The epigenetics of embryo development

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Implications

• A range of stresses experienced by the early embryo can alter the growth trajectory of the fetus and neonate and creates a predisposition to a range of metabolic diseases after birth. These effects limit an organism’s capacity to achieve its full genetic potential.
• Many of the techniques involved in the various forms of assisted reproductive technologies cause stress to the gametes and early embryo. In general, the more extreme the assisted reproductive intervention, the more severe the effect on the animal’s potential.
• These stresses appear to primarily act by changing the patterns of gene expression encoded by the epigenetic program created during embryo development. Epigenetics involves a range of mitotically heritable modifications to the structure of chromatin that govern the patterns and levels of gene expression. Each differentiation event occurring in early development involves fundamental changes in the cell’s epigenetic settings. Covalent modifications to the cytosine base of DNA, especially within cytosine-guanine dinucleotides, are one of the most important epigenetic mechanisms.
• While it is well established that assisted reproductive technologies perturb the epigenetic reprogramming of the early embryo, the molecular basis for this is yet to be fully described or understood.
• A greater molecular description and understanding of processes of epigenetic reprogramming is required to allow for adaptation of breeding and assisted reproduction technology programs that allow the full genetic potential of offspring to be realized.

Key words: blastocyst, chromatin remodeling, cytosine methylation, gene expression, germ cell, pluripotency

Introduction

Modern developmental epigenetics is the study of the processes that govern the patterns of gene expression that define each of the many cell types that go to make up the body. As such, it is central to a molecular description and understanding of embryo development. The cells of the early embryo all have the same developmental potential—the potential to form any of the cells of the body as well as the placenta and extra embryonic membranes (totipotency). As development progresses, differentiation into discrete cell populations occurs and each of these populations have a progressively restricted range of developmental outcomes. It is now recognized that cellular differentiation results from changed patterns of gene expression, and these patterns define not only the structure and function of the cell but also the range of developmental outcomes of each cell lineage. Underlying these changes in gene expression are changes in the structure and function of chromatin, which together confer these stable, but lineage-specific patterns of gene expression. The range of changes to chromatin required to achieve this are the basis of epigenetics. Importantly, these changes to gene expression occur without any change to the underlying nucleotide sequence of DNA.

It has long been recognized that the phenotype of an individual is created by the genetic code inherited from parents, but it is increasingly recognized that the manifestation of this genetic code can be modulated by plasticity in the epigenetic settings created during embryo formation. The environment in which the embryo, fetus, and neonate develops seem to create subtle alterations to epigenetic settings that can cause long-term changes in homeostasis. As a result, the environment experienced during development can have an important whole-of-life impact on the health and disease predisposition of offspring. There is also the potential for the transgenerational inheritance of some of these changes, where the environment affects epigenetic settings during germ cell formation. These effects are collectively referred to as the “developmental origins of health and disease.”

A precise description of the epigenetic processes that define critical stages of embryo development has become a key priority in the field. This understanding also offers the promise of a molecular understanding of how the multiple components of our environment influence and create phenotypic variability and how these effects might be influenced to provide the best long-term health outcomes.

The aim of this review is to provide an overview of some of the broad epigenetic features known to be involved in critical transitions in embryo development. Much of the discussion will be drawn from evidence in the mouse where most of the experimental embryology has been performed, and discussion will focus on the earliest stages of embryo development. Of the range of epigenetic mechanisms known to exist, greatest attention has been on the nature and levels of covalent modifications to the cytosine present in cytosine-guanosine dinucleotides of DNA, and this review will primarily focus on this epigenetic modification.

Embryo Development

Embryo development involves initiating a process of rapid proliferation of cells from a single-cell embryo (zygote) that is formed by fertilization. Initially, the resulting cells all have the unique capacity of totipotency (the capacity to form all of the specialized cell types of the embryo, the extra-embryonic membranes, and the placenta). As the number of cells within the embryo increases, they progressively begin to specialize in function by a process of differentiation.

In the mouse, the first differentiation event occurs several days after fertilization when embryos contain around 60 to 70 cells. This results in the formation of the blastocyst stage embryo, which possesses two distinct
Epigenetics

In classical embryology, the term epigenetics (or epigenesis) referred to the hypothesis that embryo development occurs as a progressive and gradual differentiation of the unstructured egg. This contrasts with the other classical hypothesis of preformation, whereby the embryo was argued to develop by a process of enlargement and elaboration of preexisting structures present within the egg. Modern molecular embryology shows that epigenesis is the primary developmental process, particularly in mammals.

