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Environmental and epigenetic effects upon preimplantation embryo metabolism and development

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Abstract

In vitro fertilization has provided a unique window into the metabolic processes that drive embryonic growth and development from a fertilized ovum to a competent blastocyst. Post-fertilization development is dependent upon a dramatic reshuffling of the parental genomes during meiosis, as well as epigenetic changes that provide a new and autonomous set of instructions to guide cellular differentiation both in the embryo and beyond. While early literature focused simply on the substrates and culture conditions required for progress through embryonic development, more recent insights lead us to suggest that the surrounding environment can alter the epigenome, which can, in turn, impact embryonic metabolism and developmental competence.

Preimplantation Embryonic Development and Vulnerability

Stimulated by the increasing worldwide use of assisted reproductive technologies (ART) (Text box 1), there is renewed interest in the scientific evaluation of *in vitro* culture systems during early embryonic development. While much has been learned about the metabolic processes required for embryonic development from a fertilized ovum to a competent blastocyst, increased appreciation for the vulnerability of the developing embryo to the surrounding environment during *in vitro* fertilization has sparked concern about whether the culture conditions appropriately replicate the conditions *in vivo*.

Text Box 1

Assisted Reproductive Technology (ART)

ART consists of clinical procedures that include the *in vitro* handling of human gametes or embryos for the purpose of establishing a pregnancy [97]. While the process varies for each individual or couple, the general scenario begins with controlled ovarian stimulation using gonadotropins (FSH, LH) and an oocyte maturation trigger (hCG, GnRH agonist) to induce the development of multiple ovarian follicles with the goal of obtaining

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multiple mature oocytes. After transvaginal aspiration of follicular fluid, oocytes are retrieved and either cryopreserved or inseminated to produce zygotes. Prior to fertilization, semen is processed to remove seminal plasma and inactive cells from motile sperm. While semen is most often obtained by masturbation, men with oligo- or azospermia may require procedures such as percutaneous epididymal sperm aspiration, testicular sperm extraction (TESE) or microsurgical epididymal sperm extraction (MESE). Oocytes are incubated in culture medium with 10,000 – 75,000 sperm for about 18 hours. In cases of low sperm count or function, intracytoplasmic sperm injection (ICSI) can be performed, microinjecting a single spermatozoan into the oocyte cytoplasm. Successfully fertilized oocytes are identified one day after insemination or ICSI by the presence of two pronuclei. With further culture, embryos develop through the cleavage stages and form blastocysts within about 5 days. Viable embryos can then be cryopreserved, now most often through vitrification, or transferred directly into the uterus at any stage to establish pregnancy.

The preimplantation embryo must undergo a highly organized sequence of events, including pronuclear formation, syngamy, embryonic genome activation, successive cell cleavages, compaction, lineage differentiation and blastocoel formation [1], (Figure 1). These complex activities require that the parental genomes be combined into a new and autonomous blueprint to guide cellular differentiation, which can only occur in the presence of adequate energy production and distribution. Early efforts focused on embryonic metabolism as the key to competent development, assuming that the embryo could develop unperturbed if only the appropriate combination of resources were made available [2, 3]. However, the preimplantation embryo is complex and a complete understanding of the processes that determine its normal development is lacking.

While adequate metabolism is vital, a growing understanding of epigenetics has highlighted the vulnerability of the intricate systems that regulate gene expression, metabolism, and embryo survival. It is now clear that the environment in which the embryo develops can alter its metabolism, epigenetic marks, and developmental capacity, with potentially far-reaching consequences. Here, we present an overview of the current understanding of embryonic metabolism in the context of epigenetic regulation and explore how their interdependence could influence embryonic development, fetal outcomes, and adult-onset disease risk.

Lessons from *In Vivo* Physiology

The *in vivo* physiologic environment of the early embryo is tightly regulated. The fluid composition of the oviduct and uterus demonstrates a prominent substrate gradient, with pyruvate and lactate levels significantly more abundant in the human oviduct than in the uterus, and glucose concentrations over five times higher in the uterus than the oviduct [4]. It has long been understood that the survivability of mouse embryos of different developmental stages is not necessarily the same when cultured in similar media [5], further suggesting physiological differences in metabolic requirements along the developmental journey.

