouse model confirmed that these OCT4-binding sites are indeed required both for protection of the maternal ICR1 against DNA methylation and during the maintenance stage of the imprinting cycle (Hori et al. 2012; Sakaguchi et al. 2013). This model also suggests that CTCF and OCT4/SOX2 act cooperatively (Sakaguchi et al. 2013). OCT4- and SOX2-binding sites have also been identified at the Angelman imprinting center (Kaufman et al. 2009). The mechanism of action of pluripotency factors is not clear, but OCT4 interacts with CTCF when regulating the X chromosome inactivation process (Donohoe et al. 2009). Very recently, a novel function was attributed to OCT4: negative regulation of chromatin loop formation mediated by cohesin at CTCF-binding sites (Kim et al. 2011). Whether mutations of pluripotency factor-binding sites are also involved at ICR2 is yet to be determined. It is also plausible that

mutations/deletions of other transacting factor-binding sites are involved in the pathogenesis of 11p15 imprinting disorders, and this possibility should be further investigated. However, all binding sites for ZFP57 in ICR1 overlap with CTCF-binding sites; such sites have been investigated in cases of SRS and BWS and no mutation has been found (Demars et al. 2010).

Genetic Variants in Cis-Regulatory Elements may Play a Role in Susceptibility to 11p15 Imprinting Disorders

Recent studies clearly show that genetic variants in *cis* account for allele-specific differences in DNA methylation status (reviewed in Tycko 2010), chromatin status (McDaniell et al. 2010) or transcription factor binding (Kasowski et al. 2010; McDaniell et al. 2010), which result in differences in allele-specific expression. The significance of the parental origin of alleles is also emerging in genetic studies and may be particularly relevant to imprinting disorders. Relatively little research has addressed the nature and effects of allelic diversity at imprinted loci. Zogel et al. (2006) identified preferential maternal transmission of one specific haplotype of the 15q11-13 ICR in a subgroup of Angelman patients with primary imprinting defects. Interestingly, within this haplotype, a polymorphism affects a SOX2-binding site (Kaufman et al. 2009). It would therefore be interesting to determine whether genetic variability in imprinted regions, by affecting the binding of regulatory factors at play in the establishment and/or the maintenance of 11p15 genomic imprinting, has a role in imprinting disorders.

The Multilocus Hypomethylation Disorder Indicates Abnormal Expression of a Transacting Regulatory Factor

The involvement of transacting factors in the pathogenesis of imprinting disorders was first suggested in 2006: we and others showed that a subset of BWS and transient neonatal diabetes mellitus (TNDM) patients displayed a loss of DNA methylation at loci other than the causal locus (i.e., ICR2 and *ZAC1*, respectively; Mackay et al. 2006; Rossignol et al. 2006). This finding has since been confirmed by other studies in BWS (Bliek et al. 2006; Azzi et al. 2009; Lim et al. 2009; Meyer et al. 2009; Court et al. 2013; Poole et al. 2013), SRS (Bliek et al. 2006; Azzi et al. 2009; Turner et al. 2010; Kannenberg et al. 2012; Court et al. 2013; Poole et al. 2013), TNDM (Mackay et al. 2006; Court et al. 2013) and pseudohypoparathyroidism 1B (PHP1B) (Perez-Nanclares et al. 2012; Court et al. 2013; Maupetit-Mehouas et al. 2013), but not in Prader-Willi or Angelman syndromes (Court et al. 2013; Table 2). This finding defines a new entity of imprinting disorders, now called the multilocus hypomethylation disorder (MHD). Multilocus imprinting

Table 2 Multilocus hypomethylation disorder in imprinting disorders

Disorder	MHD frequency[a]	Parental loci affected	References	Mutation of *trans*-regulatory factors
ICR2 LOM BWS	11–33 %	Mat and pat	Rossignol et al. 2006; Azzi et al. 2009; Bliek et al. 2009b; Lim et al. 2009; Court et al. 2013	One case with *NLRP2* mutation (Meyer et al. 2009)
ICR1 GOM BWS	0 %	–	Azzi et al. 2009; Bliek et al. 2009b	–
ICR1 LOM SRS	8.7–17 %	Mat and pat	Azzi *et al*. 2009; Azzi *et al*. 2010; Turner *et al*. 2010; Court *et al*. 2013; Poole *et al*. 2013	No
TNDM	50–75 %	Mat[b]	Mackay et al. 2006; Mackay et al. 2008; Court et al. 2013	*ZFP57* mutations (Mackay et al. 2008; Court et al. 2013)
PHP1B	9–50 %	Mat and pat	Perez-Nanclares et al. 2012; Court et al. 2013; Maupetit-Mehouas et al. 2013	No
PWS	0 %	–	Court et al. 2013	–
AS	0 %	–	Court et al. 2013	–

ICR2 LOM BWS Beckwith-Wiedemann patients with ICR2 loss of DNA methylation, *ICR1 GOM BWS* Beckwith-Wiedemann patients with ICR1 gain of DNA methylation, *ICR1 LOM SRS* Silver-Russell patients with ICR1 loss of DNA methylation, *TNDM1* transient neonatal diabetes mellitus type 1, *PHPIB* Pseudohypoparathyroidism 1B, *PWS* Prader-Willi syndrome, *AS* Angelman syndrome, *MHD* Multilocus hypomethylation disorder

[a]The highest frequency was reported by (Court et al. 2013) who investigated all imprinted loci

[b]Only maternally methylated DMRs are affected

disorder (MID) is not restricted to multilocus hypomethylation and, indeed, both hypo and hypermethylation at numerous imprinted genes may coexist in the same SRS (Kannenberg et al. 2012) or PHP1B (Maupetit-Mehouas et al. 2013) patient. This observation adds an additional layer of complexity to the issue of imprinting regulation and MID occurrence. MID displays mosaicism and involves both maternally and paternally imprinted ICRs, except in TNDM where only maternal loci are affected (Table 2).

Although MID is relatively frequent, mutation analyses of recently identified transacting regulatory factors, including *ZFP57*, *TRIM28* (*KAP1*), *NLRP2*, *NLRP7* and *C6ORF221*, are rare. *ZFP57* mutations (a maternal-effect gene involved in both the establishment and maintenance of imprints) have only been identified in cases of TNDM with MHD (Mackay et al. 2008; Court et al. 2013), and a *NLRP2* (member of the NLRP family of CATERPILLER proteins) mutation has been identified in a case of BWS with MHD (Meyer et al. 2009; Table 2).

Another interesting concept that emerged recently is the imprinted gene network: groups of imprinted genes can be co-regulated as parts of networks. For example, ICR1 at 11p15 interacts physically with several chromosomal regions as part of an epigenetically regulated network operating both intra- and interchromosomally (Varrault et al. 2006; Gabory et al. 2009). The existence of this network

suggests that a defect of one imprinted locus might induce perturbation at other imprinted loci. Further work is needed to assess the contribution, if any, of imprinted gene networks to the pathogenesis of MHD/MID.

Genotype-Phenotype Relationships

BWS

Various molecular and chromosomal alterations can lead to BWS (Gaston et al. 2001; Gicquel et al. 2005b; Weksberg et al. 2005; Demars et al. 2011a); only about 25 % of cases are caused by genetic defects. UPids of paternal origin (patUPiD, 20 % of cases) are segmental and always include the 11p15 region but the proximal breakpoints are diverse (Nyström et al. 1992; Henry et al. 1993; Cooper et al. 2007; Romanelli et al. 2011). Genetic mutations on the maternal allele of *CDKN1C* account only for 5 % of cases overall but are found in more than 70 % of familial cases. The most frequent mechanism is clearly epigenetic (70 % of cases): LOM at ICR2 explains more than half BWS cases (50–60 %) whereas GOM at ICR1 is less common (10 % of cases). PatUPiD and both epigenetic defects always display variable mosaicism, presumably explaining the substantial variability of the phenotype. Some of the phenotypic features of BWS can be directly correlated to particular molecular alterations (Gaston et al. 2001; Cooper et al. 2005; Weksberg et al. 2005; Brioude et al. 2013a; Table 3). Hemihyperplasia is more frequent in patients with patUPiD11p15, whereas the abdominal wall defect is strongly associated with abnormalities mapping in the centromeric domain. Moreover, exomphalos are almost constant in *CDKN1C* mutations. An important finding is that tumor risk differs substantially between molecular subtypes. The tumor risk is high (around 30 %) in patients with ICR1 GOM who only develop nephroblastoma (Wilms Tumor: WT); around 20 % of patients with patUPD11p15 develop a childhood tumor, especially nephroblastoma, hepatoblastoma and adrenocortical tumor. Fortunately, less than 5 % of patients with ICR2 LOM, the situation for the majority of BWS cases, develop a tumor and no nephroblastoma has yet been reported in these patients. It is also important that physicians be careful with patients with a positive clinical diagnosis of BWS without known molecular anomaly or isolated severe hemihyperplasia or organomegalies, because the risk of WT is high in such cases. These observations and findings can help the physician in the follow-up of patients, as diverse factors need to be considered: the type of transmission (maternal or paternal); the risk of false negative findings due to the variability of the percentage of mosaic abnormality between tissues; and the possibility of a multilocus disorder involving any of the ICR2, *SNRPN*, *ZAC*, *IGF-2R*, *DLK1/GTL2 IG-DMR* and *GNAS* loci (Rossignol et al. 2006; Azzi et al. 2009). These findings also allow better genetic counseling for families (Brioude et al. 2013a). Abdominal ultrasound scans every 3 months are

Table 3 Genotype-phenotype correlations in all patients referred to our center, between January 1991 and September 2009, as BWS with a positive molecular diagnosis (n = 407)

	CDKN1C mutation	ICR1 GOM	ICR2 LOM	PatUPD11p15	All subtypes
Macroglossia	93.9 %	85.7 %	97.6 %	86.2 %	94.0 %
Abdominal wall defect	93.9 %	28.6 %	67.2 %	48.7 %	62.40 %
Visceromegaly	13.8 %	64.5 %	39.0 %	58.3 %	43.8 %
Hemi-hypertrophy	3.0 %	40.0 %	20.2 %	81.0 %	33.3 %
Hypoglycemia	37.5 %	32.4 %	40.2 %	60.5 %	43.4 %
Ear creases and pits	90.9 %	27.2 %	65.4 %	50.0 %	38.1 %

ICR Imprinted center region, *LOM* Loss of methylation, *GOM* Gain of methylation, *IVF* In vitro fertilization, *ICSI* intra cytoplasmic sperm injection

recommended for patients with a telomeric defect (ICR1 GOM and patUPiD11p15), associated with regular physical examinations. For patients with a centromeric defect, regular physical examination seems to be sufficient.

Two observations related to BWS suggest that the preimplantation embryo is particularly prone to imprinting errors. First, the incidence of monozygotic twinning in BWS is very much higher than normal, with an unusually high proportion of female monozygotic twins. These twins are always discordant for the BWS phenotype and the only molecular defect is, in all cases, an ICR2 LOM. Weksberg's group showed that the imprinted defect is found in blood leucocytes of both twins but only in fibroblasts of the affected twin (Weksberg et al. 2002; Gicquel et al. 2005a). This is probably due to the sharing of the blood circulation, which is a common feature of monozygotic twins. Discordance between monozygotic twins may result from a failure of maintenance of methylation during a single cell cycle at or just prior to the twinning event, caused by an error in the nucleocytoplasmic trafficking of a transacting factors involved in the process (Bestor 2003; Weksberg et al. 2005). The X-inactivation process takes place at the same time and presumably involves common factors, and this may explain the female vulnerability. A second line of evidence that preimplantation is a critical period is the large proportion of BWS (DeBaun et al. 2003; Gicquel et al. 2003; Maher et al. 2003; Halliday et al. 2004), SRS (Svensson et al. 2005; Wakeling et al. 2010) and also Angelman syndrome (Ludwig et al. 2005) patients who were conceived by ART. Again, the molecular defect is a LOM at maternally (BWS, AS) or paternally (SRS) imprinted loci, implicating a factor involved in the maintenance of methylation. The underlying cause of this association remains unclear, and no particular procedure or cause of infertility has been found to be specifically associated with these abnormalities.

SRS

Most (over 50 % of) SRS patients display hypomethylation of the telomeric ICR1 domain of the 11p15 region (Gicquel et al. 2005a; Netchine et al. 2007). SRS and BWS mirror each other, both clinically and at the molecular level. In SRS patients, the paternal allele switches to a maternal epigenotype resulting in biallelic expression of *H19* and decreased *IGF2* expression. In most of these cases, the hypomethylation is partial, reflecting the mosaic distribution of the epimutation and explaining at least in part the variability of the SRS phenotype.

mUPD7, where at least two imprinted domains are located (7p11.1-p14 and 7q31), is present in about 5–10 % of SRS cases (Preece 2002). However, in around 40 % of SRS cases with a typical clinical phenotype, no abnormality is found.

