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Gene expression in the placenta: maternal stress and epigenetic responses

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ABSTRACT Successful placental development is crucial for optimal growth, development, maturation and survival of the embryo/fetus into adulthood. Numerous epidemiologic and experimental studies have demonstrated the profound influence of intrauterine environment on life, and the diseases to which one is subject as an adult. For the most part, these invidious influences, whether maternal hypoxia, protein or caloric deficiency or excess, and others, represent types of maternal stress. In the present review, we examine certain aspects of gene expression in the placenta as a consequence of maternal stressors. To examine these issues in a controlled manner, and in a species in which the genome has been sequenced, most of these reported studies have been performed in the mouse. Although each individual maternal stress is characterized by up- or down-regulation of specific genes in the placenta, functional analysis reveals some patterns of gene expression common to the several forms of stress. Of critical importance, these genes include those involved in DNA methylation and histone modification, cell cycle regulation, and related global pathways of great relevance to epigenesis and the developmental origins of adult health and disease.

KEY WORDS: *placenta, gene regulation, microarray, DNA methylation, epigenetics*

Introduction

In the Western world, cardiovascular disease, along with metabolic disorders and insulin resistance with their complications, are leading causes of death. A number of important risk factors have been associated with the virtual pandemic of these killers. These include smoking, sedentary lifestyle, high body mass index, hypertension, and so forth. Nonetheless, many individuals who develop cardiovascular and/or metabolic disease do not have these risk factors. Thus, it is clear that as yet unrecognized and underappreciated factors must be considered in the genesis of these disorders. In his monumental volume *Stress*, Hans Selye (1907-1982) observed that stress to the organism, in essentially any of its forms — dietary, environmental, disease, and others — could result in cellular, hormonal, and related damage, with the body mounting a response he termed the "General-Adaptation-Syndrome" (Selye, 1950). Writing years before the nuances of biochemical and molecular mechanisms were established, Selye envisioned an orchestrated biological defensive response to such challenges. This concept is of special relevance to the developing fetus, as during the course of gestation a number of stresses to the mother are known to affect placental, as well as embryonic/fetal development, many with life-long consequences.

Along this line, a factor that has received increasing attention is the idea of "programming" during fetal life, often as a consequence of maternal stress. Special features of antenatal programming include: critical periods of vulnerability, failure or unsatisfactory completion of specific developmental milestones, association with functional defects, the permanent nature of such sequelae, and so forth (Barker, 1992; 1994; 1995a; 1995b; 1998a; 1998b; 2004a; 2004b; Barker *et al.,* 1989a; 1989b; 1995; Nijland *et al.,* 2008). The concept of the developmental origins of adult health and disease, first articulated by David J.P. Barker in the mid-1980s, "… suggested that poor nutrition in early life increases susceptibility to the effects of an affluent diet" (Barker & Osmond, 1986a). In a related analysis, these authors noted the high correlation of cerebrovascular accidents in the 1970s, to increased infant mortality six decades earlier during the years 1911-1914 (Barker & Osmond, 1987). In

Abbreviations used in this paper: E, embryonic day; miRNA, micro RNA; PCR, polymerase chain reaction; siRNA, small interfering RNA.

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addition, in men born from 1911 to 1930, Barker and his group have shown an inverse correlation between the weights both at birth and at 1 year of age to coronary artery disease as adults (Barker *et al.,* 1989a; 1989b). A subsequent study disclosed a similar trend with birthweight among women (Osmond *et al.,* 1993).

Since this concept first was proposed, supporting evidence has been provided by a series of epidemiologic studies from a number of countries and cultures. These include studies correlating adult mortality from acute myocardial infarction with high infant mortality rates in a given population, follow-up studies correlating adult hypertension, coronary artery disease, and type II diabetes with low birth weight, the relation of increased mortality from coronary artery disease to low weight at 1 yr of age, and the relation of both newborn ponderal index [weight (g) x 10^2/crown-heel length (cm)^2] and placental-to-fetal weight ratio to hypertension in the adult (see Barker, 2003, and Barker *et al.,* 1995 for review). These associations are independent of adult life style risk factors (Barker *et al.,* 1993). Among a number of other maternal stress-induced sequelae are those of immune dysfunction (Götz *et al.,* 2007; Merlot *et al.,* 2008), cortisol secretion later in life (Reynolds *et al.,* 2007; Tu *et al.,* 2007), increased incidence of schizophrenia (Hoek *et al.,* 1996; Hulshoff *et al.,* 2000; Susser & Lin, 1992), and many others (Ham & Tronick, 2006). More recently, numerous studies in experimental animals have demonstrated a relation between intrauterine fetal stress, particularly that of maternal food deprivation and/or emotional stress, and adult disease (Gluckman *et al.,* 2008; Green & Hanson, 2004; Hanson & Gluckman, 2005; Jansson & Powell, 2007). Among the major known intrauterine stresses about which the effects on subsequent adult health are largely unknown, are maternal hypoxia and dietary imbalance.

The placenta, a fetomaternal organ joining mother and offspring during pregnancy in mammals, serves as an endocrine organ in the "maternal-placental-fetal" complex, in addition to its role in the exchange of respiratory gases, a multitude of nutrients, an immunologic barrier, and other functions. As has been recognized for many years, compromised placental function can have both short- and long-term consequences for the developing conceptus. In the present review, we examine the current state of knowledge of placental gene expression responses to maternal stress such as hypoxia, protein deficiency, and caloric excess. For the most part these studies are in rodents, however, when applicable, we also review those studies of placental gene expression in the human. (We will not review thoroughly the field of antenatal origins of adult health and disease, nor the role of environmental toxins in developmental disorders, as these topics have been reviewed *in extenso* elsewhere). Importantly, beyond mere description, we attempt to place these gene expression changes into a framework of the biochemical pathways and molecular mechanisms, by which stresses to the maternal organism can result in alterations of great biologic and epigenetic importance to the developing embryo and fetus. Finally, we consider important issues for future investigation, i.e., questions that probe the limits of our understanding.

Why study gene expression in the placenta?

Successful placental development is crucial for optimal growth, maturation, and survival of the embryo/fetus. The placenta not only nurtures the fetus, but protects it from harmful waste products by acting as an excretory route, and also presents an immunologic barrier between the maternal and fetal circulatory beds. Although the nucleus of every cell in the body carries a complete set of DNA, these cells differ in function with placental and embryological development consisting of an elegantly orchestrated switching of genes on and off in the transition from single fertilized cell to fully formed placenta and fetus. Deviation in the normal gene expression pattern may lead to altered placental phenotype, as well as a modified phenotype of the conceptus. This is evidenced by the numerous lethal embryonic null mutants secondary to placental failure. The mouse has been employed as a useful model of placental development. While the mouse placenta is not identical to its human counterpart, many studies have shown that similar cell lineages are largely conserved, and similar genes direct placental development in both species. Placental cell lineages derive from trophoectoderm precursors. The mural trophoectoderm differentiates into primary trophoblast giant cells, while the polar trophoectoderm gives rise to the extraembryonic ectoderm and the ectoplacental cone. In many mammals, including the mouse, the extraembryonic ectoderm forms the chorion that fuses with the allantois, an outgrowth of extraembryonic mesoderm, at around embryonic day 8 (E8) to form the placental labyrinthine layer. The spongiotrophoblast layer of the murine placenta derives from ectoplacental precursor cells and forms the middle layer of the placenta, also known as the junctional zone. The outermost placental cells are the trophoblast giant cell layer. In addition to the primary trophoblast cells derived from the mural trophoectoderm, secondary trophoblast giant cells are derived from the spongiotrophoblast. Later in placental development, around E12.5, glycogen-filled trophoblast cells appear in the spongiotrophoblast layer. Although their function is unclear, these cells express several important gene products, and migrate into the decidua later in pregnancy. Several reviews have detailed placental cell lineages, and some of the genes involved in their differentiation (Cross, 2005; Simmons & Cross, 2005).