The oxygen concentration *in vivo* is also tightly regulated through a dynamic process that is spatially and temporally dependent. The mean oxygen tension in the female reproductive tract of several species is approximately 40% of atmospheric oxygen (160 mm Hg) and is regulated by the endocrine status of the animal, which reduces oxygen further at the time of implantation [6]. More specifically, in rhesus monkeys, the oviductal oxygen tension is cycle-dependent and ranges from 35 – 61 mm Hg, while the uterine oxygen tension is

consistently lower at approximately 13 mm Hg. A similar reduction in oxygen tension from the oviducts to the uterus is present in rabbits [6].

Evidence that early human development occurs in a low oxygen environment has been documented in studies of implantation and first trimester fetal development. The early fetus develops at low ambient oxygen tension until 10–12 weeks of gestation when intervillous blood flow increases dramatically, as determined by radiography and measurement of intervillous oxygen tension [7]. Placental antioxidant enzyme concentrations are low in the first trimester and increase with gestational age, concurrently with placental oxygenation and the resulting production of reactive oxygen species (ROS) [7]. The low oxygen environment may be beneficial to the preimplantation embryo and first trimester fetus by reducing detrimental ROS during the period of rapid cell proliferation that accompanies development.

No Innocent Bystander: The *In Vitro* Environment and Embryo Metabolism

The culture environment impacts embryonic development in both significant and subtle ways. For example, small aberrations in the intracellular pH of mouse embryos reduces blastocyst cell number, inner cell mass, and survival, as well as fetal weight [8]. Closer examination of cellular substructures reveals that mitochondria are particularly vulnerable to the culture environment. Intracellular pH disturbances affect mitochondrial distribution and subsequent embryonic development [9].

Mitochondria are the site of oxidative phosphorylation, the primary pathway for energy production in the preimplantation embryo. Recent work with oocytes and embryos reveals that mitochondrial distribution and structure are critical to normal embryonic metabolism [10]. Hamster embryos display a notable migration of active mitochondria into dense perinuclear clusters in the cytoplasm shortly after fertilization that is positively correlated with developmental competence [11]. Murine and human oocytes and embryos also display non-uniform clustering of mitochondria that is stage-specific and includes subplasmalemmal groups of highly polarized mitochondria [12], (Figure1). Changes in mitochondrial distribution could create microdomains of specialized energy production, oxygen sensing, or pH gradation that provide optimal conditions for particular developmental activities (e.g., compaction or cavitation) [13]. The perinuclear distribution of both mitochondria and actin microfilaments were disrupted in hamster embryos exposed to glucose and phosphate, leading to a developmental block at the 2-cell stage, demonstrating that exposure to “physiologic” metabolic substrates at inappropriate stages may alter cellular metabolism and development [3]. Mitochondrial number also affects embryonic energy production and likely must reach an unknown threshold to provide sufficiently for growth and development [14, 15]. Taken together, mitochondria are key sites of embryonic metabolism and must have normally functioning proteins for oxidative phosphorylation, adequate numbers, appropriate ultrastructure and correct stage-specific distribution for developmentally competent energy production and signaling [13].

Although oxidative phosphorylation is an important metabolic pathway for embryonic energy production, the use of oxygen as an electron acceptor produces ROS. Free radicals are normally present in cells at low steady-state concentrations, and there is a delicate balance between ROS production and clearance to allow for useful signaling, while minimizing cellular damage [16]. At these low levels, ROS serve in necessary physiologic roles in a wide range of activities, including oxidative stress response pathways, redox homeostasis, control of ventilation, smooth muscle relaxation and more [16]. ROS also play key signaling roles in ovulation, fertilization, and implantation [17–19].