Many SRS patients carrying ICR1 LOM display a more severely abnormal growth phenotype than other SRS patients, and this may be associated with typical dysmorphy (relative macrocephaly, prominent forehead) and the highly evocative body asymmetry. They also more frequently have associated malformations (Netchine et al. 2007; Wakeling et al. 2010; Binder et al. 2011; Ghanim et al. 2013).

The specific features of mUPD7 SRS patients are mild developmental delay, mainly consisting of speech difficulties, predisposition to myoclonus dystonia and a putative susceptibility to developing autism traits (Hitchins et al. 2001b; Guettard et al. 2008; Binder et al. 2011). All these features are thought to be related to disruption of the expression of particular imprinted genes on chromosome 7. However, it should be noted that there is no discontinuity of the phenotypic presentation between these subgroups of patients; similarly, there is variability within subpopulations of patients with the same molecular abnormalities (Bliek et al. 2006; Murphy et al. 2012).

No molecular anomalies have been identified in about 40 % of SRS patients studied, and these cases can therefore be called “idiopathic” SRS. However, this diagnosis should be made only by clinicians with substantial experience of SRS and after various differential diagnoses have been ruled out (notably 3M syndrome, Mullibrey Syndrome, Bloom syndrome and other chromosome breakage susceptibility syndromes, and *IGF-1R* molecular anomalies). This group of patients is of particular interest because further investigations may identify new molecular etiologies of SRS, and the same follow-up and treatment guidelines used for other SRS patients may also be appropriate for this subgroup.

Conclusion and Outlook

The regulation of gene expression is under the control of the genetic codes but also of epigenetic phenomena. Abnormalities affecting epigenetic, and especially imprinting, mechanisms can result in abnormal gene expression, leading to various developmental pathologies and tumors. The BWS and the SRS syndromes are some

of the most characteristic pediatric diseases involving abnormalities of imprinting, in both cases involving the 11p15 region. These human imprinting disorders have helped to identify key *cis*-regulatory elements in imprinting centers, as well as various transacting regulatory factors. Work on these 11p15 imprinting disorders has focused mainly on DNA methylation. However, other epigenetic marks and factors, such as histone acetylation and methylation, long non-coding RNAs, small RNAs and miRNA, and genetic variations (SNP, CNV involved in chromatin organization) should be explored for their involvement in the pathogenesis of 11p15 imprinting disorders. Genetic analysis with high-throughput techniques should speed the discovery of new factors in *cis* and *trans* associated with 11p15 genomic imprinting controls. Finally, it is important to elucidate the role of environmental factors (ART, undernutrition) in the pathogenesis of BWS and SRS.

Progress in these fields should provide new diagnostic and predictive tools, but such tools must be appropriate for these diseases in which there is a high risk of false negative results; the percentage of mosaic abnormalities can differ very substantially between tissues. Progress will also allow improved genetic counseling to be provided to families.

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Epigenetic Effects of Extreme Intrauterine Growth in Humans

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Abstract There is significant interest in the potential that epigenetic dysregulation during fetal development reprograms the individual for susceptibility to adult diseases. The field of study of developmental origins of health and disease (DOHaD) is embracing the use of epigenomic assays to explore this potential mechanistic process. These DOHaD investigations represent part of the spectrum of epigenome-wide association studies (EWAS), a new field of investigation that involves a substantial increase in complexity compared with genome-wide association studies (GWAS). In this report, I describe some of the challenges in experimental design and execution that need to be taken into account to make these studies maximally productive.

Introduction

The determinism of our genes for susceptibility to disease is incomplete, especially when it comes to adult diseases with clear heritability but occurring in non-Mendelian patterns and influenced by environmental factors. A challenge is dissecting the components of the susceptibility in such a multifactorial situation. Such diseases with complex genetic underpinnings are further complicated by the potential that there could be influences acting at a level above that of DNA sequence alone, so-called epigenetic processes. These epigenetic events are, by definition, capable of being passed on from parent to daughter cell, so that even remote events during a lifetime can influence later disease susceptibility. Such mechanisms have been invoked to explain susceptibilities mediated through in

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utero events, leading to human diseases that emerge decades later. This is paradigmatically represented by dramatic and well-documented events like populations going through famine, with phenotypes like psychosis (Xu et al. 2009), coronary heart disease (Roseboom et al. 2000), atopy and lung disease (Lopuhaa et al. 2000), obesity (Ravelli et al. 1999) and type II diabetes mellitus (Ravelli et al. 1998); all of these are linked to poor nutrition in utero, with some preliminary evidence that there may even be grandparental effects on longevity (Kaati et al. 2007).

It is difficult to think of another mechanism apart from epigenetic regulatory processes that could mediate such long-term susceptibilities. There are some rodent models that suggest DNA methylation to be a critical mediator of intrauterine events that "imprints" a molecular memory upon the developing fetus that remains throughout its life span. Some of these mouse models are based on observations in regular laboratory strains, such as the effect of paternal diet on the DNA methylation of offspring livers (Carone et al. 2010), whereas others are apparent in the context of spontaneous mutations involving transposable elements, such as the viable yellow (Wolff et al. 1998) or axin fused (Vasicek et al. 1997) mice. The latter mutational events generate targets for DNA methylation that then influence nearby genes, creating phenotypes that depend upon the degree to which DNA methylation modifies the region. These so-called metastable epialleles (Rakyan et al. 2002) are influenced by maternal diet (Wolff et al. 1998) and represent the paradigm for the kinds of events we should be looking for at endogenous loci in human subjects.

In this overview, I summarize the key elements of EWAS design and execution, focusing on the application of these approaches to DOHaD questions. We have previously performed such studies in rodent models (Thompson et al. 2010a, b) and have an ongoing investigation of human subjects that applies the insights described in this report. The goal is to help guide similar future studies by other groups so that the lessons that we have learned can be applied to allow the maximum benefit to be gained from such future work.

Which Epigenetic Regulators to Study and Assay Choice

The epigenetic regulator mentioned above, DNA methylation, is only one of a large number of transcriptional regulatory processes in human cells. It has the advantage of being well studied, with mature assays that have base pair resolution and a reasonable understanding of its functional properties. It is also potentially extremely quantitative, capable of measuring the fractional proportion of methylated alleles in a population to a few percent resolution. DNA methylation is by far the most studied epigenetic regulator in human disease, but it should be recognized that there are many other regulatory mechanisms in the cell, including post-translational histone modifications (Delaval and Feil 2004), nucleosomal remodeling (Hartley and Madhani 2009), small RNA effects (Beiter, et al. 2009), and higher-order chromatin structure (Dostie et al. 2006). DNA methylation refers

to 5-methylcytosine, usually in the context of a CG dinucleotide in human cells, but it should also be noted that some DNA methylation occurs outside the CG context (Lister et al. 2009) and there are other DNA modifications such as 5-hydroxymethylcytosine (Kriaucionis and Heintz 2009).

There are now assays based on massively parallel sequencing for most of the known epigenetic regulators. The desirable characteristics of the assays are that they should test as much of the genome as possible, as we remain uncertain where to look for informative changes. The assay should also be reasonably cost effective; otherwise, the number of subjects defined in the next section discussing power calculations will not be feasible. The assay should have robust quantitative discrimination, as the degree to which DNA methylation has been observed to alter in EWAS has proven to be relatively small in most studies to date. When a genome-wide assay has been performed, an orthogonal verification of a number of loci to test the predictions of the genome-wide assay should then follow.

Some of the assays above do not allow the necessary quantitative discrimination. Chromatin immunoprecipitation allows peaks of occupancy to be identified but cannot discriminate between situations where, for example, 30 % of the alleles have the chromatin constituent in one sample and 60 % in another sample. The cytosine variants and transcriptional profiling studies are quantitative and represent the preferred approaches in the short term. For studies of intrauterine growth influences on the epigenome, the studies cited earlier all used DNA methylation assays, demonstrating that this epigenetic mark is informative for such studies.

Choosing the Cell Sample

It was recently demonstrated that DNA methylation patterns are very sensitive to the proportions of cell subpopulations in the sample (Houseman et al. 2012), which can lead to a major source of variability when mixed cell types are used, such as peripheral blood leukocytes. Any systematic tendency towards a certain cell population being over- or under-represented in one of the groups studied will yield DNA methylation patterns characteristic of that cell type.

It is therefore desirable to isolate a cell type so that the same cell type is being compared in all subjects. It should be noted that even purified cells probably have underlying heterogeneity of function, so the goal is to make the samples studied as comparable as possible, even while recognizing that this still allows potential for subpopulation effects.

As an EWAS is asking whether an epigenomic dysregulation is associated with a disease, the cell type that needs to be studied is that mediating the disease. There are situations when this is not practical, when the mediating cell type is inaccessible, leading to the question of whether surrogate cells can be used instead. There is no straightforward answer to this question; it all depends upon the difficulty of getting the mediating cells and the candidacy of the surrogate cells for having similar epigenomic changes.

If the use of mixed cell populations is unavoidable, at the very least there should be a measurement of subpopulation proportions in each sample so that, in the statistical modeling of the results, this factor can be accounted for.

For DOHaD studies, one desired characteristic of the cell type chosen is that it can mediate the memory of the remote intrauterine event. A short-lived, terminally differentiated cell type is probably less informative than a self-renewing cell. In our ongoing studies of extreme fetal growth, we have focused on the use of hematopoietic stem and progenitor cells (HSPCs), as they have the self-renewing capability and are progenitors for the inflammatory cells that play a major role in the type II diabetes mellitus for which these babies are at increased risk.

While many genome-wide assays are now quite robust, they still require the equivalent of up to millions of cells to generate enough material for sequencing, which is a further limiting factor in terms of getting appropriate cells, prompting attempts to modify epigenomic assays to use smaller amounts of materials (Oda et al. 2009; Adli et al. 2010). Our HSPC studies use cord blood-derived CD34+ cells, which we can harvest in reasonable quantities, but other studies will have less capability to get comparable numbers of cells.

Effect Sizes and Power

We (Thompson et al. 2010a, b) and others (Carone et al. 2010) have noted that the degree to which DNA methylation changes as a response to perturbation is generally limited, which imposes quite a burden upon an EWAS, as it requires an assay capable of detecting these moderate changes, as described earlier. It also drives the need to collect samples from enough subjects to allow the study to be adequately statistically powered, and it makes the studies more susceptible to the confounding effects described below.

While many EWAS to date have used limited numbers of subjects, there will be a need to expand these numbers in more definitive studies, which will further compound the issue of getting enough of the appropriate, purified cell type. Prospective sample collection with future epigenomic studies in mind is a valuable current strategy.

The Confounding Effect of Genetic Variability

It is now being increasingly recognized that DNA sequence variability can influence the epigenome locally (Gertz et al. 2011). When studies are performed in inbred rodent lines, this influence is abolished, but when studying human subjects there is substantial potential for this influence. While the outcome of epigenetic differences remains valid, the causative role of DNA sequence polymorphism needs

to be recognized, as it would not fit with a hypothesis that the effects of the intrauterine environment are mediated by epigenetic changes.

To address this issue, at a minimum the cohorts should be matched in terms of self-reported race and ethnicity. In an ideal situation, this matching would be confirmed through genotyping, or even whole-genome sequencing, which may become the standard in the future as sequencing costs drop.

Other Potential Confounding Influences

The metadata collected for an EWAS are extremely important as the number of possible confounding influences is substantial, and, as much as possible needs to be described about the subject and sample so that these influences can be recognized and accounted for when modeling the results. For example, the patient's sex (Sarter, et al. 2005) and age (Heyn et al. 2012) are likely to be influential, whereas diet, cigarette smoking, and other exposures are also potentially influential. The way samples are collected, stored and assayed is a well-known source of variability for molecular assays in general, epigenomic assays being no exception. There are many reasons why babies can be born unusually small (intrauterine growth restriction) or with large birthweight (large for gestational age). It is therefore important to collect as much clinical information as possible about the clinical subjects in these studies, so that known causes of these patterns of growth (such as chromosomal abnormalities or maternal hyperglycaemia) can be identified, allowing decisions to be made whether to include these subjects in the cohort studied.

The analysis of data is a surprisingly common source of variability. Especially when studies are performed over the course of many years, it becomes possible that the analytical approaches used earlier in the study are not the same as those employed subsequently, which can contribute a major source of variability.

We do not yet know the full scope of sources of variability upon the epigenome. It is therefore critically important that a wealth of metadata be collected when performing these kinds of studies, so that the influence of each parameter can be assessed retrospectively to understand its role and to account for it when performing data analysis.