Recent studies reveal some of the fundamental mechanisms underlying placental development (Cross *et al.,* 2003; Daoud *et al.,* 2005; Gheorghe *et al.,* 2006; Hemberger, 2007; Sood *et al.,* 2006; Tanaka *et al.,* 2000). Numerous genes are required for proper development of the placenta, and their number has increased greatly, in part, due to the discovery of numerous lethal embryonic null mutants secondary to placental failure (Adams *et al.,* 2000; Schorpp-Kistner *et al.,* 1999; Schreiber *et al.,* 2000; Yamamoto *et al.,* 1998). For example, the disruption of many genes, including growth factors, transcription factors, extracellular matrix proteins, and proteins involved in cell signaling, leads to embryonic lethality secondary to placental failure (Rossant & Cross, 2001). In human trophoblast *in vitro*, several gene classes are strongly up- and down-regulated in the course of differentiation (Aronow *et al.,* 2001). Another study compared differentially expressed genes between the murine placenta and the embryo itself at E12.5 (Tanaka *et al.,* 2000). Microarray analysis has provided insights into aspects of the genetic mechanisms of development, cell growth both normal and abnormal, responses to stress, and numerous other processes (Chu *et al.,* 1998; Gasa *et al.,* 2004; Iyer *et al.,* 1999).

To what extent is placental gene expression altered during gestation?

The molecular basis of placental development remains incompletely understood. Recent studies have begun to shed some light on this process, and numerous genes have been demonstrated to

be essential for the proper differentiation of placental cell lineages and fetal survival. Unfortunately, the details of the interactions and effects of these genes are unclear. In an effort to understand this process at a more fundamental level, we examined gene expression patterns in the developing murine placenta at days E10.5 E12.5, E15.5, and E17.5, testing the hypothesis that from E10.5 until E17.5, numerous placental genes are up- or down-regulated to a significant degree, and that specific functional groups of genes are regulated at the different developmental ages (Gheorghe *et al.,* 2006). To examine gene clustering and functional analysis of pathways, we focused on those genes most highly regulated by development. At E10.5, several functional categories were over-represented, including genes involved in angiogenesis and blood vessel development, morphogenesis, and organogenesis, and genes involved in lipid metabolism and transport. At E12.5, over-represented gene categories were involved in cell cycle control and RNA binding proteins. At E15.5, notably over-represented were genes involved in cellular transport and cell growth and maintenance. At E17.5, we noted the up-regulation of an over-abundance of genes involved in the regulation of transcription and numerous proteins that localize to the nucleus.

This study identified several subsets of genes highly regulated during placental development. Clustering according to their expression patterns, suggests that at crucial times during placental ontogeny particular subsets of diverse genes are induced or repressed in concert (Fig. 1). Genes up-regulated early in placental development"clearly underlie the rapid tissue growth, cell proliferation, and vascular development occurring during this period. At E10.5, genes involved in several key processes were strongly up-regulated. These include angiogenesis, lipid metabolism, and cell cycle regulation. Genes such as ELK3, c-fos induced growth factor, plasminogen (the precursor to angiostatin), serine (or cysteine) proteinase inhibitor clade F member 1, all are involved in blood vessel morphogenesis, and were up-regulated at E10.5. At both E10.5 and E12.5 cyclin, D1, cyclin E2, cyclin C, MAD 2, pleiotrophin, BRCA 2, which are involved in cell cycle control also are up-regulated strongly. At E12.5, ribosomal genes were notably up-regulated, as were several genes involved in lipid transport and metabolism including: apolipoprotein B, apolipoprotein C-II, lysophospholipase, microsomal triglyceride transfer protein and adiponectin receptor 1. As must be evident, lipid transport and metabolism are important for the proper fetal development (Shekhawat *et al.,* 2003) and disruption of lipid transporters leads to embryonic lethality (Farese *et al.,* 1996; Gimeno *et al.,* 2003). Previous null mutant experiments have identified several of these genes as embryonic lethal, further confirming their importance in development. For instance, Cops2 mutants died soon after implantation (Lykke-Anderson *et al.,* 2003), Pten mutants died at E9.5 secondary to placental failure (Yamamoto *et al.,* 1998), and connexin 43 mutants died shortly after birth, due to cardiac and vascular abnormalities (Reaume *et al.,* 1995).

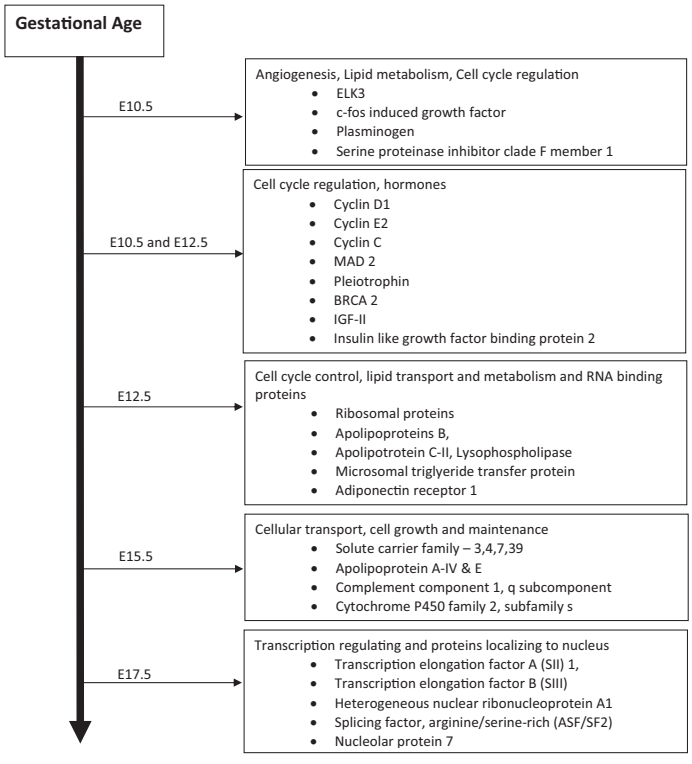

Fig. 1. Over-represented gene functional classes throughout mouse placental development. *Listed are the major groups of upregulated placental genes expressed from Embryonic day 10.5 to 17.5.*

Genes such as growth hormone releasing hormone prolactin-like protein I, secretin, and chorionic somatomammotropin hormone 2 also were upregulated during the course of placental development. Recent studies in the sheep suggest that growth hormone releasing hormone regulates the expression of both placental growth hormone and lactogen (Lacroix *et al.,* 2002). Insulin-like growth factor II (IGF-II) and Insulin-like growth factor binding protein 2, genes have been shown to be expressed in the placenta (Zollers *et al.,* 2001), were up-regulated from E10.5 to E12.5. Previous studies have shown that after E12.5 IGF-II is mainly produced by trophoblast glycogen cells (Redline *et al.,* 1993); however, in our study it was up-regulated at E12.5 and later (Gheorghe *et al.,* 2006). IGF-II also appears to have key functions in placental transport and permeability (Sibley *et al.,* 2004). A number of prolactin-like proteins have been shown to be regulated with development, such as: prolactin-like protein C 1, prolactin-like protein F, prolactin-like protein I, prolactin-like protein K. The prolactin gene family in the mouse has at least 26 identified members (Wiemers *et al.,* 2003), and several studies have shown that in the placenta this gene family performs key reproductive and regulatory functions (Ain *et al.,* 2003; 2004). In the near-term human placenta, mRNA for a number of factors associated with angiogenesis (vascular endothelial growth factor and annexin V) and homeostasis (plasminogen activator factor, thrombomodulin, and others) are widely distributed (Chinni *et al.,* 2008). Circulating fetal fibrocytes, and perhaps other cells, also play a role in the development of the placenta and the umbilical arteries and vein (Kim *et al.,* 2008).

To what extent is placental gene expression altered by maternal hypoxia?