ROS can severely damage cells when present at supraphysiologic concentrations and inflict cellular injury through lesions or breaks in the DNA. DNA damage may be especially detrimental to preimplantation embryos during epigenetic modification to the genome (discussed in more detail later) and DNA replication. Antioxidant vitamins and enzymes that scavenge the oxygen-containing molecules generally minimize ROS damage. However, cultured embryos lack the full range of anti-oxidant defense mechanisms available to somatic cells [20], (Figure 1).

Embryo culture at atmospheric oxygen tension has been associated with increased production of ROS and impaired developmental competence [20, 21]. Atmospheric oxygen delays cleavage, as shown by time-lapse microscopy [22] and ROS levels on embryonic day 3 have been negatively correlated with blastocyst development and pregnancy [23]. Studies that evaluated embryonic development under physiological oxygen concentrations (5–7%) have noted an increase in blastocyst development and embryo cell number among multiple species, although the mechanism is unclear [21, 22, 24, 25]. Furthermore, physiological oxygen tension preserves pluripotency in cultured human embryonic stem cell lines, as demonstrated by the maintenance of both X chromosomes in the active state [26]. Conversely, insufficient oxygen tension can be detrimental, as mouse embryos cultured at 2% oxygen exhibited increased fetal wastage and lower fetal weights [27].

Metabolic studies demonstrated that after fertilization embryos prefer pyruvate as a carbon source, with glucose becoming an important substrate only in later development, (Figure 1). Further investigation revealed that many species have little to no carbohydrate metabolism through the Embden-Meyerhof pathway prior to the eight-cell stage, with the human embryo lacking activity prior to the morula stage [2]. The early absence of glycolysis may be secondary to a lack of activity of key enzymes, such as phosphofructokinase or hexokinase, although the mechanism that controls initiation of glycolysis is unclear [28]. Glucose uptake by preimplantation embryos through a large family of glucose transporters was reviewed recently, and the expression of transporter proteins is subject to regulation by glucose concentration and other factors in the environment [29]. Non-physiological disturbances of the intracellular pH alter metabolic pathways through disruption of key enzyme activity, such as phosphofructokinase, and may further inflict damage as the embryo uses metabolic resources to restore its pH [2].

Many factors in the ambient environment directly or indirectly impact embryo metabolism, and deviations from the *in vivo* environment could potentially have pathologic consequences. Attention has increasingly turned toward non-invasive methods of testing embryonic competence by screening embryonic metabolites to improve embryo selection for increased implantation and delivery rates in women undergoing ART [30]. Metabolomic profiling of conditioned culture media with near infrared spectroscopy generated a viability index that was independent of morphology and correlated with implantation and clinical pregnancy rates [31]. The association between metabolites and embryonic competence is now being tested in relation to genomic integrity, highlighting the complex interdependence of these systems [32].

The Key Role for Epigenetics in Preimplantation Development

Although it is understood that the culture environment influences embryonic development, the exact mechanisms are unclear. However, epigenetic modifications to DNA and associated histones, some of which are stable during replication, can serve as a link between the environment and the genome [33, 34]. This could explain how *in utero* environmental exposures can have long-term effects on later development, adult life and even subsequent generations. The role of epigenetic reprogramming of the genome in the transgenerational

effects of environmental factors during embryogenesis has been reviewed recently, with a focus on adult pathologies that derive from *in utero* exposure to environmental toxins, such as endocrine disruptors [35].

Epigenetic programming and modification serves two important functions in the developing embryo: 1) to control the expression of genes that determine cellular differentiation in a stage-specific manner; and 2) to establish and maintain monoallelic gene expression at predestined loci. The first of these represents a complex system of epigenetic reprogramming that initially removes the parental epigenetic marks on each gamete to result in totipotency. Further, epigenetic modifications to the genome might then guide cellular differentiation and development and some have lifelong plasticity. The second role for epigenetics, that is imprinting, ensures an appropriate gene dosage in a parental-specific manner for a small number of loci that require parental-specific monoallelic expression for normal function. The precise timeline of embryonic genome activation and gene expression suggests the presence of a “zygotic clock” that ensures sequential degradation of maternal transcripts and production of zygotic transcripts [36], another process likely governed by epigenetic modifications [37].