Analyzing and Interpreting Data Generated

Although there have been many analytical approaches applied to DNA methylation data, a recent development appears to hold a lot of promise. The "bump hunting" approach developed by the Irizarry group (Jaffe et al. 2012) represents a significant advance for a couple of reasons. Firstly, it uses a surrogate variable analysis as a pre-processing step to look for potential confounding influences upon the desired comparison, thereby eliminating even some unrecognized sources of variability. Secondly, it asks for multiple adjacent loci to show a concordant shift in

methylation changes between comparison groups, which not only reduces the chances of a single CG dinucleotide causing a false positive but also makes it appear more intuitively likely that functional consequences would arise from such changes in DNA methylation.

Single CG dinucleotides are an especially problematic source of variability, as they are unusually likely to be polymorphic sequences in the genome because of the propensity of cytosines at CGs to mutate by deamination (Duncan and Miller 1980). So while a reference genome may define a cytosine at a specific position, in a given individual the C may be mutated to a T, which will not be recognized as a mutational event by methylation-sensitive restriction enzymes or by bisulphite sequencing. This can cause erroneous identification of the site as being methylated when using methylation-sensitive restriction enzymes (which will fail to cut the site) or as unmethylated when using bisulphite sequencing (which will see a T at the position and assume this results from conversion of an unmethylated C).

What has received little attention to date is the innate variability of epigenetic regulators between individuals. These were described as variably methylated regions by Irizarry and colleagues (Feinberg and Irizarry 2010) and, more recently, as epipolymorphisms (Landan et al. 2012), in both cases focusing on their relevance in cancer epigenetics. The more recent study noted that DNA methylation variability was more prevalent at loci with intermediate methylation. As a cell can only have 0 %, 50 % or 100 % methylation at a locus, depending on whether neither, one or both alleles are methylated, intermediate methylation indicates the presence of a cell subpopulation with a distinctive epigenetic profile within the larger pool of cells. Variability of methylation between individuals at such loci would therefore represent differences in the proportion of such cell subpopulations between subjects. How we address this issue depends upon whether these variably methylated loci are likely to be functional or merely represent noise in the genome.

Substantial computational resources are needed for the management and analysis of genome-wide data sets. We describe our own solution of the Wasp System, an open source piece of software that allows analytical modules to be embedded and used as pipelines, capturing metadata simultaneously, and harnessing grid computing resources (McLellan et al. 2012). The Wasp System is being developed as an operating system for genomic data analysis, embedding pipelines that can be shared and distributed with the software throughout the research community.

Finally, in common with GWAS, we have the issue of how to interpret findings when they are not easily linked to recognizably functional sites. If a DNA methylation change occurs within a well-defined transcriptional start site, it becomes relatively easy to interpret, but increasingly as we move from a cancer and CpG island paradigm to genuinely genome-wide studies, we are observing the most significant associations at other, non-promoter loci. The resources being generated by the ENCODE and Roadmap in Epigenomics projects are going to be especially valuable in this regard, as their intent is to assign function to as much of the human genome as possible, defining enhancers and other *cis*-regulatory loci in a cell type-specific manner. We may therefore be able to prioritise certain loci based on function defined by these initiatives, and link these loci to specific genes located nearby or more distally on the chromosome.

Conclusions

We are at an interesting juncture in this emerging era of EWAS. There is obvious promise in the approach but we are simultaneously recognizing unanticipated pitfalls that require our attention. There is emerging consensus within the field that a set of rigorous guidelines for EWAS design and execution is needed, which this review attempts to summarize, with an emphasis on how studies looking at the effects of intrauterine events should be designed. With sequencing costs dropping and with the development of strong assays and analytical resources, the feasibility of bringing these studies to fruition is improving constantly, which will hopefully result in robust insights into the role of the epigenome in these conditions.

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Developmental Origins of Diabetes: The Role of Epigenetics

Rebecca A. Simmons

Abstract The "thrifty phenotype" hypothesis proposes that the fetus adapts to an adverse intrauterine milieu by optimizing the use of a reduced nutrient supply to ensure survival, but, by favoring the development of certain organs over that of others, this strategy leads to persistent alterations in the growth and function of developing tissues. This concept has been somewhat controversial, however, as recent epidemiological, clinical, and animal studies provide support for the developmental origins of disease hypothesis. Underlying mechanisms include reprogramming of the hypothalamic-pituitary-adrenal axis, islet development, and insulin signaling pathways. Emerging data suggest that oxidative stress and mitochondrial dysfunction may also play critical roles in the pathogenesis of type 2 diabetes in individuals who were growth retarded at birth. Epigenetic modifications may be one mechanism by which exposure to an altered intrauterine milieu or metabolic perturbation may influence the phenotype of the organism much later in life. Epigenetic modifications of the genome provide a mechanism that allows the stable propagation of gene expression from one generation of cells to the next. This review highlights our current knowledge of epigenetic gene regulation and the evidence that chromatin remodeling and histone modifications play key roles in adipogenesis and the development of obesity. Epigenetic modifications affecting processes important to glucose regulation and insulin secretion have been described in the pancreatic β-cells and muscle of the intrauterine growth retarded (IUGR) offspring, characteristics essential to the pathophysiology of type 2 diabetes (T2DM). Epigenetic regulation of gene expression contributes to both adipocyte determination and differentiation in in vitro models. The contributions of histone acetylation, histone methylation, and DNA methylation to the process of adipogenesis in vivo remain to be evaluated.

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Introduction

It is becoming increasingly apparent that the in utero environment in which a fetus grows and develops may have long-term effects on subsequent health and survival (Hales and Barker 1992; Kermack 1934). The landmark cohort study of 300,000 men by Ravelli and colleagues (1976) showed that fetal exposure to the Dutch famine of 1944–1945 during the first half of pregnancy resulted in significantly higher obesity rates at age 19. Subsequent studies demonstrated a relationship between low birth weight and the later development of cardiovascular disease (Barker et al. 1989) and impaired glucose tolerance (Hales et al. 1991; Phipps et al. 1993; Fall et al. 1995) in men in England. Those men who were smallest at birth (2.5 kg) were nearly seven times more likely to have impaired glucose tolerance or type 2 diabetes than were those who were heaviest at birth. The investigators found a similar relationship between lower birth weight and higher systolic blood pressure and triglyceride levels (Barker et al. 1993). Subsequent studies in diverse populations through the world have demonstrated a significant correlation between low birth weight and the later development of type 2 diabetes (Hales and Barker 2001; Valdez et al. 1994; Curhan et al. 1996; Lithell et al. 1996; McKeigue et al. 1998; Leger et al. 1997; Jaquet et al. 2000; Egeland et al. 2000; Forsen et al. 2000; Rich-Edwards et al. 1999). More recent studies controlling for the confounding factors of socioeconomic status and lifestyle factors have further strengthened the association between low birth weight and increased risk of coronary heart disease, stroke, and type 2 diabetes (Curhan et al. 1996; Rich-Edwards et al. 1999). In 1976, the Nurses' Health Study was initiated and a large cohort of U.S. women born between 1921 and 1946 was established. The associations with low birth weight and increased risk of coronary heart disease, stroke, and type 2 diabetes remain strong even after adjusting for lifestyle factors such as smoking, physical activity, occupation, income, dietary habits, and childhood socioeconomic status and occur independently of the current level of obesity or exercise (Rich-Edwards et al. 1999). In a study of 22,000 American men, those born lighter than 5.5 lb had a significantly higher incidence of adult hypertension and type 2 diabetes compared with average birth weight adults (Curhan et al. 1996). Similar to the Nurses' Health Study, the association between birth weight and later disease is largely independent of the lifestyle risk factors (Curhan et al. 1996).

Recent observations have shown that impaired growth in infancy and rapid childhood weight gain exacerbate the effects of impaired prenatal growth. The highest risk for the development of type 2 diabetes is among adults who were born small and become overweight during childhood (Forsen et al. 2000; Eriksson et al. 2000; Bavdekar et al. 1999, 2004).

The mechanisms underlying the association between size at birth and impaired glucose tolerance or type 2 diabetes are unclear. A number of studies in children and adults have shown that non- or pre-diabetic subjects with low birth weight are insulin resistant and thus predisposed to developing type 2 diabetes (Hales and

Barker 2001; Lithell et al. 1996; McKeigue et al. 1998; Leger et al. 1997; Jaquet et al. 2000; Bavdekar et al. 1999, 2004; Hoffman et al. 1997; Li et al. 2001; Yajnik et al. 1995; Clausen et al. 1997; Flanagan et al. 2000; Phillips et al. 1994). Intrauterine growth retardation (IUGR) is known to alter the fetal development of adipose tissue, which is closely linked to the development of insulin resistance (Jaquet et al. 2000; Widdowson et al. 1979; Lapillonne et al. 1997). Other studies have shown that the adverse effect of intrauterine growth retardation on glucose homeostasis was mediated through programming of the fetal endocrine pancreas (Hales and Barker 1992; Van Assche et al. 1977; Jensen et al. 2002). Jensen and colleagues (2002) measured insulin secretion and insulin sensitivity in a well-matched Caucasian population of 19-year-old, glucose-tolerant men with birth weights of either below the 10th percentile (small for gestational age; SGA) or between the 50th and 75th percentile (controls). To eliminate the major confounders, such as "diabetes genes," none of the participants had a family history of diabetes, hypertension, or ischemic heart disease. There was no difference between the groups with regard to current weight, body mass index (BMI), body composition, or lipid profile. When controlled for insulin sensitivity, insulin secretion was reduced by 30 %. However, insulin sensitivity was normal in the SGA subjects. The investigators hypothesized that defects in insulin secretion may precede defects in insulin action and that, once SGA individuals accumulate body fat, they will develop insulin resistance (Jensen et al. 2002).

What Animal Models Can Tell Us

Animal models have a normal genetic background upon which environmental effects during gestation or early postnatal life can be tested for their role in inducing diabetes. For a comprehensive survey of the numerous animal models of fetal growth retardation, the reader is referred to two excellent reviews (Fowden and Forhead 2004; McMillen and Robinson 2005). The most commonly used animal models are caloric or protein restriction, glucocorticoid administration, or induction of uteroplacental insufficiency in the pregnant rodent. In the rat, maternal dietary protein restriction (approximately 40–50 % of normal intake) throughout gestation and lactation has been reported to alter glucose homeostasis and hypertension in the adult offspring (Dahri et al. 1991; Snoeck et al. 1990; Ozanne et al. 1996; Berney et al. 1997; Wilson and Hughes 1997; Burns et al. 1997). Offspring are significantly growth retarded, remain growth retarded throughout life and, in some cases, develop mild β-cell secretory abnormalities (Dahri et al. 1991; Snoeck et al. 1990; Ozanne et al. 1996; Berney et al. 1997; Wilson and Hughes 1997) and, in others, insulin resistance (Ozanne et al. 1996; Burns et al. 1997). Aged rats develop hyperglycemia characterized by defects in insulin signaling in muscle, adipocytes, and liver (Burns et al. 1997; Ozanne et al. 2003, 2005; Petry et al. 2001; Fernandez-Twinn et al. 2005).

Fetal overexposure to glucocorticoids either via maternal administration or by inhibition of placental 11beta-hydroxysteroid Dehydrogenase Type 2 (11ßHSD2) in the rat induces hypertension, glucose intolerance and abnormalities in hypothalamic-pituitary-adrenal (HPA) function after birth (Benediktsson et al. 1993; Lindsay et al. 1996a, b; Niyirenda and Seckl 1998).

To extend these experimental studies of growth retardation, we developed a model of uteroplacental insufficiency (IUGR) induced by bilateral uterine artery ligation at day 18 of gestation (term is 22 days) in the rat that restricts fetal growth (Simmons et al. 2001; Boloker et al. 2002). Growth-retarded fetal rats have critical features of a metabolic profile characteristic of growth-retarded human fetuses: decreased levels of glucose, insulin, insulin-like-growth factor 1 (IGF-I), amino acids, and oxygen (Ogata et al. 1986; Simmons et al. 1991; Unterman et al. 1990). By 6 months of age, IUGR rats develop diabetes with a phenotype remarkably similar to that observed in the human with type 2 diabetes: progressive dysfunction in insulin secretion and insulin action. Thus, the studies in various animal models support the hypothesis that an abnormal intrauterine milieu can induce permanent changes in glucose homeostasis after birth and lead to type 2 diabetes in adulthood.

Cellular Mechanisms: Mitochondrial Dysfunction and Oxidative Stress

The intrauterine environment influences development of the fetus by modifying gene expression in both pluripotential cells and terminally differentiated, poorly replicating cells. The long-range effects on the offspring (into adulthood) depend upon the cells undergoing differentiation, proliferation, and/or functional maturation at the time of the disturbance in maternal fuel economy. The fetus also adapts to an inadequate supply of substrates (such as glucose, amino acids, fatty acids, and oxygen) by metabolic changes, redistribution of blood flow, and changes in the production of the fetal and placental hormones that control growth.