Today, epigenetics refers to the range of changes to the structure and function of chromatin that governs the pattern and levels of gene expression in a cell lineage-specific manner. To be considered epigenetic, the change to chromatin structure must be mitotically heritable within a given lineage of cells. This definition of epigenetics distinguishes it from the range of acute changes in gene expression that occur in all cell types in response to changes to their circumstance. An easily understood example is the many changes in gene expression that occur in a cell on its exposure to a hormone to which it is responsive. These changes are typically transient and persist for only a short time after hormone exposure.

Interestingly though, there seems to be considerable overlap in the molecular mechanisms that drive stable epigenetic features and acute changes in gene expression, and this creates much confusion in the field. The key feature that defines a change in epigenetics is that they encode specific chromatin structures which induce patterns of gene expression that are mitotically heritable and define a given cell lineage. Thus, each cell lineage seems to have a well-defined unique pattern of gene expression. An emerging paradigm holds that a relatively small set of transcription factors create and define each of the major cell lineages, and it is likely that the epigenetic control of these core transcription factors at major embryonic transitions control differentiation. The resulting patterns of gene expression create the structures that we recognize as each of the specific cell types that make up the body, and in so doing, define the range of functions of each cell line.

Epigenetic Transitions in the Early Embryo

It is generally considered that two major rounds of epigenetic reprogramming occur during embryo development. The first of these reprogramming events occurs during the formation of the PGC (Kobayashi et al., 2013). The second round of reprogramming occurs in the early embryo soon after fertilization (Smith et al., 2012). Both of these events are considered to reset the epigenetic landscape to a ground state, from which differentiation to progressively more advanced lineages can occur. In both cases, profound changes have been observed in a range of epigenetic properties, and there is some similarity in both reprogramming events.

Reprogramming to these two epigenetic ground states, however, results in profoundly different developmental outcomes. The PGC differentiate into the gametes (sperm and eggs) while the pluripotent cells of the early embryo differentiate into all of the cells of the organism. These different developmental fates may suggest that the epigenetic programs created during these two reprogramming events are profoundly different. Yet, experimental results suggest this may not necessarily be the case. The most powerful finding is that when mouse ICM or PGC cells are isolated and then reintroduced into the early embryo, both cell types are capable of becoming incorporated into all the tissues of the body of the resulting offspring (Wood et al., 1993; Stewart et al., 1994). The chimeric individuals formed (including germ cell chimerism) are considered the gold-standard proof of the pluripotency of the tested cells. Furthermore, embryonic stem cells (ESC) derived from the ICM can be induced to form PGC-like cells, which when transferred to the gonads, readily differentiate into gametes (Hayashi et al., 2012).

Such results argue that the epigenetic ground state of both the PGC and ICM cells of the early embryo are sufficiently similar to allow the same developmental outcomes when the cells are placed within the same environment. Thus, the different developmental outcomes that normally derive from PGC and ICM may result primarily from their different developmental context. A pluripotent cell within the gonad receives the positional information that directs the epigenetic landscape to be altered to induce the cascade of differentiation events that leads to the formation of the gametes, yet when placed within the early embryo, these same cells receive different positional information that results in different epigenetic settings and their consequent differentiation along a normal embryonic lineages. The molecular nature of this positional information is yet to be fully defined but will include exposure to paracrine mediators and the differing nature of the extracellular matrix and cell-cell contacts present within each context. These results demonstrate that the interface between the epigenetic settings created within a cell and their developmental fate, depends on the environmental cues normally provided by their position within the space-time dimension of embryo development.
Epigenetic Mechanisms

