Published in final edited form as:

Birth Defects Res A Clin Mol Teratol. 2011 August ; 91(8): 770–780. doi:10.1002/bdra.20807.

Diabetic embryopathy: A role for the epigenome?

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Abstract

Embryonic development under adverse conditions, such as maternal diabetes or obesity during pregnancy, constitutes a major risk factor for birth defects, as well as for long-term health consequences and disease susceptibility in the offspring. While contributions from epigenetic changes have been invoked previously to explain the long-term changes in terms of developmental programming, we here review how maternal metabolism may directly affect the embryonic epigenome in relationship to teratogenic processes. We consider four epigenetic modalities – DNA methylation, non-coding RNA, transcription factors, and histone modifications – and their contribution to epigenetic memory, and discuss how epigenomic changes may mediate the altered control of embryonic gene expression brought about by maternal diabetes. In combination, the epigenomic modalities serve to define transcription-permissive domains of the genome, resulting in distinct epigenomic landscapes in different developmental cell types. We evaluate experimental approaches to characterize the epigenome in adverse pregnancy conditions, highlighting the role of next-generation sequencing on the technological side, while emphasizing the necessity to study defined cell populations in terms of biologic impact. Finally, we outline the challenges in moving from findings that correlate epigenomics to developmental phenotypes to scenarios that establish teratogenic causality.

INTRODUCTION

Pregnancy under adverse conditions

In the past few years, the catch phrase “you are what your mother eats” has been gaining widespread attention in the public, most likely due to its simplicity, and owing to the prospect of offering better outcomes via a better diet. However, the underlying mechanistic relationship of maternal nutrition and metabolism to the well-being of the developing embryo, or the health of the offspring, has proven to be rather complex. Yet, it is clear that in cases where metabolism of the mother is compromised by diabetes or obesity, there are negative consequences for the offspring, either in form of direct teratogenicity, or in form of long-term disease susceptibility. For the purpose of this review, we will focus on the relationship of maternal metabolic disease and birth defects.

Diabetes and pregnancy—Diabetes during pregnancy has well-documented teratogenic effects, and significantly increases the risk for major birth defects (Mills, 1982; Buchanan and Kitzmiller, 1994; Greene, 1999). Congenital malformations are up to 10-fold more frequent with preexisting type I (Kucera, 1971; Casson et al., 1997), type II (Chung and Myrianthopoulos, 1975; Brydon et al., 2000), or gestational diabetes (Kousseff, 1999). Diabetic embryopathy can affect many organ systems (Mills, 1982; Goto and Goldman, 1994; Martinez-Frias, 1994; Casson et al., 1997; Aberg et al., 2001), but the most common

malformations affect the heart (Loffredo et al., 2001) or the neural tube (Kucera, 1971; Cleves and Hobbs, 2004). The effects of maternal diabetes on the developing embryo (Buchanan and Kitzmiller, 1994; Hod, 1996; Reece and Eriksson, 1996) have been experimentally studied in various models, including mice (Ellington, 1997; Sadler, 1997), rats (Cockroft and Coppola, 1977; Reece et al., 1994; Hod, 1996), and hamster (Gale, 1991) embryos in culture (Moley, 1999; Pampfer, 2000), and in diabetic pregnancy in chemical induction and genetic models (Formby et al., 1987; Tatewaki et al., 1991; Phelan et al., 1997; Rajaratnam et al., 1997; Machado et al., 2001; Maeyama et al., 2001). Teratogenesis may directly result from hyperglycemia (Eriksson et al., 1991; Baker and Piddington, 1993; Corrigan et al., 2009), but indirect mechanisms have also been suggested, such as aberrant glycosylation (Mills, 1982), elevated metabolites (Stephens et al., 2001) or deficiency in the arachidonic acid pathway (Goldman et al., 1985; Reece and Eriksson, 1996). The concept of “fuel-mediated teratogenesis” (Freinkel, 1988) involves a metabolic imbalance (Hay, 2006) brought about by high glucose levels while ‘starving’ the embryo of critical nutrients (Carver et al., 1995; Kousseff, 1999; Reece, 1999) during development. Furthermore, oxidative stress has been implicated (Sakamaki et al., 1999; Wentzel et al., 1999; Wentzel et al., 2001; Zaken et al., 2001), and anti-oxidants can reduce the occurrence of neural tube defects in offspring of diabetic rats (Cederberg et al., 2001; Wentzel and Eriksson, 2005). On the other hand, supplementation of folate, a methyl donor, to diabetic rodents can prevent neural tube defects (Wentzel et al., 2005; Oyama et al., 2009), demonstrating a dynamic interaction of nutrition on the detrimental effects of maternal diabetes for the embryo. However, the range of biological, and ultimately pathological changes in the developing embryo that are brought about by the systemic condition – high glucose, or diabetic milieu (Greene, 2001) – is not well understood in its entirety.

Obesity and pregnancy—In recent years, it has also become evident that obesity of the mother during pregnancy constitutes an adverse condition for the development of the embryo, even in the absence of maternal diabetes (Ehrenberg et al., 2004). Maternal obesity is correlated with a significantly higher risk for stillbirth (Nohr et al., 2005), and is associated with an increased frequency of birth defects. Offspring of obese women are at increased risk for neural tube defects (Waller et al., 1994), heart defects (Cedergren and Kallen, 2003), and omphalocele (Watkins et al., 2003). Similar to maternal diabetes, obesity of the mother presents as a significant adverse condition for the development of the embryo. Yet again, comparable to maternal diabetes, molecular mediators of this adversity to the embryo are not well understood.