As noted above, a number of stressors can lead to altered placental and fetal growth and development. Of great importance in this regard, is the less than optimal supply of oxygen (O_2), e.g.,

hypoxia. Hypoxia has been identified as a major stressor in development, and is believed to be a contributing cause to placental pathology such as that associated with preeclampsia (Austgulen *et al.,* 2004; Challier & Uzan, 2003). Hypoxia can lead to low birth weight and intrauterine growth restriction (IUGR) and disease of the newborn such as persistent pulmonary hypertension (Zamudio, 2003). Little is known, however, about the adaptive mechanisms involved in the placental responses to suboptimal oxygen availability. Several studies have attempted to harness the power of microarray and proteomic analysis to elucidate responses to hypoxia in cultured human cytotrophoblasts (Hoang *et al.,* 2001), and in rat embryos and placentas (Huang *et al.,* 2004). As with other cell types, oxygen is a critical regulator of the normal trophoblast cell development, which undergo differentiation and/or proliferation in response to varying O_2 concentrations. Acting through aryl receptor nuclear transporter and hypoxia-inducible factor 1_α (HIF-1_α), oxygen regulates placental cell phenotypes and gene expression (Adelman *et al.,* 2000). Hypoxic stress can lead to placental cell death and dysregulation of vasculogenesis, which negatively affects the development of the placental vascular bed (Kingdom *et al.,* 2000). In response to exposure to high altitude, long-term hypoxia, the ovine placentomes undergo significant structural changes (Penninga & Longo, 1998), and the vasculature displays significant increases in capillary density, vessel tortuosity, and a decrease in diffusion distance from maternal to fetal blood (Krebs *et al.,* 1997). The human placenta also shows significant morphologic and morphometric changes in response to high altitude hypoxia (Zhang *et al.,* 2002). Nonetheless, essentially nothing is known about the molecular basis of these changes.

To understand in greater detail the role of hypoxia in placental gene expression, we tested the hypothesis that hypoxia-induced altered placental morphology is accompanied by significant changes in expression profiles. We compared gene expression levels between the normal murine placenta at E17.5, and that from dams exposed to 0.5 atmosphere hypoxia (10.5% O_2) for 48 hours from E15.5 to E17.5. In response to this stress, some of the most highly up-regulated genes were those related to metabolism (alpha-keto reductase family-1 member 7, mitochondrial solute carrying protein, acetyl-coenzyme A synthetase 2, and NADPH oxidase 4), oxygen transport (erythroid associated factor, hemoglobin Y beta-like embryonic chain, erythrocyte protein band 4.2), proteolysis (cathepsin G, kallikrein 4, dipeptidase 1, serine protease 32), cell death (Bcl-like 2, perforin 1, glutathione peroxidase 1), and metabolism of reactive oxygen species (Gheorghe *et al.,* 2007). Of particular note, several genes related to DNA methylation and epigenetic control were up-regulated (DNA methyltransferase 3B, methyl-CpG binding domain protein 1, RNA binding motif protein 3). Of the chromosomal distribution of genes up-regulated by hypoxia, we noted an over-abundance of genes from chromosome 14 (9.5% of the regulated genes as compared to 3.5% of genes on the array). Many of these correspond to the granzyme family of proteases.

Several of these genes have been noted previously to be regulated by hypoxia and/or involved in angiogenesis and metabolic responses. For instance, NAPDH oxidase 4 has been identified as a potential O_2 sensor and regulator of HIF-1α (Zhu *et al.,* 2002). Ferrocheloelastase is involved in heme metabolism, and has been shown to be up-regulated by hypoxia (Liu *et al.,* 2004). Aminolevulinic acid synthase 2, glutathione peroxidase 1, and peroxiredoxin 2 are involved in the metabolism of reactive oxygen species; their activity, and expression levels have been identified as regulated by hypoxia (Abu-Farha *et al.,* 2005; Mysore, 2005). Glycophorin A is involved in erythroid differentiation, and is regulated by erythropoietin (Gubin *et al.,* 1999). Lactotransferrin is an antioxidant involved in iron metabolism and in scavenging of free radicals under hypoxic conditions (Morris *et al.,* 1995). As noted above, hypoxia upregulated several members of the granzyme gene family. These are serine proteases expressed by lymphocytes, and thought to be involved in T-cell-mediated cytotoxicity. Granzymes are released along with perforin by natural killer (NK) cells and cytotoxic T lymphocytes, and trigger apoptosis in target cells through several mechanisms. A number of studies have demonstrated placental expression of granzymes, and they may play a broader role in placental development (Allen *et al.,* 1998; Hirst *et al.,* 2001). We also observed an up-regulation of perforin, which suggests that in response to hypoxia a greater number of NK cells, which have been shown to mediate a number of important functions, invade the placenta (Parham, 2004). Thus, the data suggest that granzymes not only play a role in normal placental development, but also are involved in hypoxia-mediated responses.

Again, of particular note in our study was the significant up-regulation of genes involved in DNA methylation and epigenetic control: DNA methyltransferase 3b and methyl CpG binding domain protein 1 (Gheorghe *et al.,* 2007). As noted below, epigenetic modification is an important mechanism of gene expression regulation that does not involve modification of the DNA

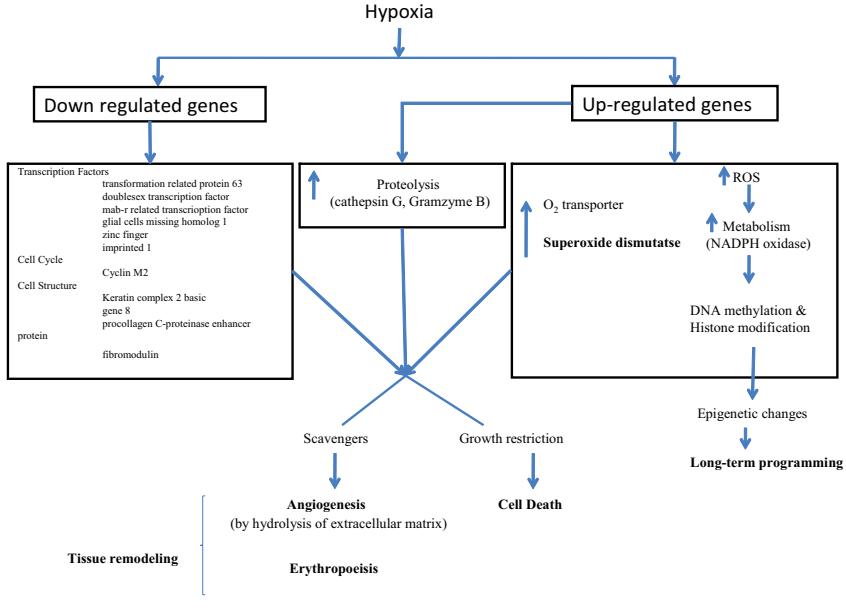

Fig. 2. Proposed mechanism of hypoxia-mediated epigenetic changes with long-term programming.

sequence, but rather DNA methylation and histone modification. These changes in expression patterns may be of importance in development, genomic imprinting, and the development of cancer (Turek-Plewa & Jagodzinski, 2005). The generation of reactive oxygen species (ROS) and the manipulation of glutathione metabolism have been shown to regulate a number of cellular processes, including DNA methylation (Fratelli *et al.,* 2005). As depicted in Fig. 2, this suggests complex genetic regulation that commences with hypoxia and leads to alternations that result in long-term programming. In turn, this activates the DNA methylation machinery, and ultimately leads to long-term changes in the organism unrelated to modification of the nucleotide sequence. A key to unraveling this mechanism will be the identification of the targets for altered methylation and subsequent long-term down-regulation of transcription.

Among the down-regulated genes, most notable were several transcription factors (transformation-related protein 63, doublesex- and mab-3-related transcription factor 1, glial cells missing homolog 1, zinc finger, imprinted 1), cell cycle (cyclin M2), cell structure (keratin complex 2 basic, gene 8, procollagen C-proteinase enhancer protein, fibromodulin). We have presented a list of the genes up- or down-regulated and their known function, and verified the regulation of several of these genes using real time PCR (Gheorghe *et al.,* 2007). In response to hypoxia, placental morphology also was altered significantly e.g. having a greater vascular density and containing many more red blood cells.

Several other studies have examined gene expression changes in response to hypoxia at the global level. One study catalogued the hypoxic-induced responses in the rat embryo to hypoxic exposure for both 24 hours and 11 days. Glycolysis-related genes, calcium homeostasis-related genes, and inflammatory genes (particularly as related to oxidative stress) were up-regulated, while cell growth-related genes were down-regulated (Huang *et al¸* 2004). Other studies also have examined human trophoblast responses *in-vitro* to low oxygen tension. Observed were up-regulation of antioxidants (superoxide dismutase) and glycolysis-related genes, and the upregulation of glutathione-S-transferase (Nelson *et al.,* 2003; Roh *et al.,* 2005). In addition, gene expression changes in placentas from pregnancies complicated by pre-eclampsia and IUGR have been catalogued. In particular, H4 histone was down-regulated in women with severe pre-eclampsia (Chen *et al.,* 2006; Soleymanloo *et al.,* 2005), and up-regulation of glutathione-S-transferase was observed in human placentas from women at high altitude (3,100m) (Chen *et al.,* 2006; Roh *et al.,* 2005). These studies have highlighted the diverse manner in which placental cells respond to hypoxic stress.

