Metaboloepigenetics: providing alternate hypotheses for regulation of gene expression in the early embryo

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Abstract

The metabolic and epigenetic landscapes of the pre-implantation embryo change and evolve rapidly as the embryo travels through the reproductive tract. The maternal and paternal genomes combine, rapid cell division is initiated, potency is re-established and eventually differentiation begins, all in the absence of a vascular supply delivering oxygen, nutrients and a functional waste removal system. In recent years, it has become clear that environmental challenges to the developing embryo, including maternal diet, stress and inflammation, alter its long-term trajectory, although the exact signaling molecules, which are recognised by the embryo, and the mechanisms by which these signals are translated into long-term outcomes, remain elusive. Recently, it has become apparent that energy or fuel-sensing metabolic pathways interact with important epigenetic regulators of chromatin structure, to regulate gene expression. While this has not yet been explored in the pre-implantation embryo, the interaction between these two key cellular systems, - metaboloepigenetics - is a plausible mechanism by which gene-environment interactions occur, and by which the embryo's trajectory is established. This review explores the metabolic and epigenetic plasticity of the early embryo, and how the two systems intertwine to propagate the next generation.

Keywords: embryo, epigenetics, metaboloepigenetics, metabolism, transcription.

Metabolism: powering pre-implantation embryo development

Metabolism in the pre-implantation embryo is the co-ordination of energy intake, production and use, which allows the embryo to sustain the rapid cell division in a highly unique a vascular environment, required for propagation of the species. In animal cells, mitochondria are the organelles, which have evolved to most effectively produce energy, and their activity has been heavily studied in pre-implantation embryos in many species. The embryo's requirements for energy substrates change rapidly as development proceeds, favoring oxidative phosphorylation in the early stages, and glycolysis closer to implantation.

The ability for the embryo to undergo pronuclear formation, syngamy, embryonic genome activation, successive mitoses, compaction, lineage differentiation and blastocoel development are based on the intrinsic capacity of the embryo to regulate the temporal and spatial distribution and consumption of energy. Decades of elegant work in several species have explored the energetic requirements of the pre-implantation embryo, both *in vivo*, and under varied *in vitro* conditions. The early cleavage embryo is almost entirely dependent on oxidation of substrates including pyruvate (Gardner and Leese, 1988; Butcher *et al*., 1998), lactate (Lane and Gardner, 2000) and amino acids (Gardner and Lane, 1993; Van Winkle, 2001) to sustain the production of ATP. A primary function of this pyruvate-to-lactate conversion is thought to be the regeneration of NAD^+ for subsequent use in glycolysis, which, although normally occurring under anaerobic conditions, occurs in the presence of oxygen in the reproductive tract (Krisher and Prather, 2012). During this period of mitotic cell division, the embryo has been proposed to have a "quiet" metabolism (Leese, 2002; Baumann *et al*., 2007), partially because although DNA replication and cell division are occurring, cellular volume decreases with each division (Turner *et al*., 1994), maintaining a moderate requirement for energy, and in turn, oxygen. In contrast, evidence in human embryos suggested that embryos which resulted in a clinical pregnancy had higher glucose consumption than those that did not (Gardner *et al*., 2011). Perhaps it is the fine balance between energy consumption and utilization, which determines the long term embryo's viability.

With an increased requirement for protein synthesis and transcription, and the necessity of blastocoel formation, there is an up-regulation from the "quiet" metabolic homeostasis, to a dramatically higher level, which is associated with a switch away from oxidative phosphorylation towards glycolysis (Leese, 1995), with the embryo demonstrating a significant capacity for aerobic glycolysis (Gardner and Leese, 1988). The mechanisms by which this switch occurs remain unclear, but it appears to be regulated by the presence of glucose, increasing the expression of glucose transporters (reviewed in Purcell and Moley, 2009). This dramatic increase in the metabolic capacity of the embryo is likely necessary to power the Na,K-ATPase, initially pumping fluid into the intracellular

spaces, and later into the extracellular spaces, forming the blastocoel cavity (Biggers *et al.*, 1977; Borland *et al.*, 1977a, b). Ion transport systems for Na$^+$, Cl$^-$, K$^+$, Ca^{2+} and Mg^{2+} concentrate ions within the blastocoel, further supporting the water movement necessary for formation of the fluid-filled cavity (Borland *et al.*, 1977a, b).