Birth defect phenotypes and variability—A common feature of birth defects resulting from adverse pregnancy conditions is that they present as phenotype of incomplete penetrance – not all individuals subjected to the exposure present with the phenotype. While an argument could be made that every individual human pregnancy is distinct from the next one, and exposure conditions of the developing embryos are, therefore, not identical, inbred rodent models support the initial argument: multiple embryos of identical genotype are exposed in the same uterine environment, yet only a fraction present with the overt birth defect phenotype. Overall, this suggests that the mechanistic etiology of phenotypes under adverse pregnancy conditions would include an element of variability (Salbaum and Kappen, 2010). The basis for this variability is presently unclear. However, we submit that understanding the source of such variability, and developing means to reduce it, could be of therapeutic importance in the quest to prevent birth defects.

One potential avenue how this variability could arise may involve epigenetic mechanisms. Epigenetics in general refers to heritable changes in gene expression without changes to the DNA sequence (Van Speybroeck, 2002). Maternal diabetes leads to significant changes in gene expression in the embryo (Reece et al., 2006; Jiang et al., 2008; Pavlinkova et al.,

2008; Pavlinkova et al., 2009), and such changes in gene expression are accompanied with higher variability of expression (Salbaum and Kappen, 2010). The wide range of observed expression changes would suggest that multiple streams of transcriptional regulation might be affected by maternal diabetes. This could be accomplished either through a transcription factor that serves as 'master regulator' (Phelan et al., 1997) and governs many downstream regulatory cascades (Loeken, 2006), or as our own studies suggest, through multiple, potentially independent transcription factors (Pavlinkova et al., 2009). How de-regulation of embryonic gene expression occurs is not clear; our results imply that a single or a dominant transcription factor cannot account for the expression changes. Glucose-dependent mechanisms via carbohydrate response elements may also not be sufficient to explain the effects: searching the genomic regions from 10,000 bp upstream to 2000 bp downstream of all annotated transcription start sites in the mouse genome for the presence of the carbohydrate-responsive element consensus sequence CAYGNG (N)₅CNCRTG (Ma et al., 2006) revealed a mere total of 477 hits associated with 418 genes; this number is approximately fivefold lower than the overall number of maternal diabetes-deregulated genes in our study (Pavlinkova et al., 2009); of these, only 32 genes present with such a consensus. An alternative possibility is that maternal diabetes may cause systemic, genome-wide effects on transcriptional mechanisms; such effects would in fact affect both transcription factor scenarios – master regulator or independent parallel factors – outlined above. It is in this scenario where the embryonic epigenome may come into play.

The epigenome and embryonic development—The establishment of the embryonic epigenome – the combination of DNA methylation, non-coding RNA expression, transcription factor activities, and histone modification – during development can be viewed as dynamic process to regionalize the embryonic genome into transcription-permissive domains. These epigenetic modifications can be passed through mitosis (Rakyan et al., 2003), and maintained in the cellular progeny.

Each of the modalities of the epigenome meets the classic definition of epigenetics – to elicit different phenotypes without changes in genome sequence. DNA methylation was long perceived as the primary means to establish, maintain, and transmit epigenetic information. Covalent addition of a methyl group to cytosine residues in the dinucleotide sequence CpG represents a stable change to the DNA, and is established during development by de novo DNA methyltransferases such as Dnmt3a and Dnmt3b (Goll and Bestor, 2005). DNA methylation is in most cases associated with gene silencing (Comb and Goodman, 1990; Nan et al., 1997). Methyl groups on DNA remain during passage of the DNA replication fork in mitosis. The maintenance DNA methyltransferase Dnmt1 establishes DNA methylation patterns on the newly synthesized DNA, using the methylation of the parent strand as template (Hermann et al., 2004; Goll and Bestor, 2005). In this fashion, epigenetic information in the DNA methylation modality can be faithfully maintained and transmitted over many cell divisions.

Non-coding RNAs are a second modality of generation and transmission of epigenetic information. Non-coding RNAs, which in fact constitute the vast majority of the transcriptional output of mammalian cells (Mattick, 2009), contribute to the realization of epigenetic information through their function on transcriptional activity, resulting in differential gene expression. A cardinal function of non-coding RNAs is gene silencing (Malecova and Morris, 2010). One of the best-studied examples is *Xist* RNA in the process of X chromosome inactivation in mammals (Chow and Heard, 2009). X chromosome inactivation is a mechanism for chromosomal dosage compensation in females. It leads to silencing of one copy of the X chromosome in any given cell, which is then clonally propagated through many rounds of cell division. *Xist* RNA mediates this transcriptional silencing of an entire chromosome by coating the inactive chromosome (Wutz et al., 2002).

Xist RNA remains attached to the inactive X chromosome throughout the cell cycle, which leads to the transmission of this epigenetic information through mitosis (Jonkers et al., 2008). Interestingly, the continued presence of *Xist* RNA on the inactive X chromosome leads to recruitment of components of the other epigenomic modalities, resulting in histone modifications and DNA methylation (Chow and Heard, 2009). Other components of the non-coding RNA realm, such as miRNAs, participate in gene silencing (e.g., by the reduction of transcripts available for translation; Guo et al., 2010). In this fashion, they also contribute to the epigenetic regulation. How, miRNA-based epigenetic information is transmitted through mitosis is less clear, as one would expect miRNAs to be diluted by cell division.