The addition of a methyl (CH₃) group to the 5′-carbon of cytosine (5′-methylcytosine) in DNA was one of the first clearly defined epigenetic mechanisms discovered in mammals. This modification is catalyzed by a class of molecules known as the DNA methyltransferases (DNMT; Bird, 2002). A fundamental role in epigenetic reprogramming is suggested by observations of a profound global loss of DNA methylation in cells of the early embryo (Smith et al., 2012) and in PGC (Kobayashi et al., 2013). Furthermore, induced loss of methylation in somatic cells (by knockout of Dnmts in fibroblasts, for example) leads to changes in the expression of a large number of genes. By contrast, the absence of methylation in ESC (due to knock-out of Dnmts) has no obvious effect on these cells, but it does prevent their normal differentiation (Brown and Robertson, 2007). It is also well established that differentiation of pluripotent cells is accompanied by the methylation of the promoter of the essential pluripotency transcription factor, Oct4 (Athanasiaidou et al., 2010). Such observations have ensured that assessment of changes in DNA methylation is a dominant area of interest in considering the processes of epigenetic reprogramming of the early embryo and suggest that global demethylation may be a key component of epigenetic reprogramming to the pluripotent ground state.

Methylation preferentially occurs at CpG dinucleotides, and some regions of the genome have a much higher density of CpG dinucleotides than expected by chance. These regions are referred to as CpG islands (CGI), and many of these occur within regulatory regions (particularly gene promoters) of the genome. Consequently, much of the attention of the last few decades has focused on the possible roles of differential methylation of these CGI in genes of interest. The hypothesis that methylation of CGI is a major regulator of gene expression has been fostered by knowledge that control of expression of imprinted loci is in large part governed by differential allelic methylation of their control regions. The methylation of the parentally silenced allele is typically associated with the repression of gene expression from that allele (Wilkins, 2005).

The added methyl group protrudes into the major groove of the DNA double helix, and as such, provides a new topological feature to an otherwise topologically bland molecular landscape. This provides a binding or docking site for a range of proteins that specifically recognize the protruding methyl group. These binding proteins can, in turn, recruit a range of effectors that together act to regulate chromatin organization and patterns of gene expression (Bogdanović and Veenstra, 2009). One striking example is the binding of MBD1, a selective 5mC binding protein. Upon binding to methylated DNA, MBD1 is capable of recruiting many further proteins, including a host of other epigenetic modifiers, such as SETDB1 (a histone 3 lysine 9 methyltransferase; Sarraf and Stancheva, 2004). The methylation of histone 3 lysine 9 can, in turn, lead to the binding with DNMT (Fuks et al., 2003), hence providing a positive feedback loop between the DNA and histone methylation pathways, and also heterochromatin protein 1 (HP1; Stewart et al., 2005), which fosters the formation of heterochromatin. These interactions provide a mechanism by which methylation of regions of DNA can lead to its recruitment to the heterochromatic regions of the genome, and of course, a key feature of heterochromatin is that it is a region of relative repression of gene expression. Thus, differential regional patterns of methylation of the genome can result in lineage-specific patterns of heterochromatin formation, and hence, gene repression.

In the last few years, it has been discovered that 5′-methylcytosine is subjected to oxidation by the action of the TET family of enzymes to form sequentially 5′-hydroxymethylcytosine, 5′-formylcytosine, and 5′-carboxycytosine (Veron and Peters, 2011). The epigenetic significance of these modifications is still rather unclear. It is noteworthy that the proteins that selectively recognize and bind methylcytosine seem to have relatively low affinity for these TET-mediated modifications. 5′-hydroxymethylcytosine tends to be associated with euchromatin and so may mark regions of DNA that are to remain in the transcriptionally active fraction of chromatin. The 5′-formylcytosine and 5′-carboxycytosine modifications may mark regions for demethylation and perhaps form part of an active demethylation pathway. Much is yet to be understood of the roles and significance of these modifications, and it is currently an area of very intensive investigation (Kohli and Zhang, 2013; Figure 1).

Another major epigenetic mechanism is the post-translational modification of histone proteins. A wide range of covalent histone modifications have been described, and several are well recognized to be major determinants of gene expressivity (Lunyak and Rosenfeld, 2008). Most prominent of these include the acetylation and methylation of specific lysine (K) residues on histone 3 (H3). For example, acetylation of H3K9 (H3K9ac) and tri-methylation of H3K4 (H3K4me3) are associated with an open, euchromatin structure that permits easier access of transcription factors and the activation of gene transcription within that region. Conversely, H3K27 and H3K9 tri-methylation (H3K27me3 and H3K9me3) generally serve as repressive chromatin modifications by the creation of a more closed chromatin conformation, and these modifications are commonly associated with the formation of repressive heterochromatin (Ringrose and Paro, 2004).