In the placenta of patients that experienced chronic hypoxic ischemia, mRNA levels of both leptin and insulin-like growth factor (IGF)-1 were upregulated significantly (Trollmann *et al.,* 2007). In near-term placental explants cultured in 1% O_2, expression of the tumor suppressor protein p53, that promotes cell cycle arrest or apoptosis, was significantly elevated (Heazell *et al.,* 2008). Exposure of human placental villous explants to 3% O_2 for 48 h, resulted in significant increase in endoglin, a co-receptor for transforming growth factor (TGF-β_3) pathway (Yinon *et al.,* 2008). In the placentae of patients with severe preeclampsia at 31±2 weeks gestation, mRNA of the antioxidant protein glutathione reductase was reduced significantly, while that for thioredoxin peroxidase was increased (Vanderlelie *et al.,* 2008). This suggests that oxidative stress may play a key role in the pathophysiology of the placentae in cases of preeclampsia.

In summary, the murine placenta appears to respond to hypoxia through several adaptive mechanisms. These include up-regulation of genes associated with erythropoiesis, increases in heme and iron metabolism, and in genes involved in proteolysis and peptidolysis. These varied responses suggest that the placenta responds by increasing its oxygen carrying capacity, increasing metabolic and antioxidant responses, and initiating tissue growth, turnover, and remodeling. Studies in both the human (Roh *et al.,* 2005) and sheep (Krebs *et al.,* 1997; Penninga & Longo, 1998) have demonstrated that the placenta undergoes multiple morphological and genetic changes in response to prolonged hypoxia. Hypoxic insults secondary to preeclampsia, maternal smoking, or exposure to high altitude can contribute to placental insufficiency and may lead to intrauterine growth restriction (Jones & Fox, 1980; Levi & Nelson, 2000; Reshetnikova *et al.,* 1994; Spira *et al.,* 1997). The exact mechanisms of these changes are not understood. Extracellular matrix remodeling, the modulation of apoptosis, altered cellular metabolism, and epigenetic changes all appear to be crucial steps in the physiological adaptations of the placenta to hypoxia. It is hoped that these, and other studies, will provide insights at the molecular level into these mechanisms and important clinical problems.

To what extent is placental gene expression altered by maternal protein restriction?

Several lines of evidence demonstrate that nutritional deprivation of the pregnant mother may have deleterious consequences for the progeny. For instance, a shocking "experiment" in humans was that during World War II of the "Hunger Winter" in Amsterdam and Western Holland from November 1944 until the Allied victory in May 1945. This tragedy provides useful lessons on the effects of caloric restriction/malnutrition on fetal development and disease prevalence in adulthood. During this seven month period, the caloric ration fell from ~2400 to 400-800 calories per day, less than 25% of the recommended intake for adults. Although children, and to some extent pregnant and lactating women, received extra rations during the early part of this disastrous famine, they too suffered severe dietary deficiency (Roseboom *et al.,* 2001a). In essence, upon reaching adulthood, the infants that were small at birth had significantly greater prevalence of cardiovascular disease, type 2 diabetes (Kyle & Prichard, 2006; Painter *et al.,* 2005a; Roseboom *et al.,* 2001a; 2001b; Stein & Susser, 1975; Stein *et al.,* 2004), and mood and personality disorders (Godfrey, 1998). Those fetuses exposed to maternal caloric restriction in mid-gestation had a much greater incidence of pulmonary disease, including bronchitis (Lopuhaa *et al.,* 2000), and renal disease as evidenced by microalbuminuria (Painter *et al.,* 2005b). Females who were conceived during the famine also had a much higher prevalence of obesity as adults (Ravelli *et al.,* 1999), and both males and females showed atherogenic lipid profiles (Roseboom *et al.,* 2000). Concommently during WWII, the people of St. Petersburg and surrounding area of Russia were subjected to severe dietary restrictions due to interdiction of food supplies by the German army. The children born under these conditions were not only small for gestational age, but also developed health problems later in life (Neugebauer *et al.,* 1999). However, records

of the long-term sequelae of these individuals are not as clear as those in Holland (Lind, 1984; Ravelli *et al.,* 1976). Importantly, the mechanisms of these in utero "programming" effects are unknown.

Epidemiologic data on the role of maternal nutrition in determining the long-term health of offspring derives largely from the studies of Barker and colleagues (Barker, 1995b; 2003; Barker & Clark, 1997; Barker & Osmond, 1986a; 1986b). Studies in several countries have correlated maternal dietary deficiencies that result in the newborn infant being small for gestational age, or growth restricted, with the prevalence of cardiovascular disease (Barker & Osmond, 1986b; Barker *et al.,* 1989a), type 2 diabetes (Hales *et al.,* 1991; Ravelli *et al.,* 1998), and numerous other conditions in the adult. Maternal nutritional deprivation may influence placental growth and morphology, alter the hormonal milieu of the developing fetus, and cause subsequent cardiovascular, hormonal, and behavioral consequences in the adult (Barker, 1992; 1994; 2003; Barker & Osmond, 1986a; 1986b; Barker *et al.,* 1989a; Gluckman *et al.,* 2008).

The epidemiologic observations made in human subjects have been confirmed in animal models, and have led to speculation regarding the cellular mechanisms of changes in the placenta, and their effects on the developing fetus (Armitage *et al.,* 2004; Hoet & Hanson, 1999). An important question is the extent to which these observed effects result from an overall caloric restriction, as opposed to a qualitative component in the diet that triggers the responses. Evidence from animal studies points to protein deprivation as a major factor in these defects. For example in the rat, the growth reducing effects of a low calorie diet can be reversed only by a dietary increase in protein levels, while vitamin supplements and caloric increases through carbohydrates did not reverse the effects observed (Hsueh *et al.,* 1967). Other studies have revealed that dietary amino acid balance is a key mediator of some of the cardiovascular and metabolic effects observed in response to protein deprivation (Boujendar, 2003). Overall, the several studies indicate that nutritional deprivation, and protein restriction in particular, can have immediate deleterious effects on the placenta and the fetus, and may result in long-term sequelae that extend into adulthood.

In a recent study in the mouse, we tested the hypothesis that moderate maternal protein deprivation would alter gene expression patterns in the placenta (Gheorghe *et al.,* 2009). We compared gene expression levels between normal placentas at E17.5, and those from pregnancies in which the mothers were exposed to seven days of protein deprivation (i.e., 10% protein by weight versus the 20% of normal chow) from E10.5 to E17.5. Of particular note, a number of genes involved in the p53 oncogene pathway were up-regulated. In addition to p53 itself, its positive regulators Zmis, Jmy, and Hipk2, as well as genes activated by p53 (Inpp5d, Cebpa), were induced (Fig. 3A). These p53 pathway proteins are important regulators of cell growth and proliferation. This pathway serves as a G1 checkpoint, and arrests growth and/or induces apoptosis in response to cellular damage. Mutations in the p53 gene have been implicated in a number of cancers and other pathological processes (Ryan *et al.,* 2001). Hipk2, an upstream regulator of p53, activates its transcriptional activity and pro-apoptotic activities through phosphorylation at Ser 46 (Hoffman *et al.,* 2002). Cebpa, is a transcription factor induced by p53, and mediates some of the downstream effects of p53 activation (Yoon