The fetus' immediate metabolic response to placental insufficiency is catabolism: it consumes its own substrates to provide energy. A more prolonged reduction in availability of substrates leads to a slowing in growth, which enhances the fetus' ability to survive by reducing the use of substrates and lowering the metabolic rate. Slowing of growth in late gestation leads to disproportion in organ size, since organs and tissues that are growing rapidly at the time are affected the most.

Uteroplacental insufficiency, caused by such disorders as preeclampsia, maternal smoking and abnormalities of uteroplacental development, is one of the most common causes of fetal growth retardation. The resultant abnormal intrauterine milieu restricts the supply of crucial nutrients to the fetus, thereby limiting fetal growth. Multiple studies have now shown that intrauterine growth retardation is associated with increased oxidative stress in the human fetus (Myatt et al. 1997; Karowicz-Bilinska et al. 2002; Ejima et al. 1999; Kato et al. 1997; Bowen

et al. 2001; Wang and Walsh 1998, 2001). A major consequence of limited nutrient availability is an alteration in the redox state in susceptible fetal tissues, leading to oxidative stress. In particular, low levels of oxygen, evident in growth-retarded fetuses, will decrease the activity of complexes of the electron transport chain, which will generate increased levels of reactive oxygen species (ROS; Esposti and McLennan 1998; Chandel et al. 1996; Gorgias et al. 1996). Overproduction of ROS initiates many oxidative reactions that lead to oxidative damage not only in the mitochondria but also in cellular proteins, lipids, and nucleic acids. Increased ROS levels inactivate the iron-sulfur centers of the electron transport chain complexes and tricarboxylic acid cycle aconitase, resulting in shutdown of mitochondrial energy production.

A key adaptation enabling the fetus to survive in a limited energy environment may be the reprogramming of mitochondrial function (Gorgias et al. 1996; Peterside et al. 2003; Selak et al. 2003). However, these alterations in mitochondrial function can have deleterious effects, especially in cells that have a high energy requirement, such as the β-cell. The β-cell depends upon the normal production of ATP for nutrient-induced insulin secretion (Panten et al. 1984; Newgard and McGarry 1995; Schuit 1997; Mertz et al. 1996; Ortsater et al. 2002; Antinozzi et al. 2002; Malaisse et al. 1980; Lenzen et al. 1986) and proliferation (Noda et al. 2002). Thus, an interruption of mitochondrial function can have profound consequences for the β-cell.

Mitochondrial dysfunction can also lead to increased production of ROS, which will lead to oxidative stress if the defense mechanisms of the cell are overwhelmed. β-cells are especially vulnerable to attacks by ROS because expression of antioxidant enzymes in pancreatic islets is very low (Lenzen et al. 1996; Tiedge et al. 1997), and β-cells have a high oxidative energy requirement. Increased ROS impair glucose-stimulated insulin secretion (Noda et al. 2002; Maechler et al. 1999; Sakai et al. 2003), decrease gene expression of key β-cell genes (Kaneto et al. 1999, 2001, 2002a, b; Jonas et al. 1999, 2001; Efanova et al. 1998), and induce cell death (Moran et al. 2000; Donath et al. 1999; Silva et al. 2000).

We have found that uteroplacental insufficiency induces oxidative stress and marked mitochondrial dysfunction in the fetal β-cell (Simmons et al. 2005). ATP production is impaired and continues to deteriorate with age. The activities of complexes I and III of the electron transport chain progressively decline in IUGR islets. Mitochondrial DNA point mutations accumulate with age and are associated with decreased mtDNA content and reduced expression of mitochondrial-encoded genes in IUGR islets. Mitochondrial dysfunction results in impaired insulin secretion. These results demonstrate that IUGR induces mitochondrial dysfunction in the fetal β-cell, leading to increased production of ROS, which in turn damage mtDNA (Simmons et al. 2005). A self-reinforcing cycle of progressive deterioration in mitochondrial function leads to a corresponding decline in β-cell function. Finally, a threshold in mitochondrial dysfunction and ROS production is reached and diabetes ensues.

Mitochondrial dysfunction is not limited to the β-cell in the IUGR animal. IUGR animals exhibit marked insulin resistance early in life (prior to the onset of

hyperglycemia), characterized by blunted whole body glucose disposal in response to insulin and impaired insulin suppression of hepatic glucose output (Vuguin et al. 2004). Basal hepatic glucose production is also increased (Vuguin et al. 2004). Oxidation rates of pyruvate, glutamate, succinate, and α-ketoglutarate are significantly blunted in isolated hepatic mitochondria from IUGR pups (prior to the onset of diabetes; Peterside et al. 2003). Rotenone-sensitive NADH-O_2 oxidoreductase activity is similar in control and IUGR mitochondria, showing that the defect responsible for decreased pyruvate, glutamate and α-ketoglutarate oxidation in IUGR liver precedes the electron transport chain and involves pyruvate and α-ketoglutarate dehydrogenases. Increased levels of manganese superoxide dismutase (MnSOD) suggest that an antioxidant response has been mounted, and 4-hydroxynonenal (HNE) modification of pyruvate dehydrogenase E2 catalytic and E3 binding protein subunits suggests that HNE-induced inactivation of this key enzyme may play a role in the mechanism of injury. These results indicate that uteroplacental insufficiency impairs mitochondrial oxidative phosphorylation in the liver and this derangement predisposes the IUGR rat to increased hepatic glucose production by suppressing pyruvate oxidation and increasing gluconeogenesis (Peterside et al. 2003).

Mitochondria in muscle of IUGR young adult rats, prior to the onset of hyperglycemia, exhibit significantly decreased rates of state 3 oxygen consumption with pyruvate, glutamate, α-ketoglutarate and succinate (Selak et al. 2003). Decreased pyruvate oxidation in IUGR mitochondria is associated with decreased ATP production, decreased pyruvate dehydrogenase activity and increased expression of pyruvate dehydrogenase kinase 4 (PDK4). Such a defect in IUGR mitochondria leads to a chronic reduction in the supply of ATP available from oxidative phosphorylation. Impaired ATP synthesis in muscle compromises energy-dependent GLUT4 recruitment to the cell surface, glucose transport and glycogen synthesis, which contributes to insulin resistance and hyperglycemia of type 2 diabetes (Selak et al. 2003).

Other animal models of fetal growth retardation also show mitochondrial abnormalities. Mitochondrial DNA content is reduced in liver, pancreas and skeletal muscle of male offspring of dams fed a low-protein diet during pregnancy and lactation (Park et al. 2003, 2004). This was associated with reduced expression of mitochondrial DNA-encoded genes (Park et al. 2003).

A number of recent studies in humans further suggest that mitochondrial dysfunction may contribute to type 2 diabetes. Studies using ^{13}C and ^{31}P magnetic resonance spectroscopy (MRS) have shown decreases in mitochondrial activity and increases in intramyocellular fat content in young insulin-resistant offspring of parents with type 2 diabetes, a group that has a strong tendency to develop diabetes later in life (Petersen et al. 2004). Expression of genes involved in oxidative phosphorylation is reduced among patients with type 2 diabetes mellitus and insulin resistance (Mootha et al. 2003), although this may be an effect rather than a cause of diabetes.

Chromatin Structure, DNA Methylation and Gene Expression

Epigenetic modifications of the genome provide a mechanism that allows the stable propagation of gene expression from one generation of cells to the next. Epigenetic states can be modified by environmental factors, which may contribute to the development of abnormal phenotypes. There are at least two distinct mechanisms through which epigenetic information can be inherited: histone modifications and DNA methylation (Berger 2007; Reik 2007).

In eukaryotes, the nucleosome is formed when DNA is wrapped around an octameric complex of two molecules of each of the four histones: H2A, H2B, H3, and H4. The amino termini of histones can be modified by acetylation, methylation, sumoylation, phosphorylation, glycosylation, and ADP ribosylation. The most common histone modifications involve acetylation and methylation of lysine residues in the amino termini of H3 and H4. Increased acetylation induces transcription activation, whereas decreased acetylation usually induces transcription repression. Methylation of histones, on the other hand, is associated with both transcription repression and activation (Berger 2007; Reik 2007). Moreover, lysine residues can be mono-, di-, or trimethylated in vivo, providing an additional mechanism of regulation (Berger 2007; Reik 2007).

The second class of epigenetic regulation is DNA methylation, in which a cytosine base is modified by a DNA methyltransferase at the C5 position of cytosine, a reaction that is carried out by various members of a single family of enzymes (Reik 2007). Approximately 70 % of CpG dinucleotides in human DNA are constitutively methylated, whereas most of the unmethylated CpGs are located in CpG islands. CpG islands are CG-rich sequences located near coding sequences, and they serve as promoters for their associated genes. Approximately half of mammalian genes have CpG islands (Reik 2007). The methylation status of CpG islands within promoter sequences works as an essential regulatory element by modifying the binding affinity of transcription factors to DNA binding sites. In normal cells, most CpG islands remain unmethylated; however, under circumstances such as cancer (Yoshida et al. 2006; So et al. 2006; Takahashi et al. 2006) and oxidative stress, they can become methylated de novo. This aberrant methylation is accompanied by local changes in histone modification and chromatin structure, such that the CpG island and its embedded promoter take on a repressed conformation that is incompatible with gene transcription. It is not known why particular CpG islands are susceptible to aberrant methylation.

DNA methylation is commonly associated with gene silencing and contributes to X-chromosomal inactivation and genomic imprinting, as well as transcriptional regulation of tissue-specific genes during cellular differentiation (reviewed in Cedar and Bergman 2009; Schübeler et al. 2000; Gopalakrishnan et al. 2008). It is not known why some genes are able to undergo aberrant DNA methylation; however, a study by Feltus et al. (2003) suggests that there is a "DNA sequence signature associated with aberrant methylation." Of major significance to T2D is

their finding that *Pdx1*, a pancreatic homeobox transcription factor, was one of only 15 genes (of 1,749 examined) with CpG islands within the promoter that were methylation-susceptible (which was induced by over-expression of a DNA methyltransferase). This study demonstrates that genes essential to pancreatic development, like *Pdx1*, are susceptible to epigenetic modifications, which could ultimately affect gene expression.

Histone methylation can influence DNA methylation patterns and vice versa (Cedar and Bergman 2009). For example, methylation of lysine 9 on histone 3 (H3) promotes DNA methylation, whereas CpG methylation stimulates methylation of lysine 9 on H3 (Schübeler et al. 2000). Recent evidence indicates that this dual relationship between histone methylation and DNA methylation might be accomplished by direct interactions between histone and DNA methyltransferases (Cedar and Bergman 2009). Thus, chromatin modifications induced by adverse stimuli are self-reinforcing and can propagate.

Epigenetic Regulation of Gene Expression in Fetal Growth Retardation

A number of studies suggest that uteroplacental insufficiency, a common cause of IUGR, induces epigenetic modifications in offspring (MacLennan et al. 2004; Fu et al. 2004; Park et al. 2008; Raychaudhuri et al. 2008). Epigenetic modifications affecting processes important to glucose regulation and insulin secretion, characteristics essential to the pathophysiology of T2D, have been described in the IUGR liver, pancreatic β cells and muscle (MacLennan et al. 2004; Fu et al. 2004; Park et al. 2008; Raychaudhuri et al. 2008).

Chromatin Remodeling in the β-Cell of IUGR Rat

Pdx-1 is a homeodomain-containing transcription factor that plays a critical role in the early development of both the endocrine and exocrine pancreas and in the later differentiation and function of the β-cell. As early as 24 h after the onset of growth retardation, *Pdx1* mRNA levels are reduced by more than 50 % in IUGR fetal rats. Suppression of *Pdx1* expression persists after birth and progressively declines in the IUGR animal, implicating an epigenetic mechanism.

Changes in histone acetylation are the first epigenetic modifications found in β-cells of IUGR animals. Islets isolated from IUGR fetuses show a significant decrease in H3 and H4 acetylation at the proximal promoter of *Pdx1* (Park et al. 2008). These changes in H3 and H4 acetylation are associated with a loss of binding of USF-1 to the proximal promoter of *Pdx1* (105). USF-1 is a critical activator of *Pdx1* transcription, and its decreased binding markedly decreases *Pdx1*

transcription (Qian et al. 1999; Sharma et al. 1996). After birth, histone deacetylation progresses and is followed by a marked decrease in H3K4 trimethylation and a significant increase in dimethylation of H3K9 in IUGR islets (Park et al. 2008). H3K4 trimethylation is usually associated with active gene transcription whereas H3K9 dimethylation is usually a repressive chromatin mark. Progression of these histone modifications parallels the progressive decrease in *Pdx1* expression that manifests as a deterioration in glucose homeostasis and increased oxidative stress in the aging IUGR animals (Park et al. 2008). Nevertheless, at 2 weeks of age, the silencing histone modifications in the IUGR pup are responsible for suppression of Pdx1 expression, since there is no appreciable methylation of CpG islands in mice at this age (Park et al. 2008). Reversal of histone deacetylation in IUGR islets at 2 weeks of age is sufficient to nearly normalize Pdx1 mRNA levels permanently, perhaps due to active β-cell replication present in the neonatal rodent (Park et al. 2008).