Genome-wide mapping of H3K4me3 and H3K27me3 in ESC demonstrates that genes that carry H3K4me3, but not H3K27me3, are generally actively expressed. These include many of the core transcription factor genes that encode pluripotency. Many genes in pluripotent ESC are associated with both the active (H3K4me3) and repressive (H3K27me3) epigenetic signatures. This bivalent state is thought to generate a condition whereby the gene is “poised” for activation and held in a transcriptionally-ready state. Genes possessing this bivalent domain are commonly those that govern specification to differentiated lineages. By contrast, genes marked by H3K27me3 but not H3K4me3 are stably repressed (Mikkelsen et al., 2007). It is considered that the resolution of this bivalent state at poised loci is a key step toward the changes in gene expression required for lineage-specific differentiation. The actions of the Polycomb group proteins (PcG) have a key role in this resolution. The Polycomb repressive complex (PRC) functions as a transcriptional repressor. PRC2 is responsible for catalyzing the repressive H3K27me3 modification (Margueron and Reinberg 2011; Wong et al., 2014). PRC2 has a role in maintaining the pluripotent state and has markedly reduced expression on the differentiation of ESC (Boyer, 2006). Conversely, the H3K27me3 demethylases, UTX and JMID3, are absent from bivalent genes in ESC and are recruited to some of these genes, on differentiation (Lan et al., 2007).

There is considerable cross-talk between the histone modifications and the DNA methylation machinery. Thus, DNA methylation can cause the recruitment of histone methylases, and the methylated histones can, in turn, recruit DNMT to induce further DNA methylation. This can, in turn, lead to the spreading of methylated regions of the genome while special insulator regions of the genome can limit this spread to specific regions. DNMT1 possesses a binding site for histone deacetylases (Rountree et al., 2001), and these enzymes are well known to be associated with repression of gene expression via their action in remodeling nucleosomes. There is also evidence for a negative association between the presence of PcG and methylation of CGI within gene promoters (Klose et al., 2013), perhaps providing an explanation of the persistent hypomethylation of these sites relative to the majority of CpG in...
the genome. A hypothesis that is gaining currency is that DNA methylation provides a lock to the repressive state formed by some histone modifications.

**Setting the Embryonic Epigenetic Ground State**

A long-standing paradigm of developmental epigenetics has been that the global demethylation of the early embryo occurred in two distinct phases (Morgan et al., 2005). The first phase involves a global, active round of demethylation of the paternally inherited genome, which occurred within the first cell cycle after fertilization. By contrast, the maternally inherited genome was proposed to lose methylation progressively by a process of failed replication of the methylated state with each subsequent cell division (failure of maintenance methylation). The two processes combined were argued to result in the global hypomethylation of the entire embryo by the blastocyst stage followed by a marked round of methylation after implantation. This remethylation was proposed to favor the pluripotent epiblast over trophoblastic tissues. While this model has dominated thinking in the field for many years, it has recently come under reanalysis, and new results indicate that global demethylation is achieved by quite different processes.

Current modeling shows that the loss of methylation from the paternally derived genome is more modest than previously believed, and rather than global loss of methylation, there is some demethylation resulting in a reduction to a level similar to that observed in the more hypomethylated maternally inherited genome (Li and O’Neill, 2012). Upon syngamy, there is some further modest remodeling of the genome over the next several cell cycles but no evidence for a global failure of maintenance meth-

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**Figure 1.** Covalent modifications to cytosine. The actions of the DNA methyltransferase class of enzymes (DNMT) convert cytosine within CpG dinucleotides to 5’-methylcytosine. This modified base acts as a docking site for a host of epigenetic modifiers and commonly causes localization of the regions of DNA to the heterochromatin fraction. The TET family of enzymes can further modify 5’-methylcytosine to 5’-hydroxymethylcytosine (which tends to localize to the euchromatic fraction) and then successively to 5’-formylcytosine and 5’-carboxylycytosine. These two products may act as substrates in a DNA repair process that leads back to cytosine, potentially providing a pathway for demethylation.
ylation in the ICM cells. Intriguingly, this general trend did not hold for enhancer elements also showed the general pattern of extensive demethylation in the ICM compared with the trophectodermal lineage. After implantation, demethylation of the epiblast leads to resetting of high levels of methylation in the various somatic cell lineages formed, but in the PGC, demethylation is re-established until such time as differentiation during gametogenesis occurs. During this process, the higher levels of methylation found in sperm are laid down.