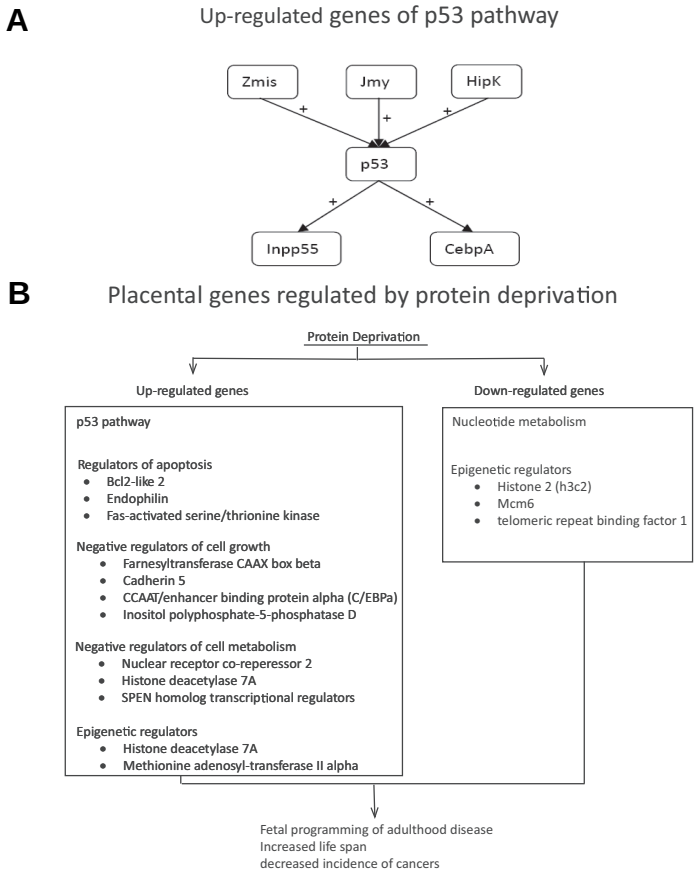

Fig. 3. Maternal stress-mediated placental gene regulation. (A) *p53 related genes up-regulated by protein restriction in the mouse placenta. + stands for induced.* **(B)** *Proposed mechanisms of protein restriction induced long-term changes in gene expression.*

& Smart, 2004). Among the gene ontology classes most over-represented in the up-regulated group, we noted the mitogen-activated protein kinase pathway, regulators of apoptosis (Bcl2-like 2, p53, endophilin, Fas-activated serine/threonine kinase), negative regulators of cell growth (farnesyltransferase CAAX box beta, cadherin 5, CCAAT/enhancer binding protein (C/EBP) alpha, inositol polyphosphate-5-phosphatase D, p53), and negative regulators of cellular metabolism (nuclear receptor co-repressor 2, histone deacetylase 7A, SPEN homolog, transcriptional regulator). Acting in concert, activation of these genes could result in growth restriction during pregnancy (Fig. 3B). Among down-regulated genes, particularly striking were those related to nucleotide metabolism**.** For selected genes, we confirmed these results using qRT-PCR (Gheorghe *et al.,* 2009).

Another potentially important finding, is that protein deprivation altered the expression of several genes involved in DNA methylation, histone acetylation, and epigenetic regulation of gene expression. The expression levels of histone deacetylase 7A and methionine adenosyltransferase II, alpha were elevated several fold. Histone acetylation triggers changes in chromatin structure, and regulates transcriptional availability of genes. In turn, histone deacetylation increases histone affinity for DNA, thereby repressing transcription (Bulger, 2005). Methionine adenosyltransferase II alpha synthesizes AdoMet the direct precursor used for DNA

methylation by methyltransferases (Mao *et al.,* 1998). Histone 2 (h3c2) is down-regulated, along with Mcm6 and telomeric repeat binding factor 1. These proteins contribute to DNA replication, stability, and structure (O'Connor *et al.,* 2004; Yu *et al.,* 2004). In the placenta of patients with preeclampsia, phosphorylation of extracellular signal-regulated kinase1/2 was significantly less frequent in the invasive trophoblasts, as compared to control (Moon *et al.,* 2008). In another study, in placentas of preeclamptic patients, contrary to expectations, polymorphisms of several enzymes associated with oxidative stress (copper/zinc superoxide dismutase, manganese superoxide dismutase, glutathione-S-transferase, and others) did not differ from controls (Zhang *et al.,* 2008). In contrast, in the placentas of patients with HELLP (Hemolysis, Elevated Liver Enzymes, Low Platelets) syndrome, genes encoding vascular endothelial growth factor receptor, leptin, and several other proteins, were up-regulated, as compared with the placenta of both normal control patients and those with preeclampsia (Buimer *et al.,* 2008).

Because the various tissues and organ systems undergo critical, often brief, periods of growth and development during fetal life (Winick & Noble, 1966), "programming" as a consequence of maternal stress should not be unexpected, with insults to the developing organism having consequences later in life's course. Studies in ruminants also have demonstrated that under-nutrition can have profound consequences for the fetus. In sheep, restricted maternal nutrition in early to mid-gestation was associated with an increase in placental weight, an increase in crown-rump length, and lower fetal to placental weight ratios (Heasman *et al.,* 1998). Maternal under-nutrition also altered cardiovascular homeostatic regulation by the renin-angiotensin system, and exposed the lambs to higher levels of glucocorticoids (Edwards *et al.,* 1999), and development of hypertension (Dodic *et al.,* 2001). Protein restriction in bovines also resulted in an increase in placental weight and altered placental morphology (Perry *et al.,* 1999).

Studies in rodents have shown similar effects. In rats, maternal protein restriction triggers hypertension in the pups in adulthood (Langley & Jackson, 1994), probably by augmentation of the pups' renin-angiotensin system. In the spontaneously hypertensive rat placenta, several proteins, angiotensin receptor type I and inducible nitric oxide synthase (NOS), were up-regulated, while angiotensin converting enzyme and peroxisome proliferator-activated receptors alpha and gamma were downregulated (Raso *et al.,* 2008). An alteration of placental glucocorticoid (GC) metabolism also was observed in placentae of rats fed a protein restricted diet, namely the activity of 11β-hydoxysteroid dehydrogenase that metabolizes glucocorticoids. This placental enzyme, which normally protects the pups from maternal glucocorticoid excess, was reduced in protein restricted rats (Langley-Evans *et al.,* 1996), thus exposing the fetus to abnormally high GC concentrations. Elevated circulating cortisol concentrations, with modified responsiveness of the hypothalamic-pituitary-adrenal axis, and elevated mean arterial blood pressure with increased left ventricular wall thickness and mass, also were observed in guinea pigs in which the dam received only 70% of normal chow during either the first or second half of gestation. Some of these changes persisted in the F_2 generation (Bertram *et al.,* 2008). Another hormonal alteration in nutritionally deprived rat pups, was an increase in somatostatin expression in the periventricular nucleus. This led to much lower levels of growth hormone, and had deleterious effects on the growth of the pups post-partum (Huizinga *et al.,* 2000). Fetal undernourishment also led to neuronal sequelae. The facial motor nucleus in pups was under-developed, resulting in decrease in the ability of pups to suckle and chew (Perez-Torrero *et al.,* 2001). These observations also may relate to the epidemiologic findings, noted above, that abnormal antenatal nutrition may be associated with the development of schizophrenia and other mental illness.

In several animal models, in addition to the potential deleterious effects referenced above, a positive aspect of nutritional deprivation in the adult is that of prolonged lifespan and reduced cancer rates. A proposed mechanism for these benefits is that nutritional restriction without severe malnutrition inhibits cellular proliferation and induces apoptosis. This effect has been shown in mice lacking p53, in which –/– and +/– mutants have lowered spontaneous cancer rates when fed a calorically reduced, but otherwise complete, diet (Hursting *et al.,* 2004). In the adult and aging animal, nutritional restriction has been shown to have beneficial effects that increased life span (Nikolich-Zugich & Messaoudi, 2005). A different picture has emerged in the fetus, however. As discussed above, caloric and protein deprivation have been shown to trigger fetal programming of adult disease, and lead to an increased prevalence of metabolic disorders in adulthood (Barker, 1995b; 1998a; 1998b; Barker & Clark, 1997). In the developing fetus, numerous animal studies have shown negative long-term effects of caloric and protein deprivation on the cardiovascular, renal and nervous systems and metabolism (for review see McMillan & Robinson, 2005). A different form of nutritional compromise, that of placental restriction in sheep by removal of the endometrial caruncles in the nonpregnant ewe prior to mating, alters the expression of a number of genes associated with adipogenesis in adipose tissue of the fetus (Duffield *et al.,* 2008). Other protein deprivation models have been studied (Waterland *et al.,* 2006b; Watkins *et al.,* 2008). These findings emphasize the interrelation of placental development and its gene expression, to development of the fetus and its repertoire of gene expression.

