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Chapter 5

Control of Embryonic Gene Expression and Epigenetics

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Abstract

Preimplantation embryo development follows a series of critical events. Remarkable epigenetic modifications and reprogramming of gene expression occur to activate the embryonic genome. In the early stages of preimplantation embryo development, maternal mRNAs direct embryonic development. Throughout early embryonic development, a differential methylation pattern is maintained although some show stage-specific changes. Recent studies have shown that differential demethylation process results in differential parental gene expression in the early developing embryos that may have an impact on the correct development. In the recent years, noncoding RNAs, long noncoding RNAs (lncRNA) and short of mRNAs and therefore their role in preimplantation development has gained significance.

Keywords: gene expression, methylation, miRNA

1. Introduction

Preimplantation embryo development follows a series of critical events. These events start at gametogenesis, formation of mature gametes, and lasts until parturition. Male and female gametes are derived from primordial germ cells (PGCs) by the processes of spermatogenesis and oogenesis, respectively. PGCs have unique properties of gene expression, epigenetics, morphology and behaviour. Once the PGCs undergo mitosis, spermatogenesis and oogenesis progress differently. In spermatogenesis, spermatogonia undergo mitosis starting at puberty until death and each primary spermatocyte produces four spermatids at the end of meiosis. In oogenesis, PGCs differentiate into oogonia, they enter meiosis and arrest until puberty. Unlike meiosis II in spermatogenesis, secondary oocyte does not complete meiosis II until fertilisation. With completion of meiosis II, each oogonia produce a single viable oocyte [1].

At fertilisation, the oocyte completes meiosis and the fertilised oocyte is called the zygote. Oocyte and sperm nuclei fuse resulting in syngamy (**Figure 1**). The zygote undergoes a series of cleavage divisions, forming two-cell, four-cell, eight-cell morula and blastocyst stages [2] (**Figure 1**). During cleavage stage divisions, programming of maternal and paternal chromosomes takes place to create the embryonic genome (embryonic genome activation, EGA) and to start the preimplantation embryo development. If the EGA fails, the development does not continue because of the inability of the embryo to have cellular functions [3]. This activation is initiated by the degradation of maternal nucleic acids, specific RNAs stored in oocytes, proteins and other macromolecules [4]. Upon EGA, which starts at the two-cell stage in mouse and four- to eight-cell stage in human [5], remarkable reprogramming of expression occurs in the preimplantation embryo. These reprogramming events are controlled by DNA methylation, histone acetylation, transcription, translation and miRNA regulation [6]. Therefore, the development of preimplantation embryos includes continuous molecular, cellular and morphological events. These events would eventually form a multilineage embryo that has a capability to implant and continue the foetal development.

In this chapter, different factors affecting gene expression during preimplantation embryo development will be discussed. Epigenetic factors, focusing on methylation profiles, of gametes and preimplantation embryos will be reviewed. The effects of noncoding RNAs on gene expression will be thoroughly evaluated.