Base-level analysis of methylation status has been possible on ~90% of all CpG within the preimplantation embryo and gametes (Wang et al., 2014). The methylation state of only 6.8% of CpG in the genome remains stable from sperm to ICM of blastocysts through to Day 7.5 embryos. Of the stable CpG, most were unmethylated (mainly in CGI and promoters) in sperm, and the smaller number of stably methylated CpG were mostly in repeats or introns. Similar trends were observed for oocyte CpG. For most CpGs, there was a general trend for average methylation levels being higher in sperm than oocytes, and the level in two cells was a little lower than in oocytes. There was a further small decline in most classes of genomic elements in four cells and a large decline in the ICM. This general trend was evident for all classes of repeat elements, but some specific elements, such as intracisternal A particle genes (IAP), showed a much higher overall level of change during development. Methylation levels in enhancer elements also showed the general pattern of extensive demethylation in the ICM cells. Intriguingly, this general trend did not hold for CpG islands, which generally showed low levels of methylation throughout development. The selective demethylation of the emergent ICM may be associated with the reduced expression of DNMT3B in ICM compared with the TE (Hirasawa and Sasaki, 2009). DNMT3B expression is negatively regulated by the PRDM14 protein, and PRDM14 expression is a key determinant of the normal formation of both the ICM and PGC lineages (Grabole et al., 2013).

Primordial germ cells also undergo global erosion of DNA methylation. Loss of DNA methylation commences by at least Day 10.5 of development during the migration of the PGC from the hindgut to the genital ridge. Demethylation seems to be largely complete by Day 13.5 (Kobayashi et al., 2013). Demethylation occurs in PGC of both male and female embryos, and this erasure includes the imprinted loci. By Day 16.5, a marked remethylation of the germ cell genome occurs as gametogenesis commences, and this is greater in male than female germ cells. The global levels achieved at this time reflect the levels detected in the mature gametes. While the demethylation affects most regions of the genome, including many of the repeat elements, some regions avoid demethylation and the level of methylation is greater in the male than female germ cells. As is the case for the preimplantation embryo, the CGI in most promoters are relatively hypomethylated throughout this reprogramming of PGC and most show relatively little differential methylation during germ cell specification and maturatation. During the global remethylation event from E16.5, new patterns of methylation at imprinted loci are laid down (Kobayashi et al., 2013; Figure 2). Unlike the situation in the preimplantation embryo, the demethylation in PGC is reported to be accounted for by a failure of maintenance methylation. This is not mediated by an absence of DNMT but is due to the absence of UHRF1, which is a recruitment factor for DNMT1 (Kagiwada et al., 2013). This process does not explain how some regions remain methylated, however. Perhaps the regions that maintain methylation utilize other DNMT that do not require UHRF1, and it has been shown that DNMT3B acts to methylate some loci during this time of global demethylation (Hackett et al., 2012).

The global hypomethylation of pluripotent PCG and ICM cells contrasts with the relatively hypermethylated state of most differentiated somatic cell types. The purpose and role of this global demethylation is yet to be fully understood. Until recently, much of the attention to CpG methylation has focused on the differential levels of methylation of GGIs. This focus stems from the knowledge of the role of differential methylation of mono-allelically imprinted loci and the clear role of promoter methylation in repressing gene expression. In this respect, the discovery that the activity of critical pluripotency specifying genes, such as Oct4, are tightly associated with their levels of methylation further focus attention to this level of control. It is noteworthy, however, that methyla-
tion of Oct4 is a late event in differentiation and occurs after the repression of its expression (Radziszewska and Silva, 2014), and it has been suggested that this reflects a mechanism for “locking” the gene in a repressed state rather than a primary repressive mark (Hackett et al., 2012). Despite this focus on the CGI, the vast majority of these islands are hypomethylated in most cell types and show relatively limited differential methylation during epigenetic reprogramming of PGC and ICM compared with the very large changes in methylation of most other elements within the genome.