To what extent is placental gene expression altered by maternal caloric excess?

Because maternal obesity poses an increased risk to the fetus during pregnancy, and has long-term consequences for the progeny, we tested the hypothesis that maternal caloric excess effects growth-related gene expression changes in the placenta. We fed female C57BL/65 mice a hypercaloric diet (20% fat, 38% sugar) or standard chow for six weeks prior to mating and throughout pregnancy. Near-term (E18), the dams were euthanized. We measured gene expression changes in the placenta, and performed pathway analysis on regulated genes.

Maternal overfeeding was associated with a two-fold increase in body fat mass, with several genes related to obesity, diabetes, DNA methylation, and the transforming growth factor-beta (TGF-β) pathway being differentially expressed. The TGF-β superfamily comprises ~30 growth and differential factors, including several TGF-βs, activins, inhibins, and other growth and cell cycle control factors (Goumans & Mummery, 2000; Kitisin *et al.,* 2007; Massague *et al.,* 2000; Roberts & Mishra, 2005; Roberts & Wakefield, 2003).

Thus, our findings may have important implications for placental growth and epigenetic regulation. In other studies in mice, the chow was supplemented with methyl supplements (Weaver *et al.,* 2005; Wolff *et al.,* 1998) or folic acid (Wehby & Murray, 2008), vitamin B-12, choline, and betaine to enhance metabolism of cellular methyl donors (S-adenosylmethionine) (Waterland & Jirtle, 2003). These interventions resulted in altered coat color phenotype with concomitant increase in DNA methylation at the A^{vy} locus. Conversely, in mice fed a methyl-donor-deficient diet that lacked folic acid, vitamin B-12, and choline the imprinted Igf2 gene was down-regulated with altered DNA methylation (Waterland *et al.,* 2006a). Human studies also have demonstrated effects in the placenta on maternal dietary supplementation (Rush *et al.,* 1984).

What is the role of epigenetics in placental gene expression?

During the course of life and reproduction, cells store information that has been handed down from their ancestors, and that will be transmitted to their descendents. For the most part, this "memory" is encoded in the sequence of nucleic acids that comprise the DNA of the genome, the genotype or entire compliment of genes that provides the stability and accurate heritability from generation to generation. Much traditional research has explored the combined effects of genetics and the environment in germline mutations of the coding and promoter regions of genes. In addition, cells can inherit and transmit information that is not part of the genomic sequence. This *epigenetic* [from Greek, above, upon, over, or beyond conventional genetic], cellular memory involves the heritable transmission of gene expression patterns that persist through cell division, but do not involve an alteration in DNA sequence. Epigenetic processes act in a cell specific, temporally-regulated manner to direct development, differentiation, organogensis, and related processes. Some have compared epigenetic mechanisms to the software to orchestrate and/or modulate the DNA hardware. One major class of epigenetic mechanisms termed "cytoplasmic", is determined by *cis*-acting factors associated with DNA methylation and/or histone modification by acetylation/methylation/phosphorylation. DNA with accompanying histones are packaged in nucleosomes, the core of which contains an octamere of histone proteins. Four basic forms of histones (H2A, H2B, H3, and H4, as well as minor variants), are encircled by 146 base pairs of DNA (Finch *et al.,* 1977); a fifth histone, H1, serves as a linker protein (Bernstein *et al.,* 2007). The histone modifications noted above, and DNA methylation, confer a great increase in the regulatory capacity of each nucleosome, allowing specific functions such as DNA repair and gene activation to be modulated in the appropriate manner (Sarma & Reinberg, 2005). Enzymes critically associated with these nucleosomal modifications include: DNA methyltransferases (DMT), histone acetyltransferases (HAT), histone methyltransferase (HMT), histone deacetylases (HDAC), histone demethylases (HDM), and others (Dodd *et al.,* 2007; Klose *et al.,* 2006). It is by these nucleosomal modifications, with their influence on proximate genes, that genes may be regulated to affect phenotype by activity, chromatin structure, dosage compensation, and epigenetic memory, without changes in the nucleic acid code *per se* (Martin & Zhang, 2005; Wolffe & Matzke, 1999).

Epigenetic changes play a key role in normal cellular function, as well as the development and differentiation of various cell types (Drake & Walker, 2004; Jablonka & Lamb, 2002; Monk, 1998; Murrell *et al.,* 2005; Rahnama *et al.,* 2006; Reik, 2007). Examples include X-chromosome inactivation in female mammals, and genomic imprinting in which one parental allele is altered resulting in parent-of-origin, or random modification of gene transcription (Willard *et al.,* 1993). The epigenetic state can be disrupted by maternal environmental influences such as hypoxia, protein deprivation, caloric excess, and so forth which alter DNA methylation or modify histones. Also importantly, a wide variety of environmental toxins, including low dose radiation and psychological stress, have been demonstrated to be important in epigenetic mechanisms (Dolinoy *et al.,* 2007; Feinberg, 2007; Hertz-Piccioto *et al.,* 2008; Jirtle & Skinner, 2007; Pryce *et al.,* 2002; Szyf *et al.,* 2007). Increasingly, epigenetic changes are being recognized to be of importance in ageing, and the development of cancer and other diseases. Despite the general understanding that DNA and/or histone modifications constitute a major factor in the pathogenesis of epigenesis, little is known of the molecular mechanisms whereby these chemical reactions/changes are regulated, and/or how they are transmitted between generations (Bird, 2007).

Some historical perspectives

From an historical context, epigenetics has several facets. For the pioneer Edinburgh geneticist Conrad Hal Waddington (1905-1975), who coined the term, epigenetics was the study of how phenotypes arise from genotypes during development (Waddington, 1939; 1940; 1942; 1957). Epigenetics later was defined as heritable changes in gene expression not due to any alteration in DNA sequence (Holliday, 1987). In the mid-1970s, the concept of covalent chemical DNA modifications, including methylation was proposed to account for this phenomenon (Holliday & Pugh, 1975; Riggs, 1975). More recently, others have defined epigenesis as the study of mitotically and/or meiotically heritable changes in gene function without a change in DNA sequence (Dolinoy *et al.,* 2007; Russo *et al.,* 1996). As defined by Adrian Bird, epigenetics is "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, 2007). The latter definition focuses on chromosomes and genes, including those aspects such as DNA repair, cell-cycle phases, and those stable changes maintained from generation to generation.

In terms of perspective, epigenetics has a history that antedates understanding the genome and its regulation. The French biologist/zoologist and comparative anatomist who contributed greatly to classification of life forms, Jean-Baptiste Pierre Antoine de Mont, Chevalier de Lamarck (1774-1829), noted that organisms may inherit traits acquired during their parent's lifetime. Although discredited by many, in part, because of his view that simple life forms arose from dead matter by spontaneous generation to become more complex as they were transformed into new species, Lamarck held that organisms inherit characteristics acquired during their parent's lifetimes, evolving in a constant process of striving toward greater complexity and "perfection" (Lamarck, 1801; 1809; 1815-1822). Lamarck's "First Law …" stated that a change in the environment alters the needs of organisms in that environment, resulting in a change in behavior.

Such behavioral alteration leads to greater or lesser use of a given structure with resultant increase or decrease in the size of that structure or organ. His "Second Law" stated that all such changes were heritable as a result (For instance, that a giraffe's neck elongated as it ate from the highest leaves on a tree, and this feature would be seen in the next generation) (Haig, 2007; Jablonka & Lamb, 1995; West-Eberhard, 2007). Neo-Lamarckian biologists soundly reject such an idea. In more contemporary times, the views of the Russian biologist-agronomist, Trofim Denisovich Lysenko (1898-1976), gained considerable press for the agricultural "revolution" he promoted, in concert with Soviet collectivization policies. In essence, Lysenko held that acquired characteristics of a plant (or other organism) could be inherited by succeeding generations. An ideological-political creation, Lysenkoism held the study of classic genetics to be "bourgeois" or "fascist" pseudoscience. Lysenkoism invoked by biological determinists, as with the eugenics and scientific racism adopted by social constructivists, may be seen as the extremes to which political dogma can use science in promoting its propaganda (Roll-Hansen, 2008; Soyfer, 2001).