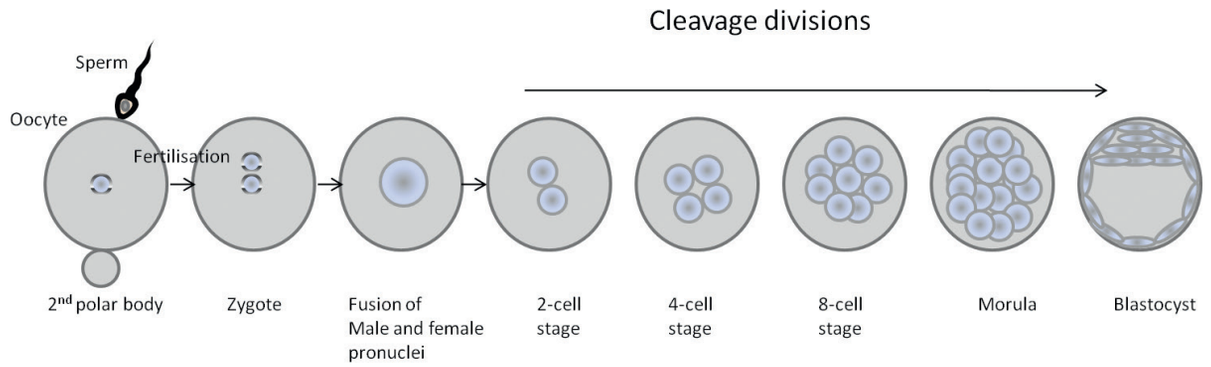

Figure 1. Schematic diagram outlining the main stages of preimplantation embryo development. Fertilisation followed by syngamy, cleavage divisions results in two, three, four, and so on cell embryos which eventually form the morula and the blastocyst.

2. Gene expression and epigenetics

For a normal developing embryo, the expression of both maternal and paternal genes is required. An intense epigenetic change occurs upon fertilisation to establish pluripotency [7]. Although there are a number of post-translational modifications within chromatin including acetylation, ubiquitination, SUMOylation and phosphorylation; methylation of histone lysine and arginine residues is the main focus in preimplantation embryos.

Methylation and chromatin modification not only play crucial roles in determining the transcriptional state but also are capable of determining the transcriptional repression

[8–10]. The mechanism leading to the changes in methylation is not well established, but it has been suggested that the reprogramming takes place by either passive or active demethylation. Indirect pathways of demethylation are associated with DNA repair [11–14]. Two main stages, PGCs and preimplantation embryos, are important in the regulation by methylation.

2.1. Epigenetic modification of the zygote and the preimplantation embryos

In mammals (human, bovine, rat, pig and mouse), the zygote undergoes genome-wide demethylation [15–17] with the exception of imprinted genes [18]. The male pronucleus of the zygote undergoes selective demethylation due to the loss of DNA replication leading to asymmetric methylated sister chromatids [15, 16, 19, 20]. These events start following the sperm decondensation in humans and in mouse with some variations [17, 21, 22]. The female pronucleus of the zygote remains highly methylated at this stage [17, 21, 22]. Demethylation of the maternal genome starts with the first cleavage divisions [19, 23, 24]. By the morula stage, the mouse preimplantation embryos become undermethylated. Polarisation and compaction of individual blastomeres start at around eight-cell stage of the developing embryo. Many factors are involved in these processes including E-cadherin (CDH1), partitioning defective homologue 3 (PARD3), PARD6B and protein kinase C zeta [25–27].

The blastocyst stage embryo has a fluid-filled cavity and two cell populations consisting of inner cell mass (ICM) and trophectoderm (TE). All the blastomeres are believed to be totipotent in cleavage embryos until four- to eight-cell stage since these cells form both the ICM and TE lineage [28]. ICM develops into epiblast, whereas TE forms the extraembryonic tissues such as placenta. ICM is composed of pluripotent cells that have the capacity to develop into any cell type of the foetus. Transcriptional and epigenetic events strictly regulate these differentiation events. A number of transcriptional factors play a crucial role in blastocyst formation. These include caudal type homeobox 2 (CDX2) for TE specification, octamer 3/4 (OCT4) and NANOG for the establishment of ICM pluripotency [29–31]. CDX2 is extensively expressed in eight- and 16-cell stage and it is expressed only in TE cells of the blastocyst [32]. Although OCT4 and NANOG are also expressed broadly at eight- and 16-cell stage embryos, they are only expressed in ICM in blastocysts [32]. A number of transcription factors are required for blastocyst formation. Embryos lacking CDX2 expression cannot form blastocoel cavity but they have the ability to implant [30]. Lack of OCT4 or NANOG expression causes failure of ICM and the development of these embryos is arrested at the blastocyst stage [31, 32]. TEAD4 is another transcription factor that has a role in blastocyst transition in which the lack of TEAD4 nuclear localisation impairs TE-specific transcriptional programme in inner blastomeres [33]. Furthermore, the aberrant expression of TCFAP2C transcription factor also leads to embryonic arrest during morula to blastocyst transition [34] and Klf5 mouse-mutant embryos arrest at the blastocyst stage [35].