Epigenetic regulation occurs through several mechanisms, including DNA methylation, histone modification, chromatin remodeling, nuclear architecture rearrangements and perhaps non-coding RNA molecules [37], (Text Box 2). Small non-coding RNAs of the small interfering RNA class, more so than microRNAs, now appear to have a central role in directing preimplantation development [38, 39].

Text Box 2

A Closer Look at Epigenetic Mechanisms

A panel of experts defined an epigenetic trait as “a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” [98]. Epigenetic mechanisms regulate temporal, tissue-specific, and parent-dependent gene expression [99].

Biological Functions

1. Differentiation – Epigenetic changes ensure cell differentiation by influencing gene expression through the resulting epigenetic marks.
2. Imprinting – Epigenetic changes ensure parent-specific, mono-allelic expression of certain genes, which is required for normal function of these genes.

Molecular Mechanisms

1. DNA methylation - Enzymatic addition of a methyl group to the carbon-5 position of the cytosine pyrimidine ring or the nitrogen-6 position of the adenine purine ring. The result is a physical blockade to transcriptional proteins and an affinity for methyl-CpG-binding domain proteins that form compact chromatin and silence genes. This modification occurs most frequently at C-phosphate-G (CpG) dinucleotides within repetitive sequences, but non-CpG methylation has been reported [100].
2. Histone modification - Histones are highly conserved proteins that regulate DNA structural organization and gene expression, see review [99]. Histones undergo myriad post-translational enzymatic modifications (methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation) on specific residues, most commonly at the amino-terminal tail, that alter their interaction

with DNA and result in gene activation or repression (the histone code hypothesis) [101].

3. Chromatin architecture remodeling – The two major chromatin domains, euchromatin and heterochromatin (HCh), are inheritable structural modifications that have significant functional consequences. Facultative HCh is condensed and transcriptionally inactive, and serves mainly to silence gene expression, as noted in studies of granulopoiesis [102]. Heterochromatinization is associated with small RNA molecules and methylated histone H3 sub-types that are recognized by heterochromatin protein 1, a fundamental component of the complex that tightly packages DNA, see review [103].
4. Nuclear organization - The 3-dimensional spatial organization of chromosomes affects their exposure to epigenetic mechanisms due to a non-homogeneous distribution of constituents within nuclear subdomains [104]. The nuclear position of a gene may secure its expression status through its interaction with specific epigenetic modifiers [105]. Studies suggest that nuclear position is conserved in mitosis, as recently reviewed [106]
5. RNA regulation - Non-coding RNA controls gene expression through small interfering RNA (siRNA) and microRNA (miRNA), which both have developmental stage- and tissue-specific expression [99]. RNA interference by siRNA silences gene activity by targeting degradation of mRNA and may also play a role in the formation of HCh by recruiting key proteins to specific chromosomal sites [106]. MicroRNA regulates gene expression by signaling mRNA degradation through interactions with the 3' untranslated region (3'UTR) of mRNA [107] and also further regulates gene expression through interactions with other epigenetic mechanisms, such as histone modification [108].

DNA methylation is a prominent and well-studied epigenetic mechanism. In mammalian development, two cycles of DNA demethylation and remethylation occur [40], (Figure 1). Global demethylation first occurs in the primordial germ cell during fetal development and is later followed by remethylation during gametogenesis to establish gender specific, *de novo* imprinting marks, as previously reviewed [41]. Interestingly, the remethylation process in male germ cells occurs in early gametogenesis, while female germ cells gradually complete this process during oocyte maturation [41, 42]. It is generally believed that this initial imprinting process preserves the integrity of parental-specific imprinted loci and is maintained after fertilization. Recent studies of imprinted genes in sheep suggest that the timeline for imprinting may not be so well established and that monoallelic expression may occur later in development [43]. The newly formed zygote then again undergoes a second demethylation of the parental genomes in a chronologically asymmetric manner and remethylation of non-imprinted sites then begins *de novo* at various developmental stages, according to mammalian species [40, 44–46] (Figure 1).