It has been proposed that this switch from oxidative phosphorylation to glycolysis prepares the embryo for the hypoxia it will face from the time of implantation until remodeling of the spiral arterioles of the human placenta at the end of the first trimester (Burton *et al.*, 2010; Cartwright *et al.*, 2010), and perhaps explaining the choice for inefficient energy production. Additionally, the glucose provides the pentose sugars for nucleic acid synthesis, and is required for both phospholipid and non-essential amino acid biosynthesis, which supports the rapid cell division (O'Fallon and Wright, 1986; Wales and Du, 1993; Cairns *et al.*, 2011; Gutnisky *et al.*, 2014). Although an inefficient means for generating ATP, perhaps this metabolic adaptation is important in biomass production and redox regulation, and has been likened to the Warburg Effect, a hallmark of highly proliferative cells, particularly cancer cells (Krisher and Prather, 2012).

Metabolic plasticity allows for adaptation to stress induced by *in vitro* culture, diet and other environmental challenges and contaminants. Prior to embryonic genome activation (which is as late as the 4th mitosis in some species), levels of active transcription are very low or non-existent, and metabolism provides a mechanism by which the embryo can still respond to altered surroundings. But is it possible that this metabolic plasticity, which promotes embryo survival in the absence of transcription, also has detrimental effects? Is altered metabolism changing the abundance of stored transcripts, or altering the epigenetic landscape, thereby changing the trajectory of the embryo forever? Is the aerobic conversion of pyruvate to lactate in the embryo necessary for the maintenance of NAD$^+$, which is known to regulate families of chromatin-modifying enzymes?

Epigenetics: a changing landscape during pre-implantation embryo development

It should come as no surprise that the epigenome of the early embryo is a rapidly changing landscape. The sperm and egg, two highly specialised cell types, come together, undergo thorough chromosomal rearrangement to fuse the maternal and paternal genetic material, and then begin to divide as a single entity, no longer highly specialised, but with the ultimate potential to form every cell type. Epigenetics, or the study of cellular traits and phenotypes that are mitotically inheritable, is the mechanism by which these highly specialised characteristics are erased, and potency re-established (Santos *et al.*, 2005; Goldberg *et*

al., 2007; Shi and Wu, 2009). This potency again becomes restricted, with each cell division progressively restricting the range of developmental outcomes. Although the concept sounds simple, the field of early embryo epigenetics has been wrought with inconsistency, controversy and challenge. Concurrent with technological advancement, the field of epigenetics has grown to include not only DNA methylation, the best described epigenetic modification in the pre-implantation embryo, but also histone modification and non-coding RNA, just to name a few.

Early studies describing the DNA de-methylation patterns of the early embryo suggested two distinct phases: a global, active (non-replication dependent) round of demethylation of the paternal genome, followed by a progressive, passive loss of methylation of the maternal genome as cell division proceeded (Mayer *et al.*, 2000; Morgan *et al.*, 2005). This active loss of paternal genome methylation was supported by studies confirming that Ten-Eleven Translocase family member, TET3, was responsible for the conversion of 5-methyl cytosine to 5-hydroxymethyl cytosine, and its subsequent removal via iterative oxidation (Gu *et al.*, 2011; Wossidlo *et al.*, 2011).

Most recently, single base resolution MethylC-seq revealed that during pre-implantation embryo development, most functional genomic elements undergo significant demethylation, except CpG islands (CGIs) and 5' untranslated regions (UTRs) whose methylation levels are already very low in gametes (Wang *et al.*, 2014). Additionally, they demonstrated that by generating single-base resolution, allele-specific whole-genome methylomes, the paternal methylome and at least a significant proportion of maternal methylome goes through active demethylation during embryonic development, based on the presence of the oxidised methyl cytosine bases (5hmC, 5fC; Wang *et al.*, 2014). With the possibilities of single cell, single base resolution methylation analysis within reach, we are likely to see increased clarity and understanding not only of the roles of DNA methylation in functional and structural genomics - including heterochromatin formation, X-chromosome inactivation and genomic imprinting - but also of epigenetic heterogeneity during early development, and the mechanisms by which loci-specific methylation alterations occur.