In IUGR, *Pdx1* is first silenced due to recruitment of co-repressors, including histone deacetylase 1 (HDAC1) and mSin3A (Park et al. 2008). These repressors catalyze histone deacetylation. Binding of these deacetylases facilitates loss of trimethylation of H3K4, further repressing *Pdx1* expression (Park et al. 2008). We found that inhibition of HDAC activity by trichostatin A (TSA) treatment normalizes H3K4me3 levels at *Pdx1* in IUGR islets (Park et al. 2008). These data suggest that the association of HDAC1 at *Pdx1* in IUGR islets likely serves as a platform for the recruitment of a demethylase, which catalyzes demethylation of H3K4.

The molecular mechanism responsible for DNA methylation in IUGR islets is likely dependent on the methylation status of lysine 9 on H3 (H3K9). Previous studies have shown that changes in methylation of H3K9 precede changes in DNA methylation (Li et al. 2006; Bachman et al. 2003; Kouzarides 2002). It has also been suggested that DNA methyltransferases may act only on chromatin that is methylated at H3K9 (Bachman et al. 2003). Histone methyltransferases specifically DNA methyltransferase 3A (DNMT3A) and DNA methyltransferase 3B (DNMT3B), bind to DNA methylases, thereby initiating DNA methylation (Li et al. 2006).

These results demonstrate that IUGR induces a self-propagating epigenetic cycle in which the mSin3A/HDAC complex is first recruited to the *Pdx1* promoter, histone tails are subjected to deacetylation and *Pdx1* transcription is repressed. At the neonatal stage, this epigenetic process is reversible and may define an important developmental window for therapeutic approaches. However, as dimethylated H3K9 accumulates, DNMT3A is recruited to the promoter and initiates de novo DNA methylation, which locks in the silenced state in the IUGR adult pancreas, resulting in diabetes.

How do these epigenetic events lead to diabetes? Targeted homozygous disruption of *Pdx1* in mice results in pancreatic agenesis, and homozygous mutations yield a similar phenotype in humans (reviewed in Bernardo et al. 2008). Milder reductions in *Pdx1* protein levels, as occurs in the *Pdx*+/- mice, allow for the development of a normal mass of ß cells but result in the impairment of several events in glucose-stimulated insulin secretion (Bernardo et al. 2008). These results

indicate that *Pdx1* plays a critical role in the normal function of ß cells in addition to its role in ß cell lineage development, which may be the reason that humans with heterozygous missense mutations in *Pdx1* exhibit early and late onset forms of T2D (Bernardo et al. 2008).

The discovery of a critical developmental stage during which aberrant epigenetic modifications may be reversed represents a therapeutic window for the use of novel agents that could prevent common diseases with late-onset phenotypes. T2D is one such disease, where predisposed individuals could be treated with agents that normalize the epigenetic programming of key genes, thus providing protection against development of the adult diabetic phenotype.

Genome-Wide DNA Methylation Is Disrupted in IUGR Islets

Epigenetic modifications are not confined to the *Pdx1* locus in the IUGR rat. We mapped DNA methylation across approximately 1,000,000 loci using the HELP assay (Thompson et al. 2010). Comparison of IUGR with normal rats at 7 weeks of age, prior to the onset of diabetes, revealed changes in DNA methylation at a number of novel loci, not limited to canonical CpG islands or promoters. We found that IUGR in the rat caused consistent and non-random changes in cytosine methylation, affecting <1 % of HpaII sites in the genome in the islet. The majority of these changes took place not at promoters but at intergenic sequences, many of which are evolutionarily conserved. Furthermore, some of these loci were in proximity to genes manifesting concordant changes in gene expression and were enriched near genes that regulate processes that are markedly impaired in IUGR islets (e.g., vascularization, proliferation, insulin secretion, and cell death).

Epigenetic Landscape in Human Islets

Recently, Kaestner and colleagues (Bhandare et al. 2010) used chromatin immunoprecipitation with massively parallel sequencing (ChIP-seq) technology to create a genome-wide map of histone modifications associated with gene activation or repression in human pancreatic islets. They mapped the genome-wide enrichment and location of four histone marks: three associated with gene activation—H3K4me1, H3K4me2, and H3K4me3—and one associated with gene repression, H3K27me3. H3K4me1, H3K4me2, and H3K4me3 are frequently found near active gene promoters, whereas H3K4me1 is also often associated with enhancers. Interestingly, there was little enrichment of H3K4me2 and H3K4me3 at the promoters of the highly transcribed insulin and glucagon genes. In contrast, there was robust enrichment of H3K27me3 at the NEUROG3 promoter, a regulator of fetal islet development that is repressed during adult life. They found 16.5 % of the H3K4me3 loci were >5 kbp from the nearest gene, indicating a large number of potentially

novel transcriptional start sites active in islets. A larger fraction of H3K4me1 (24.8 %) and H3K4me2 (24.3 %) loci was intergenic and may represent potential regulatory regions. The insulin and nearby genes in an extended 80-kb region are a part of a large, human islet-specific, open chromatin domain and share a common control mechanism. The presence of intergenic transcription in this region has been proposed to play a role in the maintenance of open chromatin structure, suggesting that a locus-specific control mechanism might be responsible for constitutive insulin gene expression in humans. The data in the Kaestner study also indicate a region (chr1:2,100,000–2,200,000 mm^8) of high levels of H3K4me1, a mark associated with regulatory regions covering the insulin gene locus. Thus the pattern of histone modifications in the islet is complex.

The epigenetic landscape in the human β-cell also appears to be markedly altered. Fuks and colleagues (Volkmar et al. 2012) carried out a comprehensive DNA methylation profiling of human T2D pancreatic islets using the Infinium 27 k Methylation Assay. This assay interrogates the methylation status of 27,578 CpG sites corresponding to 14,475 consensus coding sequences and well-known cancer genes. They identified 276 differentially methylated CpG sites that were affiliated with 254 genes. Interestingly, they found predominantly promoter hypomethylation in T2D islets that was frequently associated with increased gene expression. However, for a significant proportion of differentially methylated genes, there was no significant differential expression. Thus, for many genes the link between differential methylation and gene activity is complex. Of major importance was the finding that the methylation changes were not present in blood cells from T2D individuals; neither were they experimentally induced in non-diabetic islets by exposure to high glucose, further underscoring the cell-specificity of DNA methylation patterns.

Summary

The combined epidemiological, clinical, and animal studies clearly demonstrate that the intrauterine environment influences both growth and development of the fetus and the subsequent development of adult diseases. There are critical, specific windows during development, often coincident with periods of rapid cell division, during which a stimulus or insult may have long-lasting consequences on tissue or organ function postnatally. Birthweight is only one marker of an adverse fetal environment, and confining studies to this population only may lead to erroneous conclusions regarding etiology. Studies using animal models of uteroplacental insufficiency suggest that mitochondrial dysfunction and oxidative stress play an important role in the pathogenesis of the fetal origins of adult disease. Environmental effects can induce epigenetic alterations, ultimately affecting expression of key genes linked to the development of T2D, including genes critical for pancreatic development and β-cell function, peripheral glucose uptake and insulin resistance and atherosclerosis. Understanding the role of developmental programming of

genes crucial to the development of T2D may unveil a critical window during which epigenetic therapeutic agents could be used as a means to prevent the later development of a disease. Prior to the use of such therapeutic agents there remains much to be learned about the programming of the epigenetic code, especially on a genome-wide scale. Much of the recent progress in understanding epigenetic phenomena is directly attributable to technologies that allow researchers to pinpoint the genomic location of proteins that package and regulate access to the DNA. The advent of DNA microarrays and inexpensive DNA sequencing has allowed many of those technologies to be applied to the whole genome. It is now possible that epigenetic profiling of CpG islands in the human genome can be used as a tool to identify genomic loci that are susceptible to DNA methylation. Aberrant methylation may then be used as a biomarker for disease. The genome-wide mapping of histone modifications by ChIP-chip and ChIP-seq has led to important insights regarding the mechanism of transcriptional and epigenetic memory and how different chromatin states are propagated through the genome in yeast and in mammalian cells (Lieb et al. 2006; Kim et al. 2005). Although Bisulfite-seq (anlaysis of genome-wide DNA methylation) and Chip-Seq (analysis of genome-wdie histone modifcations) experiments are currently being performed in human tissue, obstacles such as intrinsic human epigenetic variability (including age-related changes) and tissue-specific epigenetic variability must be characterized and mapped in the healthy, non-diseased state before this information can be applied to diseases such as T2D. Eventually genome-wide epigenetic characterization will lead to specific therapies with epigenetic targets and also will allow monitoring of genome-wide epigenetic consequences of these therapies once they are applied.

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Genetic and Developmental Origins of Food Preferences and Obesity Risk: The Role of Dopamine

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Abstract Fetal growth and development associates with poor lifetime health outcomes. Despite the strength of the epidemiological evidence, there is little research that describes the functional pathways linking fetal development to brain-based disorders and metabolic health. We used a longitudinal cohort (Maternal Adversity, Vulnerability and Neurodevelopment; MAVAN) to study children of mothers recruited at mid-gestation and examine neurodevelopmental outcomes focusing on the association between birth weight and phenotypes associated with attention deficit disorder and obesity. These studies provide preliminary support for a 'thrifty' eating hypothesis that emphasizes the potential adaptive value of altered appetite regulation in the face of predicted nutritional deprivation, with impaired fetal growth as a marker for the in utero states that would produce such a prediction. We suggest that the effects might be mediated by altered activity across the mesocorticolimbic dopamine.

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Large retrospective, epidemiological studies accumulated in the late 1980s, providing increasing evidence to the deeply rooted thought that perinatal events can persistently affect an individual's functioning and health/disease patterns throughout his lifetime. Evidence of such associations can be found in the literature since the beginning of the twentieth century (e.g., Kermack et al. 1934; Pasamanick and Lilienfeld 1955), but studies from Barker, Hales and colleagues serve as an important hallmark. These authors proposed that the quality of fetal growth and development, as reflected in measures such as birth weight for gestational age, predicts the risk for obesity and related metabolic/cardiovascular illnesses (Hales and Barker 1992, 2001). Birth outcome measures are proxy measures that reflect the quality of the maternal/fetal environment and are thus limited in the degree to which they inform on causal pathways. Nevertheless, the statistical association between birth weight and health has been established in a wide range of epidemiological reports around the world (Gluckman et al. 2008). Some studies suggest that 25–63 % of adult diabetes, hypertension and coronary heart disease could be attributed to the effects of low birth weight if they are accompanied by rapid weight gain during early development (Barker et al. 2002).

Birth weight also predicts a range of pre-clinical states that promote metabolic/cardiovascular disease, including increased hypothalamic-pituitary-adrenal (HPA) activity, hyperlipidemia and insulin resistance. Such states favor energy storage and mobilization and are thus considered as potentially adaptive under conditions of adversity such as nutritional deprivation or chronic stress, both of which present severe metabolic demands for the organism (Hales and Barker 2001; Gluckman and Hanson 2007; Bateson et al. 2004). Many conditions are associated with impaired fetal growth, such as gestational malnutrition, chronic maternal diseases leading to placental insufficiency, maternal smoking during gestation, etc. Intra-uterine growth restriction occurs in response to endocrine signals (Goland et al. 1993; Meaney et al. 2007; Chapman et al. 2013), notably increased levels of the highly catabolic glucocorticoids, that both restrain fetal growth and stably alter the expression of genes in liver, muscle and fat tissues that regulate energy balance (Meaney et al. 2007). The net effect for metabolism is an enhanced capacity to mobilize energy substrates (gluconeogenesis, lipolysis), an increased ability for energy storage, especially lipids, and decreased energy expenditure. The 'thrifty' phenotype hypothesis suggests that these effects are adaptive. Across human history fetal growth restriction has been accompanied by nutritional deprivation. Since humans, until recently, were likely to live under the same environmental conditions within which they born, phenotypic alterations that favored a positive energy balance could be adaptive under recurring conditions of nutrient shortfalls. Gluckman and Hanson (2007) termed such effects "predictive adaptive responses." The risk for obesity and metabolic disease emerge among 'mismatched' individuals expressing the thrifty phenotype but living in calorically enriched environments.