Transcription factors, although typically only discussed in the context of direct gene regulation, are integral parts of the epigenome. Transcription factors are the hallmark of expression-driven change in cellular phenotype, and thereby meet the definition of epigenetics that we use here. With respect to the longevity of their actions, several ways of transcription factor-driven transmission of information across mitosis are well established. One way to transmit epigenetic information is through transcription factors that have autoregulatory properties. In such a scenario, a transcription factor protein gets evicted from the nucleus during mitosis, and becomes distributed between the two daughter cells. However, the autoregulatory capacity leads to transcriptional replenishing of the factor, thereby maintaining the epigenetic information akin to a feed-forward regulatory concept. A prime example for such a scenario is the role of the transcription factor MyoD in muscle differentiation (Tapscott, 2005). MyoD can act as a “master” transcriptional regulator for muscle differentiation, either in the normal developmental context of muscle specification, or in the experimental context of transfection of fibroblasts. In both cases, MyoD drives the developmental program for muscle specification, but also maintains its own expression (Thayer et al., 1989). In this way, MyoD transcription factor presence, once triggered, can be maintained over many cell divisions.

In addition, a direct way for transcription factor-based inheritance of epigenetic information has also been described. While most transcription factors are evicted from the cell nucleus at mitosis, it has been shown that general (Xing et al., 2008) as well as sequence-specific (Xin et al., 2007; Verdeguer et al., 2010) transcription factors have the ability to resist nuclear eviction and remain associated with their cognate DNA recognition sites at their target genes throughout mitosis. In this fashion, transcription of target genes can commence quickly, thereby contributing to the maintenance of the cellular phenotype across cell division. It is thought that this mechanism constitutes to epigenetic memory, and contributes to the process of mitotic “bookmarking” (Sarge and Park-Sarge, 2009; Zaidi et al., 2010).

Histone modifications are another modality how epigenetic information is constructed and maintained for many cell cycles. Covalent modifications of histone tails, collectively referred to as the ‘histone code’, affect transcription processes in both positive and negative ways. In this fashion, histone modifications can shape a genomic landscape of transcriptional permissiveness. Different histone modifications mark genes (Barski et al., 2007; Mikkelsen et al., 2007) as transcriptionally active – examples are histone H3 lysine 4 trimethylation (H3K4me3), histone H4 lysine 5 acetylation (H4K5Ac), or histone H3 lysine 27 acetylation (H3K27Ac) – or inactive, as in the case of histone H3 lysine 9 trimethylation (H3K9me3) or histone H3 lysine 27 trimethylation (H3K27me3). In fact, many proteins that were initially discovered as transcriptional co-activators or co-repressors (Xu et al., 1999) – factor that modulate transcription – have subsequently been described to have functions in the acetylation (Spencer et al., 1997) or methylation (Lee et al., 2006) of histones, and, therefore, contribute to the shape of transcription-permissive chromatin domains. Furthermore, histone ‘reader’ proteins recognize these modifications and contribute to the

regulation of transcription (Vermeulen et al., 2010). Patterns of histone modifications are established during processes of cell differentiation (reviewed by Cedar and Bergman, 2009), where the action of transcription factor complexes at specific promoters or enhancers can recruit chromatin-modifying factors such as histone methyltransferases or histone deacetylases (Roopra et al., 2004). The subsequent loss of histone acetylation and the acquisition of histone trimethylation may then lead to engagement of methylbinding proteins, which in turn may draft DNA methyltransferases to the complex, thereby leading to DNA methylation (Feldman et al., 2006). Such mechanisms define transcriptionally active regions, while also shaping a pattern of gene silencing across the genome of a specific cell (Ballas and Mandel, 2005) by overall reduction of transcription-permissive chromatin, with the remaining transcriptionally active genes to determine the identity of a given cell (Ballas et al., 2005). Landscapes of histone modifications can be maintained through mitosis (Kouskouti and Talianidis, 2005) despite the fact that histones dissociate from DNA during passage of the DNA replication fork. How this is achieved is best understood in the cases of histone H3 lysine 9 trimethylation and histone H3 lysine 27 trimethylation, two epigenetic marks associated with silencing of transmission. In both cases, proteins that create these marks – Suv39H1 and G9a in the case of H3K9me3 (Esteve et al., 2006) and the polycomb repressive complex PRC2 for H3K27me3 (Margueron et al., 2009) remain closely associated with the DNA replication fork (Hermann et al., 2004; Hansen et al., 2008). Nucleosome reassembly after the replication fork has passed is typically achieved by utilizing both ‘old’ histones – which carry the modifications on their tails – as well as newly synthesized histones. One would expect that such a mechanism leads to the dilution of modified histones, or to an uneven distribution of modified and non-modified histones on the newly replicated DNA. The presence of the histone modifying enzymes at the replication fork is thought to ensure that the modifying mark is propagated to the daughter cells in a manner that is conceptually analogous to the maintenance of DNA methylation. On a mechanistic basis, it is not entirely clear how presence of an H3K9me3 or H3K27me3 mark is read and re-established. However, one property of the silencing marks is that they are not confined to a small number of adjacent nucleosomes, but are typically present on long regions of chromatin. It is possible that the abundance of those repressive marks in long contiguous stretches may be sufficient to retain the repressive tone through mitotic dilution. Replenishment would come from the respective enzyme complexes, either directly at the replication fork or shortly after the fork has passed. In contrast, transcription-activating histone marks such as histone H3 lysine 4 trimethylation are typically present only on a small number of adjacent nucleosomes in the vicinity of an active transcription start site. Dilution after mitosis or uneven distribution of mark-carrying histones could jeopardize the transcriptionally active status of the DNA region in question. It has recently been shown that the histone methyltransferase Mll, which generates the transcription-activating H3K4me3 mark, can also remain associated with DNA at the replication fork (Blobel et al., 2009). This may provide a means for a ‘read-and-write’-style of regenerating the mark on newly replicated DNA. However, the catalytic histone methyltransferase activity of Mll – although an essential gene (Yu et al., 1995) – is genetically dispensable for Mll function (Terranova et al., 2006). Currently, there is no clear understanding how activating histone marks on short nucleosome stretches are passed through mitosis. One possibility how transmission of a transcription-activating histone context may be achieved without a replication fork-associated ‘read-and write’ mechanism is based on the finding that newly synthesized histone H4 proteins may already come ‘pre-marked’ (Sobel et al., 1995): they carry acetylation at lysine 5, a modification associated with activation of transcription. Incorporating these histone proteins in nucleosomes after replication may be a way to retain the transcription-activating tone long enough until the original pattern of histone marks can be re-established. On the other hand, failure to remove transcription-activating marks (e.g., such as in the case of a lysine demethylase mutation; Katz et al., 2009), can lead to

transgenerational effects, suggesting that there are mechanisms to propagate activating histone marks through mitosis and meiosis, and contribute to epigenetic memory.