Epigenetic control of mammalian development is multifaceted and emerging data about histone modifications demonstrates their important role in this process [47]. Histone modifications of the imprinting control regions (ICRs), which are important in the allele-specific expression of genes, can activate or silence imprinted genes [48]. For example, differential histone acetylation regulates allele-specific expression of the ICR between *DLK1* and *GTL2* [49]. Histone modifications can also result in monoallelic gene expression independently of DNA methylation, as demonstrated by the repression of paternally derived genes by di- and tri- methylated histone H3 in the murine placenta [50]. Thus, histone modifications function as epigenetic alterations of genomic DNA that are established during embryogenesis, likely during the morula or blastocyst stages [48], (Figure 1).

Multiple epigenetic mechanisms function concurrently to optimally induce and maintain gene expression profiles (Text Box 2). X chromosome inactivation (XCI) is a notable example of how early in development epigenetic pathways converge and are sustaining throughout life to ensure a balanced gene dosage in XX females [51]. Non-coding *Xist* RNA regulates the choice of X chromosome to undergo XCI in each cell by coating the future inactivated X chromosome to trigger the cascade that results in the formation of facultative heterochromatin [52]. Specifically, XCI involves repressive histone modifications, incorporation of specific histone variants such as macrohistones and methylation of CpG dinucleotides, as discussed by others [37, 52, 53]. Ultimately, the inactivated X chromosome is relocated to the nuclear periphery, a compartment generally associated with heterochromatin, and the epigenetic pattern is maintained within each cell line (Text Box 2).

The Potential Interdependence of Epigenetics and Metabolism

The “quiet embryo hypothesis” was first introduced by Henry Leese in 2002 [54]. It proposes that viable embryos are associated with a “quiet” rather than an “active” metabolism. The idea is that less viable embryos metabolize more substrates due to the repair or rescue of molecular and cellular damage. The metabolic behavior of frozen-thawed embryos, which are increasingly important in ART, also appears to follow the “quiet embryo hypothesis”, according to the elevated metabolic activity of arresting, compared to non-arresting, embryos [55]. Leese noted that increasing metabolic energy resources are required as development progresses to support the morphogenetic activities (e.g., compaction and cavitation) of later stage preimplantation embryos. The average oxygen consumption by the preimplantation embryo is 3.65 QO₂ through the morula stage and increases to 10.77 QO₂ in blastocysts, consistent with prior reports of increased oxygen consumption at the blastocyst stage [56, 57]. Closer evaluation of oxygen consumption in the earliest hours of embryonic development revealed minute peaks of oxygen-consumption with each cleavage [19].

The “quiet embryo hypothesis” could explain the link between embryonic metabolism, epigenetics and viability - to what degree do epigenetics affect metabolism, and how might this interaction relate to developmental competence? It is plausible that the changing epigenetic marks during early development guide stage-specific metabolism by regulating gene expression in a time-sensitive manner. If the epigenome serves as a link between the environment and the genome, then *in vitro* culture may impact embryo metabolism and development by way of alterations in the epigenetic marks that regulate pertinent metabolic pathways. Embryos produced *in vitro* have shown a perturbation in DNA methylation patterns across multiple species [58–61]. While some investigators reported a genome-wide (global) increase in DNA methylation in embryos produced by IVF [62], others found global hypomethylation [63]. In support of this notion, culture media alter DNA methyltransferase expression, with a variable impact of culture conditions on embryonic epigenetic patterns [64]. In particular, trophoblast stem cells appear most vulnerable to epigenetic modification *in vitro* [50, 65]. Considering the Developmental Origins of Health and Disease (DOHaD) [66], commonly known as the Barker hypothesis [67], this observation suggests that metabolic stresses to embryonic (trophoblast stem) cells destined to form the placenta could impact development later in life.