So this raises the question of whether CGI should be the primary focus in considering epigenetic programming. If the role of these two major rounds of global demethylation is not primarily to reprogram CGI, what is their primary purpose? Induced demethylation of somatic cells by knockout of Dnmts results in marked changes in gene expression, showing that hypomethylation does have a critical role in regulating gene expression. The observation that the enhancer regions (and particularly the super-enhancers, which are important in lineage specification; Whyte et al., 2013) show the general pattern of a marked hypomethylated state during embryo reprogramming (Wang et al., 2014) suggest that these may be important targets of epigenetic reprogramming rather than the CGI within promoters.

A small number of loci have been identified that do rely primarily on DNA methylation for their silencing. These are enriched for genes that are associated with the genome defense against transposable elements (either by piRNA or other mechanisms). These genes are known to be expressed in the germline, and it is therefore likely that on global demethylation, their expression is increased, thereby protecting the genome from expression of transposable elements that might otherwise be expressed because of their hypomethylated state. These methylation-dependent genes, therefore, act as part of a fail-safe mechanism to repress transposable element activity upon global demethylation (Hackett et al., 2012). This fail-safe is necessary because about 40% of the mammalian genome is composed of transposable elements (Waterston et al., 2002). The expression of these elements can lead to transposition and, hence, potentially harmful mutations. In somatic cells, the typically high levels of methylation of these elements are considered to be fundamentally important to the integrity of the genome by ensuring the transcriptional silencing of these elements (Yoder et al., 1997). If this is true then the developmentally associated rounds of global demethylation in the pre-implantation embryo and in PGC present a particular challenge to genomic integrity (Hackett et al., 2012).

The fact that the pluripotent cell populations within the embryo are the subject of global demethylation seems to expose them to the clear and present danger of genetic harm, for which a range of fail-safe rescue mechanisms are required to be activated. The significant nature of these risks provide a compelling argument that to warrant such a risk of harm, these demethylation events must provide essential epigenetic information to the developing embryo. One possibility may be that the cell does not have a mechanism that can allow it to precisely target loss of methylation to specific loci, including the imprinted loci in PGC, required for reprogramming to pluripotency. If so, a global loss of methylation may be the only mechanism capable of ensuring loss of methylation at all the required loci. A mechanism that allows active demethylation is yet to be unequivocally identified, and no clear mechanism for precisely targeting demethylation to multiple specific genomic sites has yet been described. Perhaps the only mechanism available to the cell for such large-scale reprogramming is to rather non-selectively wipe the epigenetic slate clean at these key embryonic transitions, which then allows the setting of a new epigenetic blueprint to be etched onto chromatin with the first differentiation events.

It is noteworthy that whole genomic methylome analyses of species such as the zebrafish embryos do not reveal similar rounds of global demethylation during development (O’Neill, 2013). It has been proposed that the presence of global demethylation in mammals may be an evolutionary adaptation required for placenta formation with viviparity. Specifically, it is suggested that global demethylation may have evolved in parallel with the evolution of parent-of-origin imprinting (Wang et al., 2014). If so, this would be consistent with a hypothesis that global erasure is required to allow removal of methylation at specific regions, such as the imprinted loci.

Perturbation of Epigenetic Reprogramming within the Embryo

It is increasingly recognized that exposure of the embryo to a range of stresses during the period of its epigenetic reprogramming results in a marked increase in abnormal developmental outcomes. One of the earliest
and most profound manifestations of this was evident with the advent of assisted reproductive technologies (ART), whereby a proportion of progeny show what became known as the “large offspring syndrome” (LOS; Behboodi et al., 1995). This is manifested as fetal and perinatal overgrowth and is generally associated with a range of congenital malfunctions and poor health outcomes and is commonly associated with changes in placental structure and function (Chavatte-Palmer et al., 2012). Large offspring syndrome is particularly evident in assisted reproduction in ruminants but has occurred to some extent in all mammalian species studied. In general, it seems the more extreme the intervention, the greater the risk and degree of fetal overgrowth. For example, in somatic cell nuclear transfer, where an unnatural process of epigenetic reprogramming occurs, there is commonly a high incidence of large offspring syndrome. With more routine interventions such as in vitro fertilization (IVF) and embryo transfer, the incidence is now becoming rare. Yet, reprogramming errors are still evident, and in human IVF programs where significant efforts at optimization have been applied, elevated rates of fetal overgrowth syndromes occur (Halliday et al., 2004).