It is important to note in this context that specificity of transcription (i.e., which genes are actively transcribed) originates with transcription factor interactions; however, it is equally important to keep in mind that the different modalities of the epigenome interact very strongly, and should not be viewed as independent processes. Transcription factor complexes contain histone modifying enzymes (Xu et al., 1999) in the form of co-activators or co-repressors, polycomb repressive complexes are recruited to bind to non-coding RNA (Bracken and Helin, 2009) in conjunction with their histone modifying activities, and histone modifying complexes can interact with DNA methyltransferases (Esteve et al., 2006; Wang et al., 2009a). The epigenomic modalities should, therefore, not be considered isolated entities, but molecular realms of a highly interactive nature.

Transcription factors serve as ‘nucleation’ events for the establishment of epigenetic marks on DNA and chromatin (Roopra et al., 2004); together, the combination of the other epigenomic modalities has a strong influence on the efficacy of transcription (i.e., to what extent is a gene transcribed) at any given gene locus. Under this paradigm, a salient consequence is that the epigenome in the embryo would be distinct between different cell types. This means that the patterns of DNA methylation, non-coding RNA expression, and landscapes of histone methylation or acetylation would be expected to be different between, for example, neuroepithelial cells and cells of mesodermal origin because of the different suites of transcription factors that are active in those cell types. Consequently, if maternal diabetes could in fact alter the embryonic epigenome, one would expect wide-ranging, yet cell-specific changes on the transcriptome level.

Epigenetics and plasticity—Epigenetic marks were typically considered stable and long-lived. As discussed before, all marks of all epigenomic modalities can be transmitted through mitosis, and thereby propagate an epigenetic status, or ‘memory’, to daughter cells after cell division. However, the long-held notion that DNA methylation was irreversible (Razin and Riggs, 1980) has changed in recent years, as more evidence accumulates to support the view is that DNA methylation is in fact reversible (Ma et al., 2009; Ito et al., 2010). The mechanism for active DNA demethylation does not simply reverse the methylation and remove the methyl group from the DNA, but appears to involve removal of the cytosine base via the base excision repair machinery (Hajkova et al., 2010). Transcription factors and non-coding RNA may be subjected to active degradation, and histone modifications are orchestrated by the enzymatic activities of histone acetyltransferases, histone deacetylases, histone methyltransferases and histone demethylases (Lehrmann et al., 2002; Agger et al., 2008; Shilatifard, 2008; Wang et al., 2009b), and are, therefore, dynamic.

Besides these active mechanisms, epigenetic marks can also suffer erasure via passive means. In this case, marks are simply diluted and not maintained during cell division. For example, failure of the DNA maintenance methyltransferase Dnmt1 to faithfully copy methylation patterns onto newly replicated DNA can lead to extinction of the DNA methylation pattern, and similar ‘failure to maintain’ may apply to all other epigenomic modalities as well. In fact, cell division represents a significant opportunity to change the epigenomic landscape by diluting the epigenetic information. Such a trajectory is of particular significance for rapidly dividing cells, and may play an important role in the developing embryo: with low efficiency of the active maintenance mechanisms in a particular cell type, the epigenetic landscape may be completely changed within a few cell divisions. Therefore, the epigenetic landscape in a given cell, and thereby the potential for modulation of gene transcription, would result from the balance of all active and passive

processes. Rather than static signs on DNA or chromatin, epigenetic marks may represent the outcome of a highly dynamic process. That process may remain in equilibrium over many cell divisions, and thereby convey the notion of long-term stability.

The presence of the diverse counter-acting chromatin-modifying functions implies the possibility for plasticity of the epigenetic landscape. Evidence for such plasticity has accumulated from several lines of investigations. Genome-wide studies on the distribution of epigenetic marks from embryonic stem cells compared to differentiated cells have shown that the epigenome indeed changes during cell differentiation (Bernstein et al., 2006; Mikkelsen et al., 2007). Yet, these studies were performed on cultured cells, and the relationship to differentiation and development *in vivo* is not fully understood. The strongest support for epigenomic plasticity has come from nutrition studies. Treatment of mice with methyl donor diets leads to diet-dependent DNA methylation patterns at specific gene loci, with concomitant differences of gene expression (Wolff et al., 1998; Waterland and Jirtle, 2003; Waterland et al., 2006). Dietary choline, a methyl donor, can influence DNA methylation, histone methylation, and expression of histone methyltransferases such as G9a (Davison et al., 2009). These exemplary studies show that maternal biology can exert effects on the embryonic epigenome, and that the epigenome exhibits plasticity.