It is important to recognise, however, while DNA methylation is the epigenetic mechanism, which has been most extensively researched in the early embryo, many others are active and function to regulate chromatin structure, transcription and cell division. Histone modification has been explored in a number of species, at all stages of pre-implantation embryo development (reviewed in Beaujean, 2014a, b). Many modifications on H2, H3 and H4 have been described, although the technologies to date have been predominantly via immunohistochemistry (IHC), which allows for the global analysis of only one single

modification at a time. Current technologies for exploring loci-specific changes in multiple histone modifications aren't yet possible for single cell analysis, and consequently very little is known about the loci-specific changes in adjacent modifications during development. What is clear is that when the paternal genome enters the oocyte at fertilisation, the protamine is removed and replaced with oocyte-derived histones (McLay and Clarke, 2003). Although the histones are maternally-derived, the maternal and paternal DNA display dramatic asymmetry of a number of described histone modifications (Reik *et al.*, 2003; van der Heijden *et al.*, 2005), which is detectable until the four cell stage for some modifications.

It is clear that the most active time for epigenetic remodelling corresponds with the embryo's greatest sensitivity to the health of the maternal and paternal milieu, through the environment created within the reproductive tract, which may present both metabolic and immune challenges. The question that remains poorly answered is 'how'? Here we outline the evidence for how metabolism links in with epigenetic stability mechanisms, enabling the plasticity necessary for development, as well as long-term adaptation to the peri-conception environment.

Metaboloepigenetics: can metabolism alter the epigenetic landscape of the pre-implantation embryo?

In recent years, a number of hypotheses have emerged that propose an interaction between the epigenome and cellular metabolism which results in alterations to cell phenotype. If this is the case, stimuli or environmental changes that alter metabolism during a period when the epigenetic landscape of the embryo is naive, could alter the long-term trajectory. Epigenetics involves alterations of the DNA and chromatin by a range of enzymes which add and subtract a number of chemical modifications including methyl, acetyl and phosphate groups (Bannister and Kouzarides, 2011). The wide variation in modification types as well as the high number of potential target sites on both DNA and histones lends plausibility to the likelihood that at least some of them may be influenced by alterations in metabolites or their by-products. Adding further support to this hypothesis, a number of direct links have been identified between the enzymes and processes responsible for alterations in chromatin structure, and metabolism. For example, both DNA methyl transferase enzymes (which actively methylate DNA) and histone methyl transferase enzymes require S-adenosyl methioinine (SAM), a product of 1-carbon (1C) metabolism, to carry out their functions. Flavin adenosine dinucleotide (FAD) and alpha-ketoglutarate (α-KG) are essential co-factors for the reverse reaction, removing the methyl groups (Iyer *et al.*, 2009). FAD in its reduced state ($FADH_2$) carries energy; when

oxidised, it is utilised in oxidation reactions within the tricarboxylic acid (TCA) cycle, also known as the Krebs or citric acid cycle, which forms a key part of aerobic respiration. Similarly, α-KG is an intermediate in the TCA cycle, and is derived from glutamine. Along with Fe^{2+}, it acts as a necessary co-factor for the TET family of enzymes, which actively remove methyl groups from DNA (Wossidlo *et al.*, 2011). It is well documented that certain stressors, such as *in vitro* culture, during early development alter the metabolism of the embryo. Given the evidence discussed above, that both the maternal and paternal genomes are targets for active DNA demethylation by the TET family of enzymes in early development, it is perhaps no surprise that there are changes in DNA methylation status following stressors known to change metabolism (eg. *in vitro* culture (Stojanov and O'Neill, 2001; Morgan *et al.*, 2008).

This interaction between metabolism and epigenetics is not unique to the methylation modification. Acetylation, recently shown to be as abundant as phosphorylation on the proteome, results from the covalent addition of an acetylCoA molecule to a lysine residue. When this modification occurs on histone tails, acetylation alters higher-order chromatin structure, whilst also serving as a docking feature for histone code readers (Choudhary *et al.*, 2009). In mitochondria, acetylCoA is derived from citrate, which is in turn synthesised from glucose in the TCA. AcetylCoA, however, cannot cross the mitochondrial membrane, so for nuclear acetylCoA to be derived, citrate diffuses across the mitochondrial membrane, enters the nucleus via nuclear pores, and can then be converted to acetylCoA via adenosine triphosphate (ATP)-citrate lyase (ACL), promoting histone acetylation through increased substrate availability (Reytor *et al.*, 2009; Wellen *et al.*, 2009). Interestingly, there is a reduction of acetylation around genes critical for regulating glycolysis, resulting in a down-regulation of their transcription, and an inhibition of glycolysis, supporting intrinsic metaboloepigenetic interactions (Wellen *et al.*, 2009; Martinez-Pastor *et al.*, 2013). This provides an example of how "fuel-sensing" pathways involved in glucose metabolism can regulate chromatin, and in turn, transcription.