The remethylation process starts shortly after implantation [16, 22, 23, 36]. This *de novo* methylation occurs asymmetrically, such that ICM is hypermethylated possibly due to the Dnmt3b methylase [37], whereas TE remains hypomethylated due to the active demethylation by enzyme catalysis and passive demethylation [11, 14, 22]. Alteration of the methylation profiles in embryos has been shown to cause alterations of ICM and TE differentiation.

Variations of the H3 arginine 26 residue (H3R26me) were shown to lead to changes of TE and ICM differentiation of a blastomere [38].

X-chromosome inactivation is an epigenetic phenomenon in which the activity of X chromosomes is strictly regulated to equalise X-chromosome expression and gene dosage between males and females and relative to autosome chromosomes [39]. For correct development, X-chromosome dosage compensation is crucial. The inactivation of X chromosome occurs in at least two phases: initiation and maintenance. X-inactivation mouse model systems have shown that the inactivation of X chromosome takes place during early embryogenesis of the female embryo by undergoing transcriptional silencing of genes along the X chromosome [40]. In human preimplantation embryos, it has been shown that the reduced expression of X chromosomes in females ensures the dosage compensation [41]. LncRNA *XIST* expression activates the X-chromosome inactivation by engaging proteins functioning in chromatin remodelling [3, 42]. With the advanced technologies, including single-cell RNA sequencing, it has emerged that lncRNAs *XACT* and *XIST* are expressed on the active X chromosome in the early human preimplantation embryos [43]. Furthermore, the expression of these two RNAs has never been shown to overlap. Introducing *XACT* into heterologous systems caused the accumulation of *Xist* RNA in *cis* and therefore it may be involved in the control of *XIST* association to chromosome in *cis* and may temper its ability of silencing. It is also possible that *XACT* functions in balancing the X-chromosome inactivation at the early stages of preimplantation embryo development [43, 44]. Recently, the dosage compensation was shown to be driven by a CAG promoter of a new Xist allele (Xist(CAG)) [45]. Furthermore, Xist(CAG) upregulation in preimplantation embryos showed variation depending on the parental origin and the paternal expression was suggested to be preferentially inactivated with the paternal Xist(CAG) transmission [45].

2.2. Epigenetic modification of the gametes

In germ cells, methylation is maintained in a sex-specific manner. Methylation in PGCs diminishes as they migrate to the gonads. Studies suggest that in females, remethylation occurs after birth when the oocytes are in the process of development. When demethylation is completed, the PGCs either enter mitosis in males or arrest at meiosis in females [46].

Reprogramming of the methylation in the embryo is necessary for parent-specific expression of genes [14]. Gene expression varies during preimplantation embryo development due to these reprogramming events and appropriate gene expression determines the survival of the embryo [6]. Recently, short noncoding RNAs, microRNAs (miRNAs) and long noncoding RNAs (lncRNA) have gained importance in their potential function to affect numerous pathways by targeting multiple genes [47, 48].

3. Gene expression and small noncoding RNAs: microRNAs

MiRNAs are a large family of short noncoding RNAs between 17 and 25 nucleotides (nt) in length [49]. MiRNAs were first identified in *Caenorhabditis elegans* over two decades ago

[50] and since then many have been identified in multiple organisms, such as worms, flies, fish, frogs, mammals and plants, by molecular cloning and bioinformatics [51]. Most miRNA sequences are conserved among a wide range of mammalians [52], though there are some that differ from each other only by a single nucleotide [53]. The conserved miRNA sequences among different species can be distinguished by the nomenclature such that when only the first three letters differ this indicates the same sequence in different species, that is, hsa-miR-145 in *Homo sapiens* and mmu-miR-145 in *Mus musculus* [54].