As noted, environmental influences may have profound effects on gene regulation. This is clearly evident in the cells of multicellular organisms; although being genetically homogeneous, they are structurally and functionally heterogenesis. Many of these differences in gene expression arise during development and are retained through mitosis. Such stable, epigenetic changes, although heritable in the short term, are not a consequence of DNA mutation. Rather, as recent studies are demonstrating, to a great degree epigenesis appears to be a consequence of DNA methylation and histone modification (Cooney *et al.,* 2002; Nanney, 1958). The term "epigenomics" has been applied to the study of altered chromatin structure, such as complex folding, altered nucleosome configuration, and related phenomena (Murrell *et al.,* 2005), while "nutri (epi)genomics" applies to those sequelae following nutritional alteration (Tost, 2008a). Importantly, several lines of evidence indicate that, in addition to maternal to fetal transfer, epigenetic modifications may be inherited across generations (Anway *et al.,* 2005; 2006; Crews *et al.,* 2007; Lane *et al.,* 2003; Morgan *et al.,* 1999; Pembray *et al.,* 2006; Rakyan *et al.,* 2003).

What are the roles of DNA methylation and histone modification in placental gene expression?

As noted above, both DNA methylation and histone modification play important roles in development. These changes also may be important aspects of the ageing process and the development of cancer. In this review, we concentrate on the epigenetic influences of maternal diet, hypoxia, and related stress as observed in the placenta and fetal tissues, and that may have long-term consequences in the fetal origins of adult health and disease.

As with most phenomena of biology and life, the molecular mechanisms whereby genes are repressed or active in a stable manner are exceedingly complex, and new insights into this hitherto poorly understood subject appear daily. The best studied of these epigenetic modifications is that of DNA methylation, which first was suggested in 1975 by two groups (Holliday & Pugh, 1975; Riggs, 1975). This post-replication, covalent methylation occurs predominantly in repetitive genomic regions, on the 5 2-carbon of cysteine residues that are followed by a guanine residue, i.e., "CpG methylation" which induces gene repression or "a silent chromatin state", (The "p" in CpG refers to the phosphodiester bond between cytosine and guanine). Occurring at or around promoter regions, forming CpG islands, this can occur directly by inhibiting the binding of specific transcription factors, and indirectly by recruiting methyl-CPG binding proteins, with their associated repressive chromatin-remodeling activities (Razin & Riggs, 1980). A seeming paradox in this scenario, is that methylation of some specific DNA sequences may permit expression of neighboring genes. Both intrinsic factors and environmental/nutritional factors can determine the activity of methyltransferases upon which DNA methylation are dependent (Bestor, 2000). Because of the requirement for a high DNA synthesis rate during both gametogenesis and early embryogenesis, considerable activity in DNA methylation/demethylation patterning occurs during both this period of development, a time during which the cells are vulnerable to abnormal environmental factors. Nonetheless, nuances of molecular regulation of DNA methylation and histone modification during embryogenesis are beyond the scope of this synopsis, and a number of reviews on this topic are available (Bird, 2007; Jaenisch, 1997; Jaenisch & Bird, 2003; Jones & Takai, 2001; Paulsen *et al.,* 2008; Santos *et al.,* 2002; Tost, 2008a; 2008b).

During the course of mammalian development, a wave of DNA demethylation occurs during cleavage, followed by genome-wide *de novo* methylation following implantation (Jaenisch, 1997). Although the male genome is widely demethylated shortly after fertilization (Mayer *et al.,* 2000; Oswald *et al.,* 2000), the maternal genome is only partly demethylated with subsequent cleavage divisions (Li, 2002). In the gastrulating embryo, the extent of methylation is high, decreasing in various tissues during the course of differentiation (Ehrlich *et al.,* 1982). For the developing embryo and fetus, the methylation/demethylation patterns while being of great significance, are enormously complex.

Additionally, gene expression is determined by the biochemical organization of the histones in the nucleosomes around which the DNA is wrapped. Several post-translational covalent modifications occur on the amino acids that constitute the histone N-terminal tails that modify their interaction with DNA and/or other nuclear proteins. Acetylation, methylation, phosphorylation and/or ubiquitination alone, or in combination play a key role in the regulation by repression or expression of contiguous genes (Jenuwein & Allis, 2001; Strahl & Allis, 2000; Turner, 2000). Again, the regulation of histone modification by acetylation and/or methylation is highly complex, has been shown to be specific for essentially every cell type, and may act with DNA methylation to constitute a system of cellular memory (Bird, 2007; Sims *et al.,* 2008). The combination of the several epigenetic modifications of genes as well as non-coding sequences, the so-called "epigenome" or "epigenotype", determine the extent to which a given gene is maintained repressed or active, and influences the phenotype at birth. A recent study has described the differences in gene expression, DNA methylation, and histone H3K9 acetylation between acute myeloid leukemia and acute lymphocytic leukemia. By integrating genetic and epigenetic information, the divergent nature of the two leukemias was described in more detail, and additional insights were revealed with regards to the gene pathways affected (Figueroa *et al.,* 2008). This study demon-

strates that in combining both genetic and epigenetic information, more accurate and insightful information can be obtained with regards to gene expression regulation and ultimately biological phenotype.

What is the role of microRNAs in placental gene expression?

MicroRNAs (miRNA) have emerged as important players in DNA methylation and post-transcriptional gene regulation (Lujambio *et al.,* 2007; Saito *et al.,* 2006). These are subtypes of small, non-coding RNA, which are 21-25 nucleotides in length. These miRNAs are capable of base pairing with mRNA, and fine-tuning gene expression during development and differentiation, by suppressing their expression in sequence specific manner. Following the discovery of first miRNA "lin4" in 1993, as a small temporal RNA (Lee *et al.,* 1993), there has been enormous growth in this family, and identification of their targets. Although miRNAs are similar to small interfering RNA (siRNA) in their generation pathway and molecular characteristics, unlike siRNA, miRNA does not degrade the target mRNA. Rather, they target the 3' untranslated regions of mRNAs with which they share partial sequence complementarily, thereby silencing post-transcriptional gene translation. In this way, the biological system increases or decreases miRNA production to up- or down-regulate gene expression according to the developmental need, producing desired morphologic and physiological changes. Moreover, placental miRNA (miR-141, miR-149, miR-229-5p, and miR135b) are secreted in maternal plasma, and their concentration decreases significantly after parturition (Chim *et al.,* 2008). This suggests that placental miRNA, in addition to regulating gene expression in placenta, may be playing an important role in maternal conditions with obscure etiology, such as preeclampsia or related hypertensive disorders. Studies reveal differential expression of miRNA (miR-210 and miR-182) in placenta from patients with preeclampsia and with small for gestational age newborn infants (Pineles *et al.,* 2007). As must be evident, additional studies will be vital to examine and understand the complexity of placental genetic regulation, and their contribution to fetal and maternal health and disease.

What are the human correlates?

In several human population studies, it has been reported that the nutritional state of individuals may have phenotypic consequences for their grandchildren (Kaati *et al.,* 2002; Lumley, 1992). An example of the role of diet in progeny DNA methylation status and phenotype is evident in patients with hyper-homocysteinemia (Ingrosso *et al.,* 2003). This disorder is characterized by excess cellular adenosylhomocysteine, a potent inhibitor of S-adenosylmethionine-dependent methyltransferases. This suggests the possibility of significantly altered DNA methylation. In these patients, dietary supplementation with folate restored global methylation levels, as well as that of the imprinted IGF2-H19 locus (Ingrosso *et al.,* 2003). Several earlier studies have indicated the developmental importance of folic acid as a dietary factory *in utero*, and the manner in which it modulates disease risks later in life (Torrens *et al.,* 2006). It remains to be determined whether, as in the case of hyper-homocysteinaemia, these phenotypic effects occur through altered DNA methylation (McKay *et al.,* 2004).

An optimal uterine environment has been shown to be essential for establishment and maintenance of embryonic epigenetic patterns (Vickaryous & Whitelaw, 2005). Because embryo culture and manipulation are employed in contemporary assisted reproductive technologies (ART), the question arises as to the extent to which ART or related procedures alter DNA methylation patterns, thereby inducing epigenetic changes in the developing organism (Brar *et al.,* 2001; Feil, 2006; Khosla *et al.,* 2001a; 2001b; Vickaryous & Whitelaw, 2005). Normally DNA methylation is confined to only one of the two parent alleles, thus imprinted gene loci allow minor alterations to be detected. An issue of great importance is the extent to which the chemical composition of culture medium, the duration of culture, or other factors, play a role in effecting changes in DNA methylation or histone modification (Doherty *et al.,* 2000; Khosla *et al.,* 2001a; 2001b; Mann *et al.,* 2004; Young *et al.,* 2001).