Research on the developmental origins of obesity and metabolic disease focuses on the relation between fetal growth restriction and peripheral metabolism. Likewise, research at the level of mechanism has linked increased exposure to glucocorticoids during fetal life to patterns of gene expression in liver, muscle and fat that

favor a positive energy balance (Meaney et al. 2007; Chapman et al. 2013). However, glucocorticoids and other metabolic signals (e.g., leptin, insulin, GH/IGF-1) also exert potent effects on neural systems during fetal development, especially within corticolimbic structures such as the nucleus accumbens, hippocampus, prefrontal cortex and amygdala that regulate attentional systems, affect regulation, impulse control and addictive behaviors. These same neural systems are important mediators of metabolic states that predispose to obesity through direct effects on appetite and feeding behavior, as well as indirectly through the regulation of counter-regulatory systems such as the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. This finding raises the intriguing possibility that at least part of the association between fetal adversity and later obesity and diabetes (thrifty phenotype) is mediated via central mechanisms and increased eating behavior rather than metabolic changes on their own. Nevertheless, to our knowledge, effects at the level of neural development and function have never been fully considered within the context of fetal growth and metabolic health.

MAVAN Research Program

Here we review a series of recent findings from the Maternal Adversity, Vulnerability and Neurodevelopment study (MAVAN; O'Donnell et al. 2014, submitted), a longitudinal birth cohort study that implicates the link between birth weight and brain-based phenotypes in the study of the prenatal origins of metabolic dysfunction. MAVAN permits an internationally unique opportunity to study the developmental origins of individual differences in neural and metabolic function. The study sample is comprised of children and their mothers from the cities of Montreal, Quebec, and Hamilton, in Ontario, Canada. Eligibility criteria for mothers included age $\geq$18 years, singleton pregnancy, and fluency in French or English. Mothers were excluded from the study if they had severe chronic illness, placenta previa, a history of incompetent cervix, or impending delivery, or had a fetus/infant born at gestational age $<$35 weeks or born with a major anomaly. Birth records were obtained directly from the birthing unit. The sample was not recruited with respect to any particular risk or outcome and can best be considered as a community sample.

The MAVAN project examines the relation between fetal growth, neural development and metabolic health by examining mechanisms that link key aspects of executive dysfunction (i.e., impulsivity, negative affectivity and sensitivity to reward) with overeating and increased body weight. These processes are highly relevant to both neurodevelopment and specific developmental disorders (e.g., Attention Deficit Disorder, ADD) and obesity and might explain the high co-morbidity between these two syndromes (Cortese et al. 2008). Furthermore, primary risk factors for ADD include maternal stress, tobacco and alcohol intake in pregnancy, and maternal undernutrition, all of which are risk factors for fetal growth restriction. Birth weight also strongly predicts the risk for ADD (Breslau

et al. 1988; also see O'Callaghan and Harvey 1997; Botting et al. 1997; Mick et al. 2002; Sasaluxnanon and Kaewpornsawan 2005; van den Bergh et al. 2006) as well as disorders associated with emotional regulation (Costello et al. 2007). Importantly, the link between birth weight and behavioral disorders cuts across the range of normal birth weights and is not unique to individuals born at extreme weights. While ADD entails more extreme deficits in impulsivity, affect dysregulation and reward sensitivity, our studies are not limited to categorical diagnosis; rather, we study links between overeating, obesity and executive dysfunction/reward sensitivity using a more dimensional approach.

Our general working hypothesis is that, among developing children, interactions between fetal adversity (as reflected by low birth weights) and hypofunctional dopamine gene variants will alter dopamine activity within the mesocorticolimbic system in a manner that promotes impulsive eating and increased caloric intake, and specifically an increase in calorically dense, highly palatable foods. This interactive influence will lead to a variety of cognitive/emotional changes that we will broadly refer to as "executive dysfunction," manifested behaviorally as impulsivity, affect dysregulation and increased sensitivity to reward. These behaviors will increase vulnerability to overeating and weight gain over time, due to increased meal size and rate of intake (unrestrained eating), use of food to reverse negative mood states (emotional eating), and greater intake of highly palatable, high caloric foods that stimulate the brain's reward areas. Importantly, the critical feature of our research agenda is the hypothesis that the relation between executive dysfunction, feeding behavior and metabolic health *will cut across the entire population and thus explain*, *in part*, *individual differences in the risk for obesity and metabolic disorders*.

The increased prevalence of obesity in children continues unabated despite significant public health initiatives. One possible explanation is that, in an ample food environment, processes tied to food reward simply overwhelm current medical and behavioral approaches to weight gain (Mietus-Snyder et al. 2008). Children are particularly vulnerable, given their wide exposure to food reward stimuli (television advertisements) and the basic impulsivity of an undeveloped cognitive capacity for affect regulation and impulse control. However, not all children exposed to an obesogenic environment overeat and become overweight. We propose that individual differences in food reward processes and impulsivity, mediated by variations in the development and function of central dopamine systems, determine such individual differences. Our objective here is not simply to define the existence of the relevant co-morbid conditions but to examine the mechanisms that link executive function to feeding behavior and metabolic health and to examine the importance for such relations across the population.

Co-morbidity Studies of Obesity and ADD

A systematic review of clinic-based studies of obesity/ADD co-morbidity (Cortese et al. 2008) concluded (1) that clinically obese adults present with an increased rate of verified ADHD, with most studies reporting an impressive—four- to nine-fold increase in ADHD compared with non-obese controls, and (2) that ADHD populations show significantly increased body mass index (BMI) compared with controls across various age groups. Studies based on community samples support these associations. In a sample of 6,735 U.S. residents in the Collaborative Psychiatric Epidemiology Surveys, Pagoto et al. (2009) found higher rates of overweight and obesity in adult ADD probands than in non-ADD participants. Mediation analyses suggest that binge eating disorder mediated these associations, underscoring the potential relevance of impulsivity. Using data from a large cross-sectional analysis of over 60,000 children and adolescents in the National Survey of Children's Health, Lapane and Waring (2008) found that unmedicated probands with ADD had a 50 % greater risk of being overweight than did child and adolescent controls. These authors concluded that longitudinal studies were needed to better understand this association and, we would suggest, its relevance for obesity across the population as a whole.

The evidence for obesity/ADD co-morbidity raises the question of underlying mechanisms. Inattention, memory problems and impulsivity are more strongly associated with obesity than is hyperactivity per se, suggesting that cognitive/emotional rather than motoric processes are key. Based on structured equation modeling, Davis et al. (2007a) concluded that ADD symptoms relate to several aspects of overeating such as eating in response to negative mood, eating in response to external cues, and binge eating, which in turn correlates with a higher BMI. Deficient inhibitory control, delay aversion and self-medication appear critical. One or more deficits in central dopamine function merit strong consideration as mediators (e.g., Levitan et al. 2004a, b; Davis et al. 2007b; Campbell and Eisenberg 2007; Pagoto et al. 2009). Thus, dopamine genomic variants associated with ADD, such as the 7-repeat allele of the dopamine-4 receptor gene, link with obesity (Levitan et al. 2004a, b, 2006; Kaplan et al. 2008). One such study showed a relation between a hypofunctional dopamine variant, childhood inattention and adult obesity in the same individuals (Levitan et al. 2004a, b).

Obesity and ADD also share a common developmental origin related to fetal adversity. The probability of obesity and metabolic illness is influenced by fetal development. Individuals born small for gestational age are at significantly greater risk for obesity, type II diabetes and hypertension. Obesity is critical as low birth weight without increased BMI carries little increase in the risk for metabolic disorders (Barker et al. 2002). The major predictors of fetal growth restriction are maternal stress, infections, malnutrition either in the form of protein deprivation or caloric restriction, and tobacco/alcohol consumption. Strikingly, these same factors also predict ADD, as supported by the strong relationship between birth weight and ADD risk (Breslau et al. 1988; also see O'Callaghan and Harvey 1997; Botting

et al. 1997). Case-control studies reveal a significantly lower birth weight among individuals with ADD (Mick et al. 2002). The relation between the risk for ADD and birth weight is not limited to individuals born at very low birth weights (McCormick et al. 1990, 1992); there is a significantly increased risk even among children born with moderately low birth weight (Breslau and Chilcoat 2000; Indredavik et al. 2005; McCormick et al. 1990, 1992). Thus, the relation between fetal growth and the risk for ADD is apparent across the normal distribution of birth weights. While studies examining birth outcome and ADD commonly report only global ratings, there is evidence that attentional systems per se are significantly disrupted in children born at low birth weight (Glover and O'Connor 2002). Thus, intrauterine growth restriction (IUGR) children are impaired on tests of attention and more impulsive than controls (van der Reijden-Lakeman et al. 1997). In sum, there is a strong rationale to hypothesize that, in addition to a common genetic diathesis, ADD and obesity share a common environmental origin related to the effects of maternal-fetal stress on the developing brain.

Dopamine and Feeding

There are two major brain dopamine pathways: (1) the mesocorticolimbic pathway from the ventral tegmental area (VTA) to the limbic system (amygdala, hippocampus and nucleus accumbens (NAc)) and the prefrontal cortex and (2) the mesostriatal pathway from the substantia nigra to the striatum. Dopamine responses define the salience or incentive value of a stimulus (Berridge 2007) and shape cognitive processing and behavior directed towards more salient stimuli. Drugs of abuse activate the central dopamine pathways, as do stimuli associated with food (Wise 2006). Thus, VTA-NAc circuits appear to be crucial in promoting the intake of palatable foods as well as regulating meal duration (Meguid et al. 2000; Saper et al. 2002; Shimura et al. 2002; Palmiter 2007). Sweet and fatty foods potentiate dopamine release, induce more pleasurable subjective feelings, and are more rewarding (e.g., Grigson 2002; Martel and Fantino 1996). Almost any mammal, including humans, will eat beyond homeostatic caloric needs if presented with highly palatable food (Saper et al. 2002).

An important contributor to increased food intake seems to be an altered sensitivity to the rewarding aspects of food, a phenomenon observed in both adults (Appelhans et al. 2011; Davis et al. 2008) and children (van den Berg et al. 2011; Verbeken et al. 2012). Both animal and human studies suggest that alterations in brain dopamine activity and/or receptor sensitivity play a role in food reward processes and eating behavior (Grigson 2002; Saper et al. 2002; Stice et al. 2008). Food-related cues activate the brain areas involved in the synthesis and release of dopamine or that are targets for dopamine projections (Killgore et al. 2003; Rothemund et al. 2007). Activity in these areas is proportional to the subjective pleasure associated with food (Demos et al. 2012; Small et al. 2003). Sweet and fatty foods potentiate a greater release of dopamine, inducing more

pleasurable subjective feelings than their less palatable counterparts (Grigson 2002; Martel and Fantino 1996). Functional magnetic resonance imaging in response to imagined intake of palatable foods shows that future increases in body mass can be predicted by weaker brain activation of specific brain areas, particularly in individuals carrying low functioning variants of dopamine receptor genes, such as the DRD4-7R allele (Stice et al. 2008, 2010).

The neuropeptides that regulate energy intake and expenditure (homeostatic processes) through the hypothalamus also modulate the activity of dopamine cells and their projections into regions involved in the rewarding processes underlying food intake. This could be a mechanism by which overeating and the resultant resistance to homoeostatic signals impairs the function of circuits involved in reward sensitivity, conditioning and cognitive control. Evidence suggests that dopaminergic neuronal activity in the VTA that projects to the NAc can be modulated by peripheral energy status signals including leptin, insulin and ghrelin (Figlewicz 2003; Abizaid et al. 2006; Fulton et al. 2006; Hommel et al. 2006; Jerlhag et al. 2006; Abizaid 2009; DiLeone 2009; Figlewicz and Sipols 2010; Narayanan et al. 2010; Opland et al. 2010; Perry et al. 2010; Davis et al. 2011; Domingos et al. 2011; Trinko et al. 2011; Dunn et al. 2012; Mebel et al. 2012; Overduin et al. 2012; Labouebe et al. 2013; Thompson and Borgland 2013), and both leptin and insulin are associated with a decrease in the response of the NAc to food cues (Farooqi et al. 2007; Figlewicz et al. 2008).

Glucocorticoids also modulate feeding behavior and food intake. The effects of the acute activation of the hypothalamic-pituitary-adrenal (HPA) axis through stress exposure on food consumption are well known, increasing the intake of highly palatable foods in humans and animals (Oliver et al. 2000; Wardle et al. 2000; Epel et al. 2001; la Fleur et al. 2005; Foster et al. 2009), even in the absence of hunger (Foster et al. 2009; Rutters et al. 2009; Gibson 2012). Emotional or stress-induced food consumption seems to be characterized by impulsivity and altered reward sensitivity, associated with dopamine dysregulation underlying incentive salience (Gibson 2012).

Chronic stress and repeated food restraint seem to have independent and possibly synergistic effects on increasing the reward value of highly palatable foods (Adam and Epel 2007). When given the option (la Fleur et al. 2005; Foster et al. 2009), individuals will favor foods with high fat and/or sugar content—so-called "comfort foods"—during times of exposure to stress (Dallman et al. 2005). Elevated HPA axis activation, palatable food intake and the consequent accretion of abdominal fat may serve as feedback signals that reduce perceived stress (Pecoraro et al. 2006), thus reinforcing the stress-induced food intake.