Embryonic epigenetics as a potential target of maternal metabolism—The question then arises how maternal diabetes might affect the embryonic epigenome. The nutrition studies show that changes in the supply of substrates for the enzymes that generate epigenetic marks may alter the balance of the respective enzymatic reactions. In this fashion, excess dietary methyl donors affect DNA or histone methyltransferases, and result in higher levels of DNA and histone methylation (Waterland, 2006). Recent progress supports the idea that a similar situation may exist in the case of diabetes. The salient finding is that excess glucose can lead to a high availability of Acetyl-CoA in the nucleus of cells. Acetyl-CoA is the direct enzymatic substrate for histone acetylation, and in fact, the presence of excess glucose has been linked to higher levels of histone acetylation (Wellen et al., 2009). The enzyme responsible for nuclear generation of Acetyl-CoA, ATP-citrate lyase, is essential for embryonic development: a targeted mutation in the *Acly* gene, which is widely expressed in the embryo, leads to early embryonic lethality (Beigneux et al., 2004). Given the intricate developmental link between histone acetylation, methylation, and DNA methylation, this suggests a mechanism by which maternal hyperglycemia can have a direct effect on the status of the embryonic epigenome.

Besides the direct effects via substrate availability, there are further avenues how maternal diabetes may affect embryonic epigenetics. One such potential pathway is through the deregulation of those genes whose products contribute to shape and status of the embryonic epigenome. A priori, one would not expect that such genes incur a specific deregulation, but be subject to the same systemic effects brought about by substrate-driven mechanisms. However, deregulation of genes for chromatin-modifying factors would lead to compound, or feed-forward downstream effects on the epigenome in the embryo. Our own data on gene expression in embryos from diabetic pregnancies suggest that such a scenario might indeed take place. We find decreased expression of prominent chromatin-modifying factors, such as G9a, Kdm1, Hdac5, Mll1, Setd1a and others. (Salbaum and Kappen, 2010). Such altered expression could have significant consequences for the shape of the epigenome in the embryo, as it may exacerbate the initial effects of substrate-driven mechanisms. Yet, as we noted previously, we find that the expression changes affect genes for enzymes that generate epigenetic marks in similar fashion than they influence genes for proteins that remove them, thereby rendering predictions difficult how exactly the epigenome would be altered by these expression changes (Salbaum and Kappen, 2010).

In this context, it is necessary to remind ourselves that the above-mentioned transcription factor-driven expression changes and any epigenomic effects cannot be entirely separated. Genes encoding transcription factors are just as likely to be modulated in their transcriptional output by epigenomic changes as other genes in the genome. Similar to the de-regulation of genes encoding chromatin factors, altered expression of transcription factors would likewise lead to feed-forward downstream transcriptional changes due to the regulatory nature of these proteins. It is, therefore, highly likely that transcription factor-driven processes and chromatin-dependent modulation act in concert, and are affected simultaneously.

While it appears a case can be made for an interaction between maternal metabolism and DNA or chromatin modification, the situation is less clear whether or how small interfering RNAs might be affected in embryos of a diabetic pregnancy. A retrospective analysis of our own transcriptome profiling experiments suggests that more than 1200 of the genes deregulated by maternal diabetes (Pavlinkova et al., 2009) are potential targets for microRNAs, and approximately 800 of these genes could be targets for two different microRNAs. Targeting by microRNAs typically results in reduced mRNA levels. Consistent with that notion, we find that the majority of maternal diabetes-deregulated genes show in fact decreased expression. It is further of interest that three of the top 20 microRNAs associated with the suite of de-regulated genes – *miR-26a*, *miR-107*, and *miR-16* – have been shown to be glucose-responsive, and increase in their expression under conditions of high glucose *in vitro* (Tang et al., 2009). Whether this is also the case in the developing embryo, or under conditions of maternal diabetes, is not known. In fact, the field of microRNA expression patterns in the developing mouse embryo is largely unexplored, although recent landmark studies (Mukhopadhyay et al., 2010) have advanced this field. However, the interaction of maternal diabetes with the realm of embryonic microRNAs represents unknown territory, and, therefore, predictions are currently premature.

Together, this suggests an overall picture where on the one hand the epigenome – at least as the modalities of DNA methylation and histone modification are concerned – may be dynamically altered to accommodate the process embryonic development, and yet it may be modulated by nutritional or metabolic influences that originate from the mother. In this fashion, diabetes – and potentially also obesity – of the mother during pregnancy could act to (mis-) shape the epigenome of the embryo, and thereby generate changes of embryonic gene expression as well as increased variability of gene expression; both in turn may contribute to morphogenetic defects.

The epigenome and birth defect phenotypes—The rationale above also presents an explanation how systemic changes such as hyperglycemia, which in theory could affect all cells in a similar fashion, can lead to cell-specific changes, and ultimately to specific morphogenetic phenotypes. Given the developmental emergence of the epigenome in a cell-specific fashion, any changes caused by maternal hyperglycemia would develop in concert with the cell type-specific patterns of epigenetic marks, and, therefore, affect expression of genes in a cell type-specific manner. In essence, the normal developmental program of the embryo serves to ‘translate’ epigenomic and subsequent transcriptomic changes into cell-specific outcomes.

The question still remains how alterations to the epigenome may be related to birth defect phenotypes. We can envision two possibilities. The majority of rodent embryos in diabetic pregnancies present without an overt phenotype; this would suggest that the normal developmental program is not easily overruled. Therefore, one way how deficient morphogenesis may occur would be through genome-wide, systemic changes to the epigenome that remain within the constraints presented by the normal developmental