An additional consideration of importance, is the role of environmental toxins in producing alterations in the nucleosome with epigenetic consequences. An obvious example from mid-twentieth century is the ingestion of the estrogen-receptor agonist diethylstilbestrol (DES) by women in an attempt to reduce the risk of spontaneous abortion. This was followed by vaginal clear cell carcinoma (Swan, 2000), and altered limb development in the first generation, and deafness in the second generation (Stoll *et al.,* 2003). Anticancer drugs and other environmental compounds may alter expression of specific genes, as well as the stress-related chaperone protein heat shock protein (HSP)-90, which may play a role in histone modification (Feil, 2006; Rutherford & Lindquist, 1998). A host of environmental contaminants including endocrine-disrupting chemicals are now known to demonstrate epigenetic effects on the germ line, and promote disease across several generations (Crews *et al.,* 2007; Parodi *et al.,* 2006).

In humans, a number of factors, genetic and epigenetic, can influence placental/fetal growth, development and long-term sequelae. Several hypotheses have been proposed to account for these phenomena. The "thrifty genotype" hypothesis proposes the existence of genes that influence birthweight, and determines whether an infant will experience intrauterine growth restriction (Ong & Dunger, 2000; Prentice *et al.,* 2005; Stöger, 2008). The "thrifty phenotype" hypothesis postulates that impairment of nutritional supply in early life results in permanent changes in tissue/organ function to conserve glucose, and prioritize development of the brain, heart, and other vital organs (Hales & Barker, 2001). A third hypothesis proposes that epigenetic alterations in gene expression, in the absence of altered DNA sequence, can be heritable, and may be reversible (Holness & Sugden, 2006). A challenge for our future is to develop strategies to negate the long-term consequences of these molecular alterations. A related issue of consequence is the epigenetic basis of dysregulation of gene expression as demonstrated in metabolic syndrome with insulin resistance (Lane *et al.,* 1996), neural development (Canli *et al.,* 2006; Collins & Barker, 2007; Ke *et al.,* 2006), cancer (Esteller, 2007; 2008), and other conditions (Pembrey, 2000). Rather than isolated instances, this may be a major factor in the seemingly increasing and intractable pandemic of these classes of diseases (for instance see Gal-Yam *et al.,* 2008; Palii & Robertson, 2007). Recognizing the importance of these vital

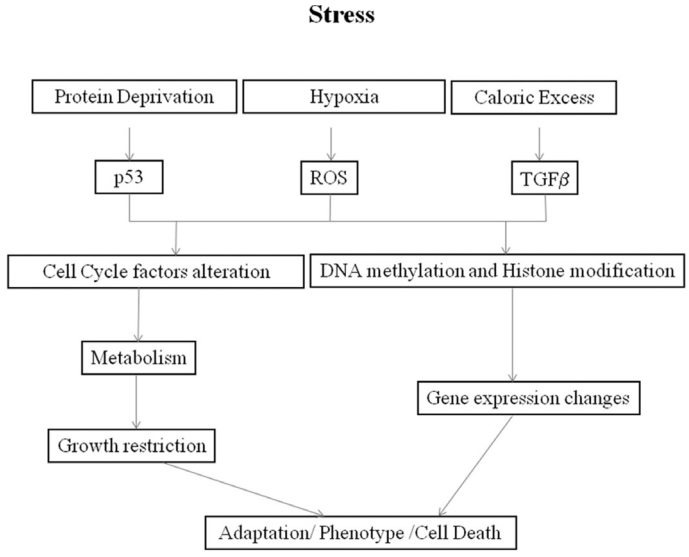

Fig. 4. Proposed model of stress-induced long-term epigenetic changes in gene expression, adaptation and phenotype.

issues, a recent National Institutes of Health initiative, as part of its "Roadmap" program, seeks applications to study the "Epigenomics of Human Health and Disease" (RFA-RM-08-017) (Jones & Martienssen, 2005).

What are the overall perspectives and critical questions to be explored?

For the long-term well-being of an individual, both optimal placental and fetal growth are essential. Thus, one would anticipate that profound inhibition of cellular growth at key time points during development would have grave long-term consequences for the embryo/fetus. This suggests that the timing of the treatment is a key determinant in the effect on the organism. Since their development, cDNA and oligo microarrays have proven to be powerful tools in the elucidation of gene expression patterns and discovery. In addition to examining cellular processes at the global gene expression level, these instruments have allowed analysis of numerous facets of normal growth and differentiation, as well as that occurring as a consequence of stress or malignant transformation. Of particular value, such studies allow analysis of gene expression by functional classes, as an aid in understanding pathways of cell metabolism, proliferation, senescence, and death.

As with most tissues and organ systems, placental development and its response to stress remains a poorly understood process. Placental malfunction or failure accounts for numerous instances of fetal mortality (Cross *et al.,* 2003), and may play an important role in the genesis of intrauterine growth restriction (Kingdom *et al.,* 2000), as well as some maternal disease (Newstead *et al.,* 2007). Numerous genes have been shown to be essential for placental function as an organ of respiratory gas and nutrient exchange, hormonal synthesis, immune function, and so forth. As is evident, maternal stresses, whether hypoxia, protein deprivation, caloric excess, or other, can result in profound alterations in placental gene expression patterns, and their consequences for growth, differentiation, and metabolism. Figure 4 presents in summary fashion established and potential pathways by which stress to the mother, whether hypoxia, protein deprivation, caloric excess, or others, can trigger changes in DNA methylation patterns and/or histone modification to effect alterations in the patterns of gene expression in the placenta and/or fetal organs.

Far from presenting a complete picture, the present review depicts but a fraction of what we need to know to understand more completely the molecular regulation of placental growth and development, and to lessen the ravages of placental dysfunction. A major challenge for the future will be to identify those portions of the genome particularly vulnerable to epigenetic modification which underlie states of health and disease, and to understand the molecular mechanisms by which these changes occur. A few of the most obvious questions follow. How are the demonstrated gene expression profiles regulated? In terms of stress, what determines the individual patterns of expression, as opposed to the up- or down-regulation of those genes common to all stressors? What are the developmental stages/times of vulnerability to environmental, nutritional, or other stress? What environmental factors alter the epigenome in a deleterious manner, and what are their dose-response relations? What are the mechanisms by which DNA methylation and/or histone acetylation/methylation are regulated? To what extent do patterns of gene expression alterations in the placenta influence gene expression in the several fetal tissues/organs? To what extent can we use the findings of gene expression responses to stress, to gain an understanding of the phenomenon of epigenesis and its various manifestations? What is the role of epigenesis in normal development, and in the etiology of disease? How is it that epigenetic changes evident at the molecular level during embryonic/fetal life, do not become manifest in the adult organism for many years or decades? What is the relative importance of epigenetic, as opposed to genetic, changes for long-term sequelae? To what extent can we develop systems using molecular signatures/adducts to detect invidious interactions in early life? To what extent can an understanding of these issues provide us effective means to contain or counteract their influence and consequences? Can epigenetic biomarkers be identified that will allow disease detection at an early stage?

These are but a few of the vital questions that must be addressed in our pursuit to improve the lives and well being of mothers and infants, and the latter's life as an adult. As biomedical scientists dedicated to betterment of the human condition, can we do less?

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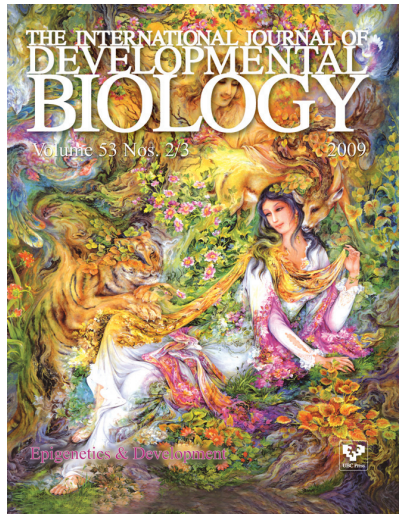

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