Animal research demonstrates the existence of stress-induced functional changes within the prefrontal cortex (PFC), such as behavioral flexibility (Cerqueira et al. 2007; Bondi et al. 2008), working memory, and the recall of conditioned fear extinction (Miracle et al. 2006; Garcia et al. 2008; Baran et al. 2009). Therefore, persistence of inappropriate behaviors such as increased food consumption could happen during exposure to chronic stress, as a result of stress-induced structural changes within the PFC.

The Thrifty Eating Hypothesis

The thrifty phenotype hypothesis of Hales and Barker (2001) proposes that low birth weight and associated long-term insulin resistance are adaptive if food supplies are scarce and likely to remain so over time. If the prediction proves inaccurate and food supplies become abundant (i.e., a mis-match; Gluckman and Hanson 2007), the thrifty phenotype becomes a risk factor for obesity, diabetes, and cardiovascular disease. Although the thrifty phenotype hypothesis focuses on programming of metabolism during fetal development, it is entirely plausible that obesogenic patterns of eating behavior per se are also programmed by early fetal adversity and low birth weights. In support of this hypothesis, we found that young adult women who experienced IUGR prefer carbohydrates over protein and have larger waist-to-hip ratios even in the absence of insulin resistance or diabetes mellitus (Barbieri et al. 2009). Other research groups report that there are specific food preferences among individuals exposed to nutritional shortage during gestation, manifesting as intake of high-fat diets (Lussana et al. 2008) and higher energy intake (Stein et al. 2009) in late middle age. Similarly, an investigation into individuals from the Helsinki Birth Cohort Study aged 56–70 showed that, as birth weight and/or ponderal index at birth decreased, the intake of fruits and berries also decreased, whereas the percent of intake from fat increased (Perälä et al. 2012). Another study comparing young adults aged 19–27 years who were born at very low birth weight (VLBW) to term-born controls demonstrated that VLBW subjects had lower mean daily intake of vegetables, fruits, berries and milk products, resulting in a lower daily intake of calcium, vitamin D and cholesterol (Kaseva et al. 2013).

Eating habits associate with obesity, diabetes, and cardiovascular disease. Therefore, in individuals who had experienced IUGR, the persistence of small energy imbalances across the life span could explain, at least in part, the increased risk of developing metabolic diseases in later life.

Although the finding of a link between IUGR in infancy and food preferences in adulthood is of great interest, the direction of causality in this relationship is unclear. It may be that IUGR triggers adaptive metabolic changes that secondarily influence eating behavior over time. One way to test the hypothesis that IUGR in itself programs eating behavior, independent of metabolism, is to study this putative phenomenon very early in life, before metabolic changes are likely to manifest. Our studies test a novel variation of the thrifty phenotype hypothesis that we refer to as the "thrifty eating hypothesis." We propose that fetal adversity establishes sustained changes in brain mechanisms that regulate eating behavior. Such changes might be highly adaptive when food supplies are low. However, as with the original thrifty hypothesis, these behaviors become a risk factor for obesity, diabetes, and the metabolic syndrome in a food-abundant environment. Impulsive eating is an excellent example of a behavior that would be adaptive when food supplies are scarce but highly obesogenic in an environment with abundant availability of high caloric foods. Interestingly, impulsivity is a core feature of ADD, which associates

with IUGR. We therefore investigated the possible association between IUGR and impulsive eating, using a snack delay task in 3-year-old Canadian children. We proposed that IUGR would associate with impulsive response toward a palatable snack at the age of 36 months. Our second hypothesis was that impulsive eating at 36 months would predict the quantum of intake of palatable fat and/or higher BMI at the age of 48 months. In support of the thrifty eating hypothesis, we found that, among 3-year-old children with normal birth weights, girls showed a greater ability to delay food rewards than boys; in contrast, among children with IUGR, there was no such differential ability between girls and boys. IUGR increases impulsivity in girls to levels normally seen in boys. In addition, in these girls, impulsive responding towards a sweet reward predicted both increased consumption of palatable fat and higher BMI at 48 months of age. These findings suggest that, in girls, the quality of fetal growth may contribute to impulsive eating, which may promote an increased intake of fats and consequently higher BMIs.

These findings are consistent with the results of studies in adults reviewed above (Barbieri et al. 2009; Lussana et al. 2008; Stein et al. 2009; Perälä et al. 2012; Kaseva et al. 2013). However, the results of these prior studies could be attributable to secondary effects of IUGR-induced metabolic changes on food choices. Given the young age of our study probands, our study suggests that IUGR is associated with obesogenic changes in eating behavior and that these changes are unlikely to be secondary to metabolic effects. Indeed, our study results raise the possibility that, in many cases, high-risk eating behavior may promote the metabolic changes previously attributed directly to IUGR.

Another piece of very compelling evidence for the fetal programming of food preferences comes from our study performed in 1-day-old preterm infants who received 24 % sucrose solution or water for evaluation of taste reactivity. Considering that the affective pattern of taste reactivity components reflects palatability or sensitivity to the hedonic signaling (i.e., pleasure) associated with the ingestion of a palatable food (Berridge 2000), we showed a highly positive correlation between fetal growth and the hedonic response to the sweet solution but not to water (Ayres et al. 2012).

Our study suggests that an increase in impulsive eating may link IUGR and food preferences in girls. Neuroimaging studies show that goal-directed decision making relies on functional interactions between the PFC and the NAc and VTA; the intensity of this functional interaction correlates with inter-individual differences in trait impulsivity (Diekhof and Gruber 2010). Impulsive personality traits are also related to executive functioning (Dolan et al. 2008). It is interesting to note that IUGR infants show poorer executive functioning (Geva et al. 2006; Leitner et al. 2007) and increased vulnerability to addictive disorders (Franzek et al. 2008) and to ADHD (Heinonen et al. 2010), which, as discussed above, is linked to obesity.

Dopamine System Genes, Food Reward and Obesity

Associations between dopamine system genes and overeating and weight gain are emerging in adults, including in several papers by our group. Most, though not all, of these studies suggest that hypo-functional dopamine gene variants are most strongly associated with overeating and obesity. Nonetheless, the possibility that hyperfunctional variants contribute to eating and weight gain in some cases, perhaps through alternate pathways, cannot be ruled out at this time. Indeed, there is an inverted U relation between dopamine levels in the PFC and attentional performance (Arnsten 2001). Of particular interest for our studies is the finding that the hypofunctional 7-repeat allele of the dopamine-4 receptor gene (DRD4) is associated with obesity. The exon III 7 repeat allele (7R) of DRD4 is associated with markedly decreased affinity for dopamine and impaired intracellular signaling in comparison to other exon III alleles (Asghari et al. 1995). Pharmacological evidence implicates the DRD4 gene in eating regulation. For example, clozapine, which binds with high affinity to DRD4, can lead to increased food consumption and weight gain (Van Tol et al. 1991). As early as 1998, Poston et al. (1998) assessed the association between the long alleles of the D4DR and obesity. Studying 115 obese subjects, they found a significant increase in the frequency of the D4DR long alleles in individuals defined as high risk using the combination of novelty-seeking-related personality traits, severe obesity (i.e., BMI $>$ 40), and any other traditional risk factor (i.e., long-term history of obesity, parental obesity, a body mass index $>$ 40), suggesting a role for the D4DR gene variation in increasing obesity susceptibility (Poston et al. 1998). This polymorphism associates strongly with ADD (Faraone et al. 2001), predicts dysphoria, binge eating and obesity in women with seasonal affective disorder (Levitan et al. 2004a, b, 2006). A specific link between the 7R allele, childhood inattention and adult obesity occurs in the same individuals (Levitan et al. 2004a, b). DRD4 7-repeat carriers also reported significantly more craving for food in a cue-elicited food-craving test (Sobik et al. 2005).

Providing further evidence that the hypofunctional 7R allele of DRD4 contributes to weight gain in women, we found that the hypofunctional 7R allele of DRD4 contributes to maximal lifetime BMI in these women with bulimia nervosa (Kaplan et al. 2008; Levitan et al. 2010). The presence of either the 2-repeat or 7-repeat allele of the DRD4 polymorphism is associated with a history of childhood ADHD in bulimia patients, suggesting that impulsivity and/or inattention (behavioral traits classically linked to the risk alleles of the DRD4 gene) may be associated with the development of bulimia in ADHD children.

Given our prior findings in female overeater populations and significant evidence for sex differences in eating behavior and obesity (Cooke and Wardle 2005; Galloway 2007), as well as for brain reward processes (Adinoff et al. 2003; Hurd et al. 1999), we hypothesized that different relationships between the 7R allele and eating behavior would emerge in girls and boys, i.e., that only girls carrying the 7R allele would exhibit a preference for highly palatable, highly caloric foods (rich in

fat and/or sugar). We examined this hypothesis in the MAVAN cohort and found significant sex by genotype interactions for fat and protein intake during the snack test. Post hoc testing revealed that in girls, but not boys, 7R carriers ate more fat and protein than did non-carriers. Based on the food diaries, across both genders, the 7R carriers consumed more portions of ice cream and less vegetables, eggs, nuts and whole bread, suggesting a less healthy pattern of habitual food consumption.

There are several potential mechanisms that could explain a link between the 7R allele and increased food intake (Kaplan et al. 2008; Levitan et al. 2004a, b, 2006), including a possible role for DRD4 signaling in satiety processes mediated at the level of the hypothalamus (Huang et al. 2005). The DRD4 receptor is predominantly localized in areas that are innervated by meso-cortical projections from the VTA, including the PFC, cingulate gyrus, and insula (Schoots and van Tol 2003). Studies have linked the 7-repeat allele to reduced dopamine functioning (Asghari et al. 1995), suggesting that it may affect reward sensitivity. Food per se is a potent activator of the brain's reward circuitry (Grigson 2002; Holden 2001; Volkow and Wise 2005). Understanding the genetic basis of food intake and food preferences in school-aged children and adolescents is extraordinarily difficult due to the major confounding effects of hormonal changes, body image concerns, and dieting (Hill 2002). As the current sample was studied at just 48 months of age, our results are likely to be independent of these factors, and they provide an excellent starting point to understand genetic contributions to eating behavior over the life span. Future studies with the MAVAN cohort will permit moderator analyses to examine the degree to which specific dopamine-related phenotypes such as impulse control and reward sensitivity contribute to individual differences in eating behaviors and to the risk for obesity.

Conclusion

These studies provide preliminary support for a 'thrifty' eating hypothesis that emphasizes the potential adaptive value of altered appetite regulation in the face of predicted nutritional deprivation, with impaired fetal growth as a marker for the in utero states that would produce such a prediction. We suggest that these effects might be mediated by altered activity across the mesocorticolimbic dopamine system. Likewise, we suggest that heritable, sequence-based genetic variants that influence dopamine function would render individuals more susceptible to the effects of fetal growth impairment on subsequent appetite dysregulation. The data for the *DRD4* gene are consistent with this idea. Finally, the coordinated influence of fetal growth and dopamine-regulating genes on the development of executive functions and appetite provide a basis for the observed co-morbidities between ADD-like states and obesity. Future research in MAVAN and other studies must address the critical issue of the degree to which altered executive function mediates the changes in appetite. This is a clinically relevant issue since executive functions

in early child are subject to intervention and might thus dampen the risk for overeating and obesity.

An interesting feature of the data from the human cohort studies is that of gender-dependent effects. This finding is not unique to the MAVAN studies or even to studies with human subjects. Studies in rodents and humans often reveal a greater effect in females of fetal growth restriction or, in the case of experimental studies, of conditions that produce fetal growth restriction. Gluckman and Hanson (2007) have noted the importance of fetal development for reproductive development as well as metabolic outcomes. In utero, developmental programming in mammals may imply greater consequences for the female, who bears the metabolic cost of reproduction.

In summary, the studies presented here as well as others in rodents (e.g., Vickers et al. 2000; Gluckman et al. 2007) suggest that neural regulation of appetite is a target for the metabolic programming that accompanies fetal growth restriction. Future studies in humans should elaborate on not only the issues associated with neural regulation of energy intake but also of energy expenditure. It is interesting that the mesocorticolimbic system is implicated not only in appetite regulation but also in a wide variety of motivational states that might associated with sustained changes in energy expenditure.