program. Epigenomic changes might occur on the quantitative level (e.g., reduced histone trimethylation at certain loci) rather than complete absence of the marks. Such changes might affect the overall precision of gene regulation, and generate variability across the entire gene expression program. The lack of precision in the developmental regulatory program would be the basis for incorrect morphogenesis in individual embryos: in those embryos, one can envision that the expression of crucial morphogenetic genes may be too low to support normal development. The second possibility is that the developmental establishment of the epigenome under hyperglycemia conditions may lead to an entirely different trajectory, with drastic, qualitative changes to the epigenome. The incomplete penetrance of phenotypes would suggest that such a derailment may not happen in every embryo of a diabetic pregnancy, but may have occurred only in those embryos that present with an overt birth defect. Such changes in the overall trajectory may stem from founder effects early in development that are subject to variability between individual embryos. One consequence of such a scenario is that one would expect that the gene expression pattern of an embryo with a birth defect would be significantly different from the pattern observed from embryos of diabetic pregnancies with normal morphogenesis. In our own transcriptome profiling experiments, this appears not to be the case: while mouse embryos from diabetic pregnancies with a neural tube defect at 10.5 days of gestation can be distinguished from embryos of a normal pregnancy based on their transcriptome, they cannot be distinguished from embryos of diabetic pregnancies with normal morphogenesis (Salbaum and Kappen, 2010). While this observation would be consistent with the first possibility of system-level quantitative changes to the epigenome, a final assessment of this question can only be made on the basis of experimental observations. We, therefore, believe that experimental determination of shape and nature of the embryonic epigenome under normal as well as diabetic conditions is imperative, particularly so to clarify what molecular changes in the embryo are caused by maternal diabetes.

Epigenetics and maternal diabetes – experimental approaches—The determination of epigenetic marks at single genes, such as measuring the presence of DNA methylation or histone modifications, has long been established. Such methods allowed precise yet limited insight at specific loci. However, the advent of genome-wide technologies, first on the basis of genome tiling arrays and hybridization methods (van Steensel and Henikoff, 2003; Mockler et al., 2005), and lately through the introduction of next-generation sequencing technology, has opened up new avenues in the field of epigenetic research. These new methods make it possible to determine the distribution of epigenetic marks across the entire genome. Genome-wide tiling arrays have been used successfully for DNA methylation studies (Hayashi et al., 2007), or mapping of transcription factor binding sites by chromatin immunoprecipitation followed by array hybridization (ChIP-Chip; Horak et al., 2002). Unfortunately, many of these arrays were focused on promoter sequences as the target for DNA methylation (Koga et al., 2009). This ‘promoter-centric’ view presents a problem for analyzing embryonic development, where tissue-specific and cell-specific gene expression is often mediated by enhancer sequences. Such enhancers may be close to the transcription start, but are often distant from the transcription initiation site (Kappen and Salbaum, 2009; Salbaum et al., 2009); therefore, assessment of promoter methylation only may miss important developmental regulatory elements.

Major progress toward the exploration of the epigenome has resulted from the application of high-throughput, next-generation sequencing technologies that permit the generation of millions of sequence reads from a sample of interest (Schuster, 2008). Sequencing-based techniques (Barski and Zhao, 2009) are particularly powerful, since they eliminate the experimental bias inherent in hybridization-based approaches. Next-generation sequencing can be successfully adapted to every modality of the epigenome. For DNA methylation, several strategies are available (Bock et al., 2010). The MeDIP method (Weber et al., 2005)

relies on randomly fractioning of genomic DNA followed by enrichment of methylated DNA via immunoprecipitation with an antibody against 5-methyl cytosine. Instead of using the enriched preparation for array hybridization (Hayashi et al., 2007), the identity of the enriched DNA fragments is determined by sequencing (Ruike et al., 2010). Avoiding potential issues associated with specificity and efficacy of the antibody preparation, the MethylMAPS (Edwards et al., 2010) approach relies on digestion of genomic DNA with restriction enzymes that have identical recognition sites on DNA, yet vary in their sensitivity to the presence of 5-methyl cytosine. Paired-end sequencing DNA fragments derived from digestion with a methylation-insensitive restriction enzyme will yield from (potentially) all recognition sites of that enzyme in the genome, whereas sequencing DNA fragments generated with a cognate methylation-sensitive restriction enzyme (which will cut only non-methylated recognition sites) will produce data where reads originating from a methylated site are missing. Comparison of those two data sets will identify the position of methylated sites in the genome. The limitation in this technology is that methylation sites can only be detected if they fall within a restriction enzyme recognition site. Finally, bisulfite sequencing, long considered as the 'gold' standard for detecting DNA methylation, can be utilized in a next-generation sequencing approach (Bormann Chung et al., 2010). In this method, half the sample is fragmented and subjected directly to sequencing, whereas the other half will be subjected to bisulfite conversion before sequencing. This conversion will yield uracil in place of cytosine, whereas methylated cytosines are not converted. The DNA methylation pattern is then generated from a computational comparison of the two sequence outcomes to the reference genome of the species in question. While the bioinformatics demands of this experimental strategy are very high, it is nevertheless feasible to determine DNA methylation patterns on a genome-wide scale.

Next-generation sequencing technologies has also proven invaluable to measure RNA expression in a tissue or cell type of interest (Wilhelm et al., 2008), whether it is the entire transcriptional output (including coding and non-coding RNA) in an RNASeq approach (Mortazavi et al., 2008), or the extent of small RNA (including e.g., miRNA) expression (Kuchen et al., 2010). The high number of sequence reads that are possible to obtain from a single sample on a next-generation sequencing platform provides the accuracy and sensitivity needed for an in-depth unbiased analysis that is not possible via microarray analysis, and that has not been captured previously through EST (expressed sequence tag) sequencing approaches.