MiRNAs have been shown to be of great importance in a wide variety of biological processes involving cell cycle regulation, apoptosis, cell differentiation, imprinting, homeostasis and development, including limb development [55], morphogenesis of lung epithelial [56], embryonic angiogenesis [57], formation of hair follicle and proliferation of T-cell [58, 59]. They play key roles in regulating transcriptional and post-transcriptional gene silencing in many organisms by targeting mRNAs for translational inhibition, cleavage, degradation or destabilisation [53, 60–64]. Each miRNA has multiple mRNA targets that may regulate up to 30% protein-coding genes and shape protein production from hundreds to thousands of genes [65–67]. MiRNAs recognise their targets through base pairing of the complementary sequence of their seed sequence (2–8 nt of miRNAs) within the open reading frame (ORF) and 3'untranslated region (UTR) of target mRNA [68]. Although the targets of miRNAs are not fully known, bioinformatics studies show a range of possible target genes [69]. The functional activities and the predicted/observed targets of miRNAs can be identified using miRNA databases. These databases can be accessed using the following URL: (http://www.targetscan.org/, http://www.microrna.org/microrna/home.do and http://mirdb.org/miRDB/).

3.1. MiRNA biogenesis

MiRNA biogenesis involves multiple important steps. MiRNAs are first transcribed from genomic DNA into primary miRNA (pri-miRNA), which contains a stem-loop structure, by RNA polymerase II. These pri-miRNAs are then processed by Drosha, which is a 30–160 kDa protein with one dsRNA-binding and two catalytic domains [70]. In the presence of DGCR8, both strands of the hairpin are cut generating a pre-miRNA product of approximately 70 nt in size [71]. These pre-miRNAs are carried from the nucleus into the cytoplasm by Exportin-5 (Exp5), which is a nucleocytoplasmic transporter in karyopherin family that has binding sites for pre-miRNAs in the presence of RAs-related nuclear protein (Ran) and guanosine triphosphate (GTP) [72, 73]. These miRNAs are further cleaved by cytoplasmic RNase endonuclease, Dicer, making 21–22 nt double-stranded structure. Although one of the strands is usually degraded, both strands of the pre-miRNA may be associated with Argonaute (Ago)-protein-containing complex and they are mediated by RISC/miRNP (RNA-induced silencing complex/mi-ribonucleoprotein) to form single-stranded mature miRNAs. MiRNAs associated with RISC mainly target mRNAs and they either inhibit their translation or cause degradation of mRNA that results in reduced protein synthesis [70, 74].

Studies showed that processing of miRNAs by Dicer was vital and any defects, such as deletion of Dicer in the developing animals, caused aberrations [75, 76]. Lack of Dicer in Drosophila germ line stem cells postponed the G1/S phase transition [77], suggesting that miRNAs may

be vital for stem cells to bypass this checkpoint. Reduced and disorganised spindles, incorrect chromosome alignment and defects in gastrulation were observed with the Dicer-mutant oocytes in mouse and in *C. elegans*, respectively [50, 78]. Injection of miR-430 in zebrafish and *C. elegans* partially repaired the gastrulation, retinal development and somatogenesis [78]. Dicer deletion in zebrafish, mouse and hippocampal initiated problems in the nervous system and led to the inability of forming mature miRNAs that resulted in variations of brain morphogenesis and differentiation of neurons [79, 80]. Although the axis formation and early differentiation of maternal-zygotic Dicer-mutant zebrafish and mouse embryos were normal, they still triggered defects in somitogenesis, morphogenesis that affected the brain formation, gastrulation, heart development and apoptosis in limb mesoderm, respectively [78, 81–83]. Apoptosis was enhanced in the developing limb mesoderm of Dicer null mouse [84]. Dicer deficiency mainly led to embryo death in mouse around embryonic day 7.5 [50, 78, 85] and in zebrafish [86] that may indicate the importance of miRNA-mediated gene silencing at maternal to zygotic transition.