DNA damage (strand breaks and alkali labile sites) induced in embryos by non-physiologic culture conditions were associated with an increase in amino acid metabolism in porcine, bovine, and human embryos [68]. Furthermore, embryos exposed to an asynchronous uterine environment exhibit abnormally excessive late-gestation growth that is associated with a shift in the expression of genes regulating differentiation [69]. While the mechanism underlying this shift in gene expression is uncertain, epigenetic changes are a plausible

pathway given the key role that they play in regulating gene expression, often in response to the environment.

While there is evidence that damage produced by supraphysiologic ROS levels affects DNA structure, there are also suggestions that ROS can impact epigenetic marks. Proteins involved in chromatin remodeling are modified by ROS to increase histone acetylation [70, 71]. The full extent of ROS impact on the epigenome remains to be determined. However, the developmental responses to altered environmental oxygen may reflect underlying changes in gene expression. Notably, when cultured at physiologic oxygen levels, the global gene expression pattern was similar to *in vivo* embryos. In contrast, culture at atmospheric oxygen altered global expression patterns and specifically perturbed the expression of genes involved in cell growth and maintenance, including *AQP8*, *CAMK1*, and *TGFBI* [64]. Given that epigenetic patterns govern gene expression, it can be hypothesized that inappropriate oxygen exposure may increase mitochondrial ROS production and perturb the epigenetic status quo, which could, in turn, alter gene expression, embryonic metabolism and ultimately developmental competence.

Beyond Embryo Metabolism: Long-term Impact of Culture Conditions on Epigenetic Patterns

The outcomes of pregnancies produced by ART have undergone intense scrutiny. Animal studies revealed a disturbing large offspring phenotype, sparking investigation of ART's potential impact on imprinting, because the animal disorders resembled Beckwith-Wiedemann (BWS) syndrome, a human overgrowth syndrome caused by imprinting errors of chromosome 11 [72, 73]. Furthermore, epidemiologic studies suggested an increased incidence of Angelman Syndrome (AS) and BWS in the offspring of ART patients who were only treated with ovarian stimulation, see review [74]. Epigenetic marks are not only erased and re-established during embryonic development, but it appears that some epigenetic modifications are dynamic throughout life and may contribute to aging and multifactorial adult-onset diseases [75, 76].

There is concern that ART may result in subtle abnormalities that could present later in life, an extension of the Barker hypothesis [67]. Evaluation of a small number of cord blood and placental samples demonstrated a modest change in the methylation level of CpG sites from *in vitro*, compared to *in vivo*, conceptions, with a fraction of the differences associated with altered gene transcription [77]. A comparison of two populations of children conceived either *in vitro* or *in vivo* found aberrant methylation of the differentially methylated region of the maternal ICR at the *IGF2/H19* locus in samples derived from *in vitro* conceptions, although evaluation of mRNA transcripts did not correlate with these aberrations [78]. Long-term offspring development in animal studies showing aberrant fetal growth have demonstrated a return to expected weights; however, more subtle abnormalities persist, such as cardiomegaly in calves, hypertension in mice and altered behavior and age-related mental decline in mice [79–82]. Murine offspring produced by ovarian hyperstimulation and *in vitro* fertilization have an increased risk of elevated systolic blood pressure [81]. Such predisposition to adult-onset disease may be a consequence of epigenetic changes occurring in very early embryonic development. Thus, ART could affect the intricate system of epigenetic controls that guide both early embryonic and life-long growth and development.

It is unclear which ART procedure has the most influence on epigenetic inheritance. Ovarian stimulation involves supraphysiologic hormonal manipulation to obtain multiple developing oocytes. The majority of current data regarding epigenetic modification focuses on altered DNA methylation of both imprinted genes and global genomic DNA in superovulated oocytes and the generated zygotes and placentas [58, 83, 84]. Murine blastocysts produced

from stimulated oocytes, as compared to those produced from spontaneously ovulated oocytes, demonstrated a dose-dependent alteration of imprinted methylation patterns at four loci (*Snrpn*, *Igf2*, *Kcnq1ot1* and *H19*) known to acquire parental-specific methylation during oogenesis [85]. The first three loci are methylated on the maternal allele and are only expressed from the paternal allele, but some stimulated oocytes demonstrated loss of maternal methylation at all three sites. Similarly, the fourth locus, for which the maternal allele is hypomethylated, demonstrated not only a gain of maternal methylation, but also a loss of paternal methylation. With high hormonal doses, all embryos display altered methylation for at least two of the four genes [85].