Sequencing strategies also have provided a break-through for the analysis of genome-wide landscapes of histone modification (Barski et al., 2007; Mikkelsen et al., 2007). Termed ChIPSeq, this technology relies on fragmentation of chromatin, enrichment of chromatin fragments containing the histone modification of interest via immunoprecipitation with specific antibodies, followed by purification of DNA from the enriched fraction. High-throughput sequencing allows the identification of recovered sequences in comparison to the reference genome of the species in question, and the number of mapped reads per genomic sequence region correlates to the presence of specific histone modifications on the DNA. Pioneering applications of this technology have been performed on embryonic stem cells (Mikkelsen et al., 2007; Goren et al., 2010) and on specific cell types of the immune system (Wei et al., 2009). The same technology – chromatin immunoprecipitation followed by sequencing the DNA in the enriched fraction – has been applied to identify transcription factor binding in embryonic stem cells (Chen et al., 2008) or tissues of the developing embryo (Gao et al., 2008), and it is possible to conduct sequential immunoprecipitation to investigate co-occurrence of DNA-binding proteins on chromatin. As discussed above, the high number of sequence reads – more than 1 billion reads are obtainable in a single instrument run – can provide a very high degree of coverage, and ensure the depth of analysis necessary to make confident conclusions from a genome-wide analysis.

One important caveat for the interpretation of any genome-wide epigenetic scan arises from the notion that the epigenome in the embryo may be cell type-specific. Similar to transcriptome profiling experiments, studies carried out on chromatin prepared from the whole embryo would be of an integrative nature. The readout would be the sum of all changes that occur, and while this approach would reveal where on the genome such changes might be evident, it suffers from the fact that not all epigenetic marks can be recognized. This is particularly important for the distinction of bivalent histone modification domains, where marks for activation as well as repression of transcription are simultaneously present at single gene loci (Bernstein et al., 2006). Such domains are deemed to be of developmental importance, and can only be recognized if the chromatin in question has been prepared from a defined cell population. Furthermore, similar to transcriptome profiling experiments on the whole embryo, specific changes could be missed if they occur only in a small population of cells in the embryo. The same considerations apply to measuring non-coding RNA expression.

In the absence of pertinent information, the determination of genome-wide distribution of epigenomic landmarks on the basis of the entire embryo represents a valid and necessary first step toward unraveling the secrets of the embryonic epigenome. However, the developmental stage of the embryo must be taken into consideration before undertaking this endeavor. Embryos at the patterning stage with a relatively small number of cell types, mostly undifferentiated progenitors, may be amenable to this analysis, whereas embryos at later stages may contain too many different cell types in various stages of differentiation, two issues that will make interpretation very difficult if not impossible. The increase in the complexity of cellular phenotypes obviously extends to the fetus. Cells of the fetus have diversified and progressed further along their differentiation path, and it can be presumed that the epigenome in each of those cell types has ‘matured’. Yet, if the notion of epigenomic plasticity is correct as discussed above, it stands to reason that the epigenome of cells at fetal stages of development would be subject to continuing maternal influences resulting from diabetes, obesity, or nutrition. In fact, it is currently unclear which stages of cell differentiation are susceptible to ‘epigenomic insults’, whether critical windows exist, or whether susceptibility is a continuum throughout development. Following the rationale above, it would be highly desirable to determine the status of the epigenome in specific, defined cell populations of interest.

For this purpose, one could envision the preparation of specific cell populations through the use of genetically manipulated mouse strains that express a reporter gene in defined cell types in conjunction with fluorescence-activated cell sorting techniques. Such an approach would yield specific cells of interest (e.g., neuroectodermal cells, mesodermal cells flanking the neural tube, or cells expressing a known ‘neural tube defect’ gene) where the effects of maternal diabetes on the epigenome could be measured without the confounding effects from other cell types. While labor-intensive, such an experimental strategy is likely to yield rich mechanistic insights into the molecular events triggered by maternal metabolic disease, and reveal a genome-wide set of target sites for future investigations.

Epigenetic changes and biological or pathological causality—The rapid technological advances in recent years have made it possible to extend the field of epigenetics research to many new paradigms. As outlined above, the new techniques are applicable on a genome-wide level, and it can be expected that a massive phase of data generation will ensue in the very near future. One can therefore expect that epigenomic findings can be strongly correlated with outcomes of pregnancies under adverse conditions.

However, one of the great obstacles to overcome in this field is the question how to prove causality of epigenomic findings for biological or pathological phenotypes. The common

principles for all four modalities of epigenetic information flow is that (1) they all can mediate pleiotropy as they can affect multiple genes simultaneously, that (2) any disturbance may act on pre-existing patterns which represent constraints on potential effects, and that (3) despite system-level alterations, outcomes may be cell type-specific. Together with potential crosstalk between the modalities, it presents a challenging problem for the biological interpretation of experiments.

In practical terms, it is now entirely feasible to characterize the primary interaction between maternal diabetes and the embryonic epigenome. One can envision that strong correlations can be established between maternal diabetes and the emergence of specific epigenetic signatures, in the form of histone acetylation patterns across the genome. The challenge is to explain how the observed changes to the epigenome (i.e., an epigenomic constellation) would relate to the phenotype of the embryo.

Traditionally, developmental mechanisms could be queried by the judicious use of loss-of-function phenotypes. The classic 'reiterative' approach consisted of generating targeted mutations, and gathering phenotypic readouts or determining effects on gene expression to identify a potential target for the next mutation approach. Information from such experiments could be integrated to reveal developmental pathways; this approach was highly successful for the delineation of cell differentiation pathways in various developmental systems such as the spinal cord (Tanabe and Jessell, 1996) or the pancreas (Sander and German, 1997). In the case of epigenetic changes, however, the problem presents how to test the biological or pathological function of a particular epigenomic-constellation-either at specific genomic sites or genome-wide that could be correlated with an upstream effect such as maternal diabetes.