Complete loss of Dicer1 in somatic cells of mouse reproductive tract not only showed reduced expression of miRNAs but also caused the female mice to become infertile with compromised oocyte and embryo integrity [50, 87]. Dicer-deficient male mice were shown to have poor proliferation of spermatogonia. Loss of Dicer1 in the germ line of male mice (homozygote Dicer1) led to decreased fertility due to abnormal spermatogenesis. The number of germ cells was reduced with abnormal spermatids, abnormal phenotype of spermatocytes with condensed nucleus, abnormal sperm motility and mutant testes with Sertoli tubules [88]. Studies suggest that the transfer of maternal cytoplasmic Dicer disguised the early abnormal phenotypes [78, 89].

Knock-out of Ago2 in mouse embryonic fibroblasts and haematopoietic cells caused decreased levels of mature miRNAs [61, 90, 91]. Ago2-deficient oocytes were observed to develop the mature oocytes with abnormal spindles and chromosomes were not able to unite properly with reduced expression levels of miRNAs (more than 80%). Loss of Ago2 function leads to embryo death around embryonic day 9.5 in mouse [92].

3.2. Expression of miRNAs in preimplantation embryos

The expression of miRNAs in preimplantation embryos has been mainly studied by knock-out experiments, by cloning experiments and by identifying individual miRNAs by microarray analysis and real-time polymerase chain reaction [93]. The expression studies have been carried out using animal models and tissues, cultured cells; that is, cancer cells and human embryonic stem cells; and mouse/bovine/human gametes and embryos. Human embryonic stem cells, which are derived from the inner cell mass of an embryo at the blastocyst stage and are characterised by their ability of self-renewal and multipotency, are the key in gene expression research since the access of human embryos is difficult and these cells are one of the closest representations of human embryos. Studying miRNA expression in stem cells not only gives insight into potential miRNAs expressed in human embryos but also may show the important role of miRNAs in the stem cell functioning [94].

MiRNA expression has been observed as early as oogenesis and spermatogenesis in mouse, bovine and human [95, 96]. Differences in the miRNA expression have been observed between

immature and mature oocytes that may represent the natural turnover and indicate that each embryonic stage is defined by a specific miRNA. Similar miRNA expression profiles in mature mouse oocytes and early developing embryos indicate that at these stages the zygote has maternally inherited miRNAs [50]. Similar to oocyte, sperm carries a range of miRNAs. Approximately 20% of these miRNAs are located in the nuclear or perinuclear part of the sperm indicating that these miRNAs are transferred to the zygote at the time of fertilisation [97]. It was suggested that the sperm-borne miRNAs may down-regulate the maternal transcripts in mammals. However, when this hypothesis was tested using microarray analysis, it was shown that none of these miRNAs in the sperm have significant importance since all of them were already present in the oocytes (meiosis II) [98].

Multiple miRNAs were involved in the formation of germ cell layers. MiR-290, which was expressed at different levels during preimplantation embryo development of mouse embryos, had a negative effect on the germ cell and mesoderm differentiation in the mouse ES cells *via* targeting Nodal inhibitors [99]. In zebrafish, however, miR-290 cluster played an important role in regulating the mesoderm induction [100]. Therefore, it is not clear if miR-290 has an inhibitory effect on the mesoderm differentiation. Other miRNAs have been shown to have an effect in mesoderm differentiation in zebrafish, such as miR-15 and miR-16 [100], which were also expressed in mouse preimplantation embryos [50].