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Developmental Epigenetics and Risks of Later Non-communicable Disease

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Abstract Non-communicable diseases (NCDs) are becoming more prevalent globally than communicable diseases and until recently there was little international attention to the need to address them. The rising incidence of NCDs is of particular concern in low and middle income countries as they benefit from socioeconomic improvement that is exacerbated by demographic changes, including changes in population age structure. Initiatives to curb the rise in NCDs have often focused on overweight and obesity, which increase risk substantially. However, the focus has largely been on individual responsibility for lifestyle choices and this approach has been met with limited success. However, predisposition to such conditions is partly set in early development, when parental diet, lifestyle and other factors influence offspring's metabolism, appetite and other physiological control systems. We are now discovering how epigenetic processes that mediate developmental plasticity influence offspring phenotype without affecting genotype. This awareness will allow the early identification of individuals at greater risk of NCDs later and will encourage the introduction and monitoring of preventative

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interventions. We present a life-course perspective on NCD risk, stressing transgenerational transmission of NCD risk and arguing that effective interventions should focus on adolescent/young adult lifestyle and health literacy for the challenge of NCDs in the next generation to be met effectively.

Introduction

Non-communicable diseases (NCDs), in particular diabetes, cardiovascular disease, chronic lung disease and some forms of cancer, are the world's biggest killers. They account for 36 million deaths per year, that is, 63 % of all deaths globally (Alwan 2008). It is estimated that someone dies of a diabetes-related illness every 7 s. Eighty percent of the deaths from NCDs occur in low and middle income countries (LMICs), particularly as such countries undergo socio-economic improvement following reductions in communicable disease. The risks of NCDs are exacerbated by Western lifestyle, urbanisation, migration and other factors associated with greater economic prosperity. In addition, demographic patterns of increased longevity and smaller family size, coupled with falling postnatal mortality, are providing a shift in the age distribution of many populations (including, in time, that of LMICs) towards middle age and, thus, the onset of NCDs. WHO projects an increase of 15 % in NCDs over the next decade globally.

Among the NCDs, diabetes is of particular concern. Over 350 million people in Asia alone now suffer from NCDs, and over 100 million in China have type II diabetes, although this may be an underestimate. Obesity and overweight are major risk factors for diabetes and these are increasing at an alarming rate in Western countries but also in the Middle East and sub-Saharan Africa (Wang and Lobstein 2006). Of particular concern is that obesity and overweight are occurring at younger ages in many LMICs, which will produce substantial costs in terms of economic productivity as well as humanitarian cost and will place a substantial burden on many health systems. Taking the likelihood and severity of such global trends in NCD incidents into account, the World Economic Forum (2011) calculates that the fiscal consequences of NCDs outweigh those of communicable disease and are, with climate change, one of the major barriers to economic development.

Childhood obesity has detrimental consequences on virtually every organ and system in the body in terms of structure and function (Han et al. 2010). Tackling this problem is therefore of great importance (Gluckman et al. 2011).

Until recently, approaches to the reduction or prevention of obesity and NCDs were dominated by one paradigm (Gluckman and Hanson 2012), namely the importance of addressing voluntary lifestyle factors. Particular emphasis was placed on diet (in reducing fat and salt intake), physical exercise and smoking cessation. As such components of lifestyle are assumed to be a matter of personal choice and as many governments shy away from seeming to act as a "nanny state,"

individuals are made to feel guilty if they cannot make such decisions about adopting healthy behaviours and become overweight or obese. This approach even extends to young children. A corollary to this approach is to blame the multi-national companies of the food industry for the provision of unhealthy food (e.g., Stuckler and Nestle 2012) and the educational system for not providing adequate opportunities for children to undertake physical exercise, although the problem is more complicated than that (see Metcalf et al. 2008). Such approaches to behavioural change and risk reduction are predicated on the model of smoking cessation, in which smokers were made to feel guilty for exposing other individuals to "secondary" smoke, and taxation and legislation were employed to discourage smoking. This model does not apply easily to obesity or other lifestyle factors.

A Life-Course Approach to NCD Risk

It is widely acknowledged that attempts to lose weight, and particularly to maintain weight loss, in adults are often unsuccessful. They are more likely to be effective if accompanied by frequent contact with a healthcare professional or support through other programmes (Cobiac et al. 2010), but such approaches are costly (see OECD 2010) and cannot easily be applied on a large scale. It is now becoming clear that part of the reason for the difficulty in sustaining such programmes on an individual basis is that the mechanisms controlling appetite/satiety do not become reset to a new level even after a sustained period of enforced weight loss (Sumithran et al. 2011). As indicated in Fig. 1, therefore, it is difficult to reduce risk factors for NCDs at a time in the life course when they have become established. There is now substantial evidence that the set-points for appetite/satiety are determined during development. A related, and clinically very important, issue is that the recent results of a randomised trial of screening for type II diabetes risk in a large number of individuals aged 40–69 years did not show any benefit in terms of mortality over a 10-year period (Simmons et al. 2012). Identification of risk does not prevent its aetiology.

There is now substantial human and animal model evidence that risk factors for NCDs, such as diabetes, are established during early life (Hales et al. 1991; Yajnik 2004; Godfrey et al. 2010). The critical periods for establishing such risk are not known for any species: initially, the focus of epidemiological studies on low birth weight suggested that late gestation/fetal growth might be critical, but it is now appreciated that such risk is established across the entire range of birth weight and that sensitivity to environmental influences operates during early embryonic development, throughout gestation and during neonatal and infant life (Gluckman and Hanson 2008). The list of organs and tissues affected is extensive but would include the heart, muscle, bone, cardiovascular, respiratory and renal systems, stress responses, and endocrine organs (e.g., the pancreas), as well as mood and behaviour, cognitive function, the timing of puberty and reproductive function and

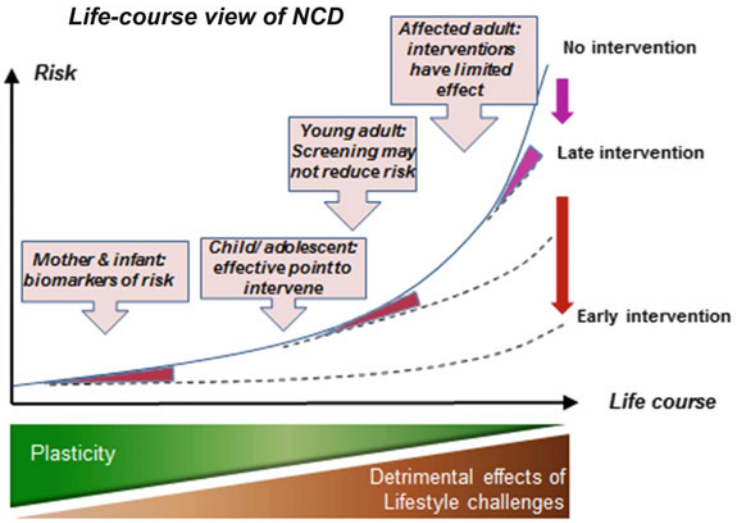

Fig. 1 Conceptual diagram to illustrate components of a life-course approach to non-communicable disease (NCD) prevention. Risk of NCDs increases in a non-linear way through life, as plasticity declines and detrimental effects of unhealthy lifestyle accumulate. Interventions in affected adults have limited effect, and screening in young adults may also not completely prevent disease because interventions at this time may be too late. Reduction of risk in early life offers an important opportunity, and the use of epigenetic and other biomarkers may allow interventions at this time to be customised and assessed. Because parental environmental effects affect the developing offspring from very early in gestation, promoting a healthy lifestyle for parents-to-be, through a focus on health literacy in adolescents and young adults, is an important priority

immune responses. Many animal studies employed an isocaloric low protein diet in pregnancy to induce phenotypic and epigenetic changes in the offspring, although this model has been criticised as of limited relevance to human disease: this criticism is unfounded in relation to poor, unbalanced diets in many LMICs. However, a range of studies is now investigating the effects of other components of relevance to developed countries, such as the quantity and quality of dietary fat (Hoile et al. 2013).

The effects are integrated into a life-course strategy aimed at maximising Darwinian fitness (Gluckman et al. 2007). As indicated in Fig. 1, the effects may be subtle and so may easily be dismissed but, even though they do not immediately signal danger, the risk becomes amplified as the life course proceeds. It is important to stress that this finding does not mean that NCDs *start* in development but that the rising trajectory of risk commences at this time. For this reason, it is important to identify early biomarkers of risk, which is where new discoveries in epigenetics are proving productive.

Epigenetic Processes and Biomarkers

There is now a very extensive literature showing that developmental environmental factors act through epigenetic mechanisms to alter the phenotype of the developing individual and that these processes influence risk factors for NCDs (see Ozanne and Constancia 2007; Burdge and Lillycrop 2010; Low et al. 2011). In animal models, where it is possible to study a range of relevant tissues, pathways have been shown from environmental cues such as maternal nutrition or behaviour, through epigenetic effects on candidate genes and downstream pathways, to phenotypic attributes in the offspring that resemble aspects of human disease (e.g., blood pressure, endothelial dysfunction, body composition or hypothalamic-pituitary-adrenal responses). The question of whether such epigenetic marks are detectable in a range of tissues, as would be expected if they were initiated during early embryonic development, has been little investigated. In humans, a combination of a target discovery approach using techniques such as arrays, followed by detailed investigation of candidate genes has been found to be most effective. This approach has allowed demonstration of effects of maternal diet on epigenetic processes measured in umbilical cord at birth and phenotype such as adiposity or bone density in 6- to 9-year-old children (Godfrey et al. 2011; Holroyd et al. 2012). It is of note that these processes were observed across the normal range of maternal exposures and that the effect size, in terms of accounting for variability in offspring phenotype, was large. In this respect, the effects are substantially larger than those associated with SNPs, even in populations at risk of NCDs (Li et al. 2012). However, the interaction between fixed genetic and epigenetic processes during development has to date been little investigated (Bell et al. 2010). In the (relatively uncommon) individuals who have a genetic predisposition to obesity, it has recently been shown that consumption of sugar sweetened beverages leads to a higher body mass index (BMI; Qi et al. 2012), although the effect size is small.

Recently, studies have been published showing links between DNA methylation levels in specific genes measured in cord blood and later adiposity in childhood (Relton et al. 2012; Perkins et al. 2012), which constitutes a promising development although there are uncertainties about the types of cells examined and the influence of other factors, such as infection, etc. The extent to which such effects measured at birth persist is unknown, although small effects on imprinted genes have been reported in adult offspring whose mothers were exposed to the Dutch Hunger Winter (Heijmans et al. 2008). To date almost all studies in humans have examined DNA methylation changes, which are thought to be more permanent, although animal studies indicate the importance of examining effects on histone proteins (Lillycrop et al. 2005; Aagaard-Tillery et al. 2008; Sandovici et al. 2011) and small non-coding RNAs (Zhang et al. 2009; Taft et al. 2010). The interaction of these epigenetic processes has been reviewed elsewhere (Gluckman and Hanson 2008; Burdge and Lillycrop 2010).

The importance of epigenetic changes in influencing offspring phenotype is underscored by the evidence that environmental factors such as maternal diet (Godfrey et al. 2011; Jiang et al. 2012), stress hormones (Harris and Seckl 2011) and aspects of behaviour such as smoking (Toledo-Rodriguez et al. 2010) all produce effects and consequently affect later risk for disease. In addition, there are now reported effects on placental epigenetic processes that may alter fetal growth (Lewis et al. 2012; Bouchard et al. 2012). From a theoretical point of view, some of the most interesting observations relate to male line transmission of epigenetic marks (Anway et al. 2006) and the reported effects in animal and humans of paternal diet (Carone et al. 2010), smoking (Marczylo et al. 2012) and endocrine disruptor chemicals (Guerrero-Bosagna et al. 2010). Moreover, data are now accumulating that epigenetic processes induced in one generation during development can be passed to subsequent generations (Drake and Liu 2010) and these may have adaptive significance in responding to a sustained environmental change (Burdge et al. 2011).

Opportunities for Intervention

The fact that epigenetic processes form a component of developmental plasticity offers the prospect that the effect of an adverse development environment on such processes may be reversed by the administration of an appropriate intervention. Proof of principle exists in studies using dietary (Lillycrop et al. 2005), endocrine (Vickers et al. 2005) and pharmacological (Stoffers et al. 2003) approaches in animals. The critical period when such interventions would have to be administered to be effective is not known, although in the rat it may extend into the pre-pubertal period (Burdge et al. 2009). However, in humans, another important approach may be the use of epigenetic biomarkers to measure the efficacy of other interventions such as caloric restriction (Bouchard et al. 2010). Adult lifestyle and educational attainment also have effects on epigenetic processes induced during development (De Rooij et al. 2012).

The prospect that early life epigenetic biomarkers will be developed to serve as a basis for selecting and monitoring interventions to promote health and disease risk is now becoming a reality. If they are to be effective, interventions may have to start before conception. Thus a new focus on lifestyle and health literacy in adolescents and young adults, as parents-to-be, may be important. This area of research will have very important implications for translation to human health and NCD prevention and, thus, major humanitarian and financial benefits.

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