There is also a well-described influence of metabolism on the removal of acetyl groups by histone deacetylases (HDACs), which include a family/class of NAD^+-dependent enzymes, the Sirtuins (North and Verdin, 2004). NAD^+ accepts electrons from other molecules as it is reduced to NADH. This change in redox state is important for mitochondrial function, where it links the TCA cycle to the electron transport chain, with NADH donating the first electron for ATP production. The Sirtuin family of enzymes (HDAC Class III) are regulated by NAD+/NADH and tightly regulate glucose metabolism, such that *Sirt6* null mice have highly upregulated glycolysis, which triggers fatal hypoglycaemia at a young age (Zhong *et al.*, 2010).

Interestingly, lactate, a product of metabolism in the post-compaction embryo, is also known to inhibit HDACs (Latham *et al.*, 2012). With the tight dependence on availability of both acetylCoA and NAD$^+$ the presumption is that small changes in mitochondrial function, and/or alterations in redox status could dramatically alter the acetylation status of the histone landscape in the early embryo. Acetylation removes the positive charge on the histone, altering the interaction of the tails with the negatively charged DNA and relaxing the chromatin structure, promoting the transition from heterochromatin to euchromatin, and subsequently, transcription (Verdone *et al.*, 2005). Given the importance of activation of the embryonic genome, and embryonic transcription during pre-implantation embryo development, it is conceivable that small perturbations to metabolism, like the ones seen during assisted reproductive technologies such as *in vitro* culture, and hormonal hyperstimulation, may alter the timing of development, as well as the activation of the embryonic genome.

Perhaps the most recently described and most poorly characterised epigenetic modification is that of O-linked glycosylation. O-linked glycosylation is a nutrient sensitive post-translational modification that involves the enzymatic addition of O-N-acetylglucosamine (O-GlcNAc) to serines and threonines in a manner akin to phosphorylation, on histone tails as well as a number of other chromatin-modifying proteins (Zhang *et al.*, 2011). O-linked glycosylation has been demonstrated to target histone tails using the TET family of DNA-modifying enzymes, which are also metabolically linked (described above; Chen *et al.*, 2013). While the function of glycosylation of histone tails is not well characterised, this O-linked glycosylation is also known to affect other key regulators of transcription including RNA polymerase II, where it competes with phosphorylation to modify the C-terminal domain (CTD), as well as targeting histonemethyl transferase and histone deacetylase complexes, and the repressive Polycomb complex (reviewed in Hanover *et al.*, 2012). The addition of this molecule via the O-linked N-acetylglucosamine transferase (OGT) enzyme is regulated by flux of activity through the hexosamine biosynthesis pathway, which converts glucose to hexoses, and which is nutrient, or glucose sensing (Obici *et al.*, 2002). This type of modification provides a direct mechanism by which availability of nutrients and metabolites can directly alter higher-order chromatin structure and organisation. In this way, alterations in nutrient availability, and modest changes in metabolism in the early embryo may dramatically alter the epigenetic landscape, and as such the trajectory of the embryo.

In addition to the covalent modifications that occur on the chromatin, non-covalent alterations, regulated by other chromatin remodelling complexes, are able to move, eject, or restructure nucleosomes.

These use DNA-dependent ATPases, therefore requiring ATP as a co-factor for regulating transcription (Varga-Weisz, 2001). With sources of ATP switching from oxidative phosphorylation in early embryo development, to glycolysis later in development, it is not yet clear how these processes are regulated in the early embryo, but will no doubt be the focus of future research.

The interactome: is metabolism altering RNA storage and stability in the pre-implantation embryo?