Mainly, the same miRNAs are expressed during the cleavage divisions of the embryo in mouse and bovine. However, their expression levels often vary during these stages. In murine embryos, the level of miRNA expression is reduced by as much as 60% between one- and two-cell stages. At the end of four-cell stage, mouse embryos have approximately twice as much miRNA compared to the two-cell stage embryo. This implies that the maternally inherited miRNAs degrade at this stage and the EGA starts between the one-cell and four-cell stages [50]. Even though the synthesis and degradation of miRNAs coexists during the preimplantation embryo development in mice, the overall miRNA expression increased towards the blastocyst stage [101].

More than 700 miRNAs have been identified in humans [87, 95, 96, 102]. The level of expression for the majority of these miRNAs stayed the same between the oocyte and the blastocyst stage [87]. More than 50% of the miRNAs expressed in human oocytes and blastocysts were shown to be involved in tumourigenesis, that is, let-7 family, miR-19a, miR-21 and miR-34 [103–109].

4. Gene expression and long noncoding RNAs

In the last few years, in addition to short noncoding RNAs, the lncRNA have gained importance in their roles to affect gene expression. The mammalian genomes consist of long intergenic noncoding RNAs (lincRNAs) that have been suggested to take a role in the regulation of pluripotency during preimplantation embryo development [110]. Human pluripotency transcripts 2, 3 and 5 (HPAT2, HPAT3 and HPAT5) were reported to adjust the pluripotency and ICM formation in preimplantation embryos. Furthermore, HPAT5 was shown to interact with let-7 family of miRNAs [110].

Implantation of embryos involves complex mechanisms and many different genetic and physiological factors are involved during the process. Developing preimplantation embryo must have a good coordinated interaction with the maternal uterine endometrium. LncRNAs were shown to be differentially expressed in endometrial tissues obtained from pigs with pregnancy and non-pregnancy with two lncRNAs, TCONS_01729386 and TCONS_01325501, with potential roles in implantation [111].

5. Gene expression and assisted reproductive technologies

In Western world, approximately 1% of children are born with assisted reproductive technology (ART) treatments. The infertile couples have the best possibility to conceive a child with these treatments. Although these techniques have been considered to be safe in terms of foetal and post-natal development [112, 113], there is an increased risk for morbidities, especially imprinting disorders [114]. Furthermore, the global gene expression profiles vary due to *in vitro* culture of zygotes [115, 116] and *in vitro* fertilisation processes [117]. Following *in vitro* culture, apoptotic and morphogenetic pathways have shown to be altered [118].

Intra-cytoplasmic sperm injection (ICSI), one of the widely used ART techniques, provides infertile couples with sperm motility problems a great chance to have a baby. ICSI is a unique process in which the sperm is injected into the ooplasm [119]. However, ICSI bypasses a number of physiological processes that would normally take place. These embryos derived from ICSI were shown to be cleaved at a slower rate. Furthermore, a reduced number of embryos become hatched with a fewer number of cells and the calcium oscillations are shorter with different patterns [120]. Mice embryos generated by ICSI were shown to be obese and have anomalies of the organs [121].

6. Conclusion

Normal development of preimplantation embryos involves complex mechanisms. For a normal developing embryo, the expression of both maternal and paternal genes is required. Several factors are involved in the regulation of parental genes in preimplantation embryos. Epigenetic modifications are one of the most important factors that are involved in the regulation of gene expression during preimplantation embryos. Extensive research studies have been performed throughout the years to establish the methylation profiles of the mammalian gametes and embryos. In the more recent years, the importance of noncoding RNAs in the regulation of genes has become clear. A handful of studies have been performed to analyse the expression of microRNAs, which have been shown to regulate mRNAs that encode up to 30% human protein-coding genes. The expression of miRNAs has been observed in mouse, bovine and human gametes and embryos. Furthermore, in the last couple of years, expression of long noncoding RNAs and their roles in embryonic development and implantation have been investigated. The extensive research studies have provided crucial understanding of the development of

preimplantation embryos and the regulation of gene expression, and with the advancing technologies more molecular studies will help to comprehend the mechanisms better.

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