While optimized ART conditions reduce the incidence of extreme abnormalities such as LOS, there is a good body of evidence that less dramatic examples of epigenetic perturbation still occur. Large offspring syndrome may result primarily from failure of the normal processes of parental imprinting of genes, yet IVF or cultured in vitro causes a change in the pattern of expression of a host of other genes that are not imprinted (Rinaudo and Schultz, 2004; Katari et al., 2009). Such changes, while not leading to neonatal overgrowth, can alter growth trajectories and abnormal allocation of tissue to organs (Mahsoudi et al., 2007). Most importantly, this effect can persist into the next generation, even after natural conception. This transgenerational effect is strongly suggestive of an epigenetic origin of these changes (Mahsoudi et al., 2007). This conclusion is supported by experiments using the agouti viable yellow (Avy) mouse; where coat color, body weight, and predisposition to diabetes in genetically identical offspring are determined by an epigenetically labile component of the promoter of the agouti gene. In this model, embryo culture from the zygote to blastocyst stage caused a 3- to 4-fold change in the rate of reprogramming of CpG methylation of this gene compared with embryos subjected to embryo transfer without culture (Morgan et al., 2008).

Even more subtle stresses during the peri-conceptual period also can cause whole-of-life changes in homeostasis. The exposure of rodent mothers during the peri-conception period to a diet low in protein, for example, alters subsequent fetal and postnatal growth trajectories and results in increased rates of hypertension and other metabolic and behavioral deficits in adults. As with more profound interventions, such as ART, a primary defect resulting from a low-protein diet in mothers are alterations to maternal and fetal nutrient transfer later in gestation (Sun et al., 2014). Thus, major consequences of disturbance of the normal pattern of epigenetic reprogramming in the early embryo are changes and defects in the normal structure and function of the placenta and extra-embryonic membranes. These changes affect fetal growth trajectories, which in turn, cause altered homeostatic settings in offspring.

**Conclusions**

DNA methylation is a key epigenetic modification essential for the maintenance of genomic integrity and has an important role in the formation of several pervasive epigenetic features, including heterochromatin formation, parent-of-origin genomic imprinting, and X chromosome inactivation (in females). The formation of the two pluripotent cell populations within the early embryo is accompanied by the profound loss of methylation across much of the genome. The formation of differentiated cell lineages from these pluripotent cells is accompanied by extensive remethylation of the genome, and this is accompanied by the setting of defined patterns of lineage-specific gene expression.

A detailed understanding of the factors that perturb the normal processes of epigenetic reprogramming is required to avoid such serious congenital abnormalities as LOS but will also allow the design of breeding programs that minimize phenotypic variability and thus maximize productive outcomes. Such programs will allow the full genetic potential of breeding stock to be realized and thus maximize economic returns.

Recent reanalysis of demethylation in the preimplantation embryo shows that it is an event more closely associated with the formation of the pluripotent state rather than the totipotent state, and this is consistent with demethylation associated with the formation of the pluripotent PGC. The association of global demethylation with the formation of pluripotent cells and remethylation with their differentiation provides a very strong prima facie case for an essential role for this epigenetic modification in embryo development. For many years, the role of methylation in the differential activity of gene promoters, such as Oct4, has been a favorite hypothetical target of regulation. Yet, recent genome-wide analysis indicates that changes in methylation of promoters is rather modest compared with most other elements within the genome. These new results indicate that new models for thinking about the processes of epigenetic reprogramming during embryo development are required.

Currently, a major limiting factor to the field is a detailed understanding of the processes for regulating demethylation, particularly active demethylation. It is not clear whether a mechanism exists that allows directed site-specific demethylation, and if so, how it would be controlled for the wide-ranging modifications thought to be associated with reprogramming to pluripotency. One possible explanation for the pervasive loss of methylation associated with the formation of pluripotent ICM and PGC cells may be to achieve demethylation at essential sites by cleansing most sites of this mark. Differentiation of the pluripotent cells could then be achieved by re-establishment of these marks as is appropriate for each new lineage. There are many questions yet to be addressed in this burgeoning field. The advent of powerful new analytical tools will ensure that it is a field of exponential growth of insights in the coming decade.

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