Cryopreservation of gametes and embryos is increasingly used for routine management of infertile patients. However, there is concern that the stress of cryopreservation may introduce an environmental insult that could affect metabolism, epigenetic marks, viability, and long-term development. Frozen-thawed embryos exhibit similar amino acid metabolism compared to fresh embryos, suggesting minimal lasting effect to their metabolism [55]. However, embryos that have undergone cryopreservation had altered gene expression profiles [86]. A recent study in a murine model reported that the process of embryo vitrification itself significantly augmented the loss of methylation in the *H19* differentially methylated domain, especially in the placenta [87]. There are very limited data regarding the possible effects of cryopreservation on human embryos.

The specific contribution of ART to adverse outcomes for the resulting offspring is difficult to ascertain due to the many confounding variables, including maternal age, inherent parental infertility, underlying parental medical conditions such as diabetes or obesity, and maternal diet [63]. Clearly, ART stresses developing embryos during a period of epigenetic vulnerability. In assessing epigenetic modification, investigators found a loss of imprinting and global alterations in DNA methylation level that depends on the type of culture medium, highlighting the complex interaction of genetic and modifiable environmental influences [88–90].

Concluding Remarks

The preimplantation embryo, while simple in appearance, contains the cellular foundations to produce a highly complex mammalian organism. During this time, epigenetic patterns are established that guide cellular differentiation and development. Embryonic metabolism is vital for survival and benefits not only from adequate access to substrates, but also from proper programming, which may include epigenetic regulation, as suggested in this review (Table 1). Clearly, additional research is needed to directly test the relationship between environmental alterations to epigenetic marks and embryonic metabolism with a focus on the resulting impact on developmental competence.

While ART has revolutionized the treatment of infertility, it is clear that the *in vitro* environment may significantly affect the delicate interdependence of the embryo's epigenetic constitution, metabolism and development. Based on existing data discussed in this review, there is an obligation to evaluate the long-term outcomes for ART-conceived offspring into adulthood and possibly for future generations. Investigators are striving toward this goal, using novel approaches to monitor and replicate the *in vivo* environment *in vitro* for healthy physiologic growth and competence [91–93]. Furthermore, continued evaluation of the optimal time for embryo transfer (cleavage stage versus blastocysts) will be informed by new data delineating the epigenetic events that occur during embryonic development. These efforts should enhance embryonic survival and implantation success, as well as long-term health, in offspring conceived by assisted reproduction.

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Glossary Box

Blastomeres	Embryonic cells derived from the mitotic divisions of a fertilized ovum
Blastocoel	The fluid filled cavity in the blastocyst stage embryo
Blastocyst	A preimplantation embryo after five mitotic divisions that consists of the inner cell mass and a thin trophoblast layer enclosing the blastocoel, which forms as the morula passes from the oviduct to the uterus
Cavitation	Formation of the blastocoel through the accumulation of fluid within the morula
Cleavage	A series of symmetrical mitotic cell divisions during which the size of the fertilized ovum remains unchanged as the blastomeres become progressively smaller with each division
Compaction	Process during which blastomeres change their shape and align themselves tightly against one another to form a morula in preparation for cavitation and blastocyst formation
Embryonic genome activation	Activation of transcription from the embryonic genome that occurs at the 2- to 8-cell stage, depending upon the species, and which takes control of development from maternal transcripts present in the oocyte
Fertilization	The fusion of two gametes become a zygote
Gamete	Reproductive cell (sperm or oocyte) that contributes a haploid set of chromosomes to the zygote at fertilization
Implantation	The formation of an attachment between the blastocyst and uterus at the end of preimplantation development that leads to invasion of the uterine endometrial tissues by trophoblast cells in humans and some other mammals to begin the formation of a placenta
Inner cell mass	A cluster of cells derived from blastomeres positioned inside the morula that become located eccentrically within the blastocyst. They are the source of embryonic stem cells and eventually give rise to all embryonic germ layers
Morula	A preimplantation embryo after three mitotic divisions that compacts into a solid, spherical mass in which individual cells are no longer visible
Placenta	A structure formed by trophoblast and mesodermal derivatives of the embryo that provides an interface for the fetus with the maternal circulatory system during gestation
Pronucleus	One of two haploid nuclei in a zygote produced by meiosis and derived from either the oocyte or sperm
Syngamy	The combination of chromosomes from two pronuclei into a diploid nucleus as the zygote undergoes its first mitotic division