Early embryonic stress can result in a number of poor outcomes, including but not limited to embryonic loss, deformity and defects and a range of adult onset disorders. However, the mechanisms by which stress negatively impacts the embryo long-term remain largely elusive. De novo transcription does not occur during oocyte maturation or the first cell cycle of the embryo in mouse. It is likely that the proteins required for the general reprogramming of the early embryo are translated from pre-existing mRNAs produced and stored during oogenesis. In particular, the zygote needs to switch from meiotic to mitotic divisions and to reprogram the haploid, specialised genome of the gametes into a totipotent diploid genome (Messerschmidt *et al.*, 2014). Additionally, the zygote must resume the mitotic cell cycle, remodel the maternal and paternal chromatin (including the protamine-histone exchange in the paternal genome), activate transcription, and initiate the embryonic developmental program. Evidence suggests that maternal mRNAs are stored during oogenesis in an inactive state until they are recruited for translation (Oh *et al.*, 2000). One mechanism by which this is proposed to occur is by elongation of the poly(A) tail (Piko and Clegg, 1982; Latham *et al.*, 1991). It is very likely that other mechanisms exist, which may include unique RNA binding proteins, RNA modification and other complex nucleic acid interactions (ie. lncRNAs).

In spite of the importance of these early events, there is very little information detailing them in the early embryo, a likely consequence of the difficulties associated with working on a single, transcriptionally inactive cell. It is clear that a fine regulatory network controlling the spatial and temporal abundance of RNAs, as well as preventing RNA loss or premature translation must be present in the early embryo, to ensure reproductive success. In addition to the vulnerability of transcriptional inactivity, the pre-implantation embryo is in a window of epigenetic naivety, with the active and passive removal of the specialised germ-cell program in order to re-establish potency (Smith *et al.*, 2014). Unable to respond by transcribing new pathways during "stressful" situations, the embryo is likely left to make the most of its active metabolic state, and mRNA reservoir, to adjust to

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hostile situations (e.g. infection, hyperglycaemic stress, oxidative stress). This idea is supported by the "quiet embryo hypothesis", which proposes that the most developmentally competent embryo is that which has an efficient, but not high, metabolic activity (Leese, 2002). An example of this is *in vitro* culture; embryos derived *in vitro* are more metabolically active, and as such, more stressed than their *in vivo* counterparts.

Several enzymes that were once characterised only as metabolic enzymes have gone on to be described as RNA binding molecules. Enzymes involved in glycolysis and the pentose phosphate pathway including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldolase, lactate dehydrogenase (LDH), phosphoglycerate kinase (PGK) and glucose-6-phosphate dehydrogenase (G6PDH) are now known to bind transcripts including but not limited to those of glucose transporters (McGowan and Pekala, 1996), immune regulators (GMCSF; Pioli *et al.*, 2002), IFNα and IL2 (Nagy and Rigby, 1995) and tRNA and rRNA (Ryazanov, 1985; Singh and Green, 1993; reviewed in Ciesla, 2006). A landmark paper in the RNA field developed and utilised "interactome capture", a process of cross-linking all the RNAs within a cell at a given time to their protein partners. Using this technology, they categorised families of RNA binding proteins which had until recently been characterised only as functional metabolic enzymes (Castello *et al.*, 2012). In addition to enzymes involved in carbohydrate, amino acid, lipid and nucleotide metabolism, eighteen of the RNA-binding enzymes used NAD^+, $NADP^+$, NADH, NADPH, FAD or $FADH_2$ as co-factors, and ten of these shared the ability to bind ATP and an anionic substrate such as succinate, L-aspartate or pyruvate (Castello *et al.*, 2012). A RNA, enzyme and metabolite network hypothesis proposed that if these interactions were functionally relevant, then these proteins could broadly connect metabolism, RNA biology and post-transcriptional gene regulation.

This raises the possibility that under an altered metabolic state, either by pharmaceutical manipulation or in response to stressors, the embryo may employ the metabolic enzymes in their roles as RNA-binding proteins, to respond metabolically to the stress. This may result in the loss of stored RNA, or immature translation. This novel concept provides a mechanism by which an acute metabolic insult could negatively influence the trajectory of embryo development both in the short and longterm, by altering the control of RNA stability and translation.

Conclusion

It is a fascinating time for pre-implantation embryo research, with evidence from many fields of biology supporting important regulatory cross-talk between metabolism and epigenetics. With technological advances allowing single cell epigenome and metabolome analysis, the field of embryo metabologenomics is likely to flourish in the coming years. Understanding the mechanisms by which these two systems intertwine is likely to reveal the means by which gene-environment interactions regulate the transgenerational inheritance of health and disease, established during the first few days of life.

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