In the case of microRNA or non-coding RNA alterations, this at least appears straightforward, as one can envision the generation of a targeted mutation of the microRNA itself, followed by testing the response of this new model in the experimental paradigm. Since most microRNAs are associated with a limited list of potential target genes, it is possible to focus the analysis of the outcome on those known target genes. The situation becomes more problematic when constellations consisting of DNA methylation or histone modification patterns are to be tested for their contribution to an overall phenotype. Such constellations may be very definitive with respect to single loci (e.g., histone trimethylation status at a promoter of interest) but it is more likely that such constellations consist of epigenetic marks at multiple sites, and potentially include several states at each of those sites. If constellations of DNA methylation changes or histone modification alterations were dependent on a single gene product, it would be feasible to test the effects by generating a loss-of-function mutation in the respective gene, and study the resulting animal model in the experimental paradigm. While examples for such targeted mutations exist in the mouse (Wagschal et al., 2008), the interpretation of such animal models was always focused on explaining the function of the gene in question; unfortunately, information on specific epigenome constellations (rather than e.g. a global effect on DNA methylation) in those loss-of-function mutants is scarce. However, neither DNA methylation, nor histone modifications are the result of single gene products, but emerge from the combination of the activities of several enzymes, and while many histone modifications have been described, the identities of the corresponding enzymes are not always known. Furthermore, it is quite possible that redundant enzyme activities exist. With respect to the use of targeted mutations, it is conceivable that mouse strains could be constructed that miss several epigenomic activities, as long as the respective targeted mutations do not affect viability or reproduction. Such strains could be highly valuable in determining functional epigenomic outcomes. Yet, in the case of mutations that cause embryonic lethality, the approach of using targeted mutations therefore becomes quickly unfeasible, as generating experimental

specimen with more than three unlinked mutations in homozygous state becomes impractical. It is also unlikely that a synthetic approach, generation of a specific epigenomic constellation *in vitro* and subsequent evaluation *in vivo*, would be possible. Therefore, the problem remains how to re-create any epigenomic constellation either for specific sites, for multiple loci simultaneously, or genome-wide, to test its biological effects.

One potential avenue might be the use of pharmaceutical compounds known to target epigenetic factors (Kirk et al., 2008; Feng et al., 2009) e.g. in the form of histone deacetylase inhibitors that are currently being tested in the field of cellular reprogramming toward induced pluripotency. Such compounds are developed with varying specificities, and could be useful for interventions with respect to basic science questions about epigenomic constellations. While it is difficult to imagine how such compounds would recreate particular epigenomic constellations, it is conceivable that interference with one or more specific enzymes could generate changes to the epigenome (i.e., new epigenomic constellations) that can then be correlated to the phenotype, and contrasted with constellations observed in embryos from normal, diabetic, or obese pregnancies.

In the absence of clear current tools for modeling epigenomic constellations, the challenge remains how to evaluate changes in the epigenome on a functional level. We believe that it might be necessary at this time to temporarily stop short of this goal and concentrate on a better characterization of outcomes on the level of the epigenome itself, as it remains a largely unexplored territory in the embryo. A prime example for epigenetic changes during development of a specific cell population is the analysis of epigenomic marks in primordial germ cells. These cells undergo dynamic changes in their content of modified histones and DNA methylation during their migration and differentiation in the developing embryo (Seki et al., 2007; Hajkova et al., 2008). While the initial reports focused on histological analyses of modified histones in primordial germ cells during their development, or on the DNA methylation status of specific genes at various stages, recent studies have extended the analyses to a genome-wide level (Daujat et al., 2009). The genome-level approach provided new insight into specific localization of histone marks on chromatin, and allowed comparisons to gene expression patterns and regions of transcriptional activity. While these studies did not address how the epigenome in this specific cell population may respond to external influences such as maternal metabolism or maternal nutrition, they illustrate how the application of genome-level technology would permit a more sophisticated biological analysis. Despite such pioneering studies, at this time, very little is known on the epigenomic status in specific cell types of the embryo or later in the fetus, how the epigenomic status might change over time during the course of development in those specific cell types, and how the epigenome can respond to maternal influences during development. Focus on such issues will not only create a rich insight into systems-level molecular mechanisms of development, but in conjunction with better transcriptome profiles, will reveal targets where maternal nutrition and metabolism impact the embryo.

CONCLUSIONS

The epigenome of the embryo is a largely unexplored territory. Yet, with its intricate contributions to transcriptional control of gene expression in the embryo and later in the fetus, it constitutes an essential element of developmental biology. Development under adverse condition such as maternal diabetes has profound effects on embryonic gene expression, and we anticipate that alterations of the epigenome comprise a mechanism to mediate the deregulation of gene expression and the loss of regulatory precision. In this context, the links between glucose and histone acetylation, or glucose and microRNA expression, are of particular interest, and suggest how epigenomic changes could arise in response to maternal hyperglycemia. With the advent of new genome-wide technologies, the

relationships between maternal diabetes, embryonic epigenome, and embryonic gene expression can now be subjected to a thorough experimental analysis. Despite these technological advances in the analytical methods, the challenge remains how to progress from establishing correlations between maternal metabolism and embryonic epigenome toward proving causality of epigenomic constellations for the molecular etiology of phenotypes, either for birth defects or for developmental programming. We believe that this is an obstacle that must be overcome, and new methods that allow simultaneous manipulation of multiple epigenomic targets are needed. While the epigenomic responses to maternal metabolism or nutrition can undoubtedly be a source of distress for the embryo, it also means that the epigenome is capable of plasticity. This plasticity may represent an opportunity for therapeutic intervention that could be used in conjunction with the control maternal diabetes, in the hope to improve pregnancy outcome. Therefore, a better understanding of the dynamics of the embryonic epigenome in specific cell populations during development is imperative.

Acknowledgments

This work was supported by NIH grants HD055528 (to JMS) and HD37804 (to CK)

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