Trophoblast	The first epithelial cells to form during preimplantation development, which are derived from blastomeres located on the outside of the morula and that eventually contribute to the placenta and fetal membranes
Zygote	Cell formed by the fusion of two gametes that contains their genetic material within two separate pronuclei

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Figure 1. Preimplantation Embryo Development, Metabolism and Epigenetic Pathways

Specific timing based on murine model and may differ between species.

Development: Preimplantation embryonic development can be categorized into three phases based on gene expression studies [47]. Phase I, from fertilization to the 2-cell stage, is the period of zygotic genome activation (ZGA). Phase II, from the 4-cell to 8-cell stage, is the phase of mid-preimplantation gene activation. Phase III, from the 8-cell to blastocyst stage, is the period of cell differentiation that most notably produces the inner cell mass (ICM) and trophectoderm (TE) cell lineages.

Metabolism: Pyruvate is the favored substrate in Phases I and II, with glucose uptake increasing gradually and surpassing pyruvate during Phase III, concomitant with increase in activity of glycolytic pathways. Normal zygote formation (2PN) is accompanied by specific sequential changes in mitochondrial distribution. Oxygen consumption, a viable marker for metabolic activity, increases significantly in Phase III. Stores of maternal mRNA encoding antioxidant enzymes provides ROS protection until ZGA, while embryonic expression becomes notable by blastocyst stage [20].

Epigenetics: DNA methyltransferase 1 (Dnmt1) targets hemi-methylated DNA and maintains imprinting marks during DNA replication. Paternal genome is actively demethylated followed by passive demethylation of maternal genome. *De novo* methylation begins in the morula and coincides with commencement of cell differentiation. Sperm nuclear protamines are sequentially replaced by acetylated histones then monomethylated histones then di- and tri- methylated histones (H3K4me3, H3K9me2, H3K27me2/3) [47]. Meanwhile, maternal pronuclear histones are modified via acetylation and methylation concomitantly. Linker histone protein H1 declines in cleavage stages likely due to ZGA, while expression of macroH2A, a constituent of the inactive X chromosome heterochromatin, increases in late preimplantation development [47]. Histone deacetylase 1 (HDAC1) appears after fertilization and is vital to preimplantation development [94]. Expression of ERG-associated protein with SET domain (ESET), a histone methyltransferase enzyme, represses *CDX2* and is vital to pluripotency of the ICM [95].

JmJD2C, a histone demethylase required for embryo development, appears at 2-cell stage and peaks at 8-cell stage and is involved in *NANOG* regulation [96].

Table 1

Epigenetics and Embryonic Metabolism

Evidence for epigenetic regulation of preimplantation embryo metabolism	References
Epigenetic marks link genome with environment via regulation of gene expression	[33, 34]
<i>In vitro</i> environment alter embryonic metabolism	[2, 9]
<i>In vitro</i> environment alters embryonic DNA methylation patterns	[56–59]
Culture substrates alter metabolic pathways	[29]
Individual culture media differentially alter Dmmt expression	[62]
DNA damage induces increased metabolic activity	[66]
ROS associated with delayed embryonic development	[23]
ROS increase histone acetylation	[68, 69]