Chapter 14

Epigenetic Instability in Embryonic Stem Cells

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1. Introduction

Embryonic stem (ES) cells constitute a very important tool for regenerative medicine today. Human ES cells, in particular, are almost all derived from embryos obtained by *in vitro* fertilization (IVF) followed by *in vitro* culture (IVC); however, such *in vitro* manipulated embryos often show epigenetic abnormalities in imprinted genes that can lead to the development of various diseases. We recently reported that epigenetic differences occurred between ES cells derived from *in vivo* developed embryos (Vivo ES) and ES cells derived from *in vitro* manipulated embryos (Vitro ES) [1]. In addition, we found that the DNA methylation state of uniparental and somatic cell nuclear transfer (SCNT) ES cells exhibits epigenetic instability during *in vitro* culture [2]. In this chapter, we review studies that have examined the epigenetic instability of ES cells during generation and maintenance cultures, and discuss the candidate factors that may be responsible for this epigenetic instability.

2. Epigenetic regulation by DNA methylation

In vertebrate genomic DNA, the 5' cytosine residues in CpG sequences are often methylated [3]. DNA methylation plays an essential role in the normal development of mammalian embryos by regulating gene expression through genomic imprinting and X chromosome inactivation, and confers genomic stability [4-7]. In this chapter, we focus primarily on genomic imprinting, which is the preferential silencing of one of the parental alleles of a gene by epigenetic DNA methylation since epigenetic modifications to some imprinted genes cause diseases such as Beckwith-Wiedemann syndrome and Prader-Willie syndrome. For example, the expression level of the *H19* imprinted gene is regulated by an upstream differentially methylated region (DMR), and epigenetic alterations to the DMR result in Beckwith-Wiedemann syndrome [8-10]. The *H19* mRNA is transcribed from the unmethylated maternal al-

lele but is not transcribed from the methylated paternal allele (Fig. 1). In contrast, DMRs of *Peg1* (Mest), *Snrpn* and *Igf2r* are methylated in the maternal allele and unmethylated in the paternal allele. Genomic imprinting is very stable except for the period when the reprogramming of genomic imprinting takes place in germline cells [11]. For the establishment and maintenance of DNA methylation, the cytosine-guanine (CpG) DNA methyltransferases (Dnmts), Dnmt1, Dnmt3a, and Dnmt3b, are the main factors that coordinately regulate CpG methylation in the genome [12-14]. Dnmt1 is involved in maintenance activity, while Dnmt3a and Dnmt3b are responsible primarily for the creation of new methylation patterns.

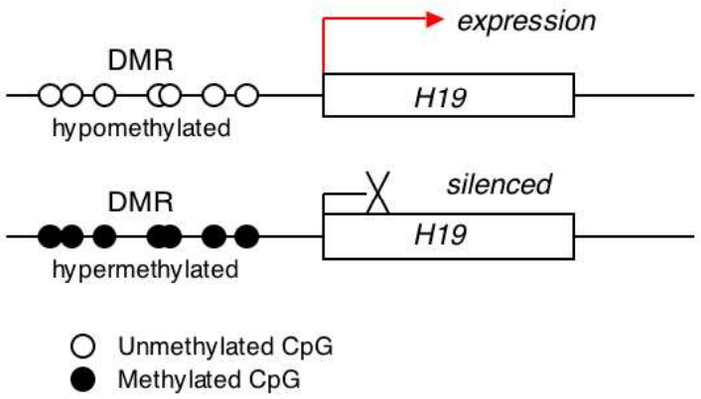

Figure 1. Regulation of gene expression in the *H19* imprinted gene.

3. Epigenetic instability in preimplantation embryos

In general, ES cells, especially human ES cells, are generated from blastocyst stage embryos that are produced by *in vitro* manipulations such as IVF and IVC. However, *in vitro* manipulated embryos may already possess epigenetic abnormalities because the culture conditions of fertilized embryos can influence the methylation state. For example, a sub-optimal culture medium (e.g., Whitten's medium) can cause aberrant genomic imprinting of the *H19* gene [15], and culture medium supplemented with fetal calf serum alters mRNA expression of imprinted genes [16]. Our recent study suggests that altered DNA methylation due to IVC conditions occurs not only in imprinted genes but also in genome-wide repetitive sequences, such as major and minor satellite sequences [17]. Thus, alteration of DNA methylation can occur in response to various factors, from the moment when embryos are collected from the oviducts or uterus.

4. Epigenetic instability in ES cells during prolonged culture

ES cells are established from the inner cell mass (ICM) of blastocyst stage embryos [18,19]. Once ES cell lines are established, they can be maintained for long periods of time and used for sever-

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al applications. However, ES cells lose their pluripotency during prolonged *in vitro* culture [20]. Several studies indicate that the accumulation of epigenetic alterations over time is correlated with the loss of pluripotency in ES cells. Dean *et al*. reported that epigenetic alterations that occur in ES cells persist to later developmental stages and are associated with aberrant phenotypes in completely ES cell-derived mice [21]. Humpherys *et al*. show that variation in imprinted gene expression is observed in most cloned mice derived from ES cell donors, even those derived from ES cells of the same subclone [22]. Such epigenetic drift of imprinted genes was also observed in our experiments during prolonged culture of mouse ES cells (Fig. 2): DNA methylation of four imprinted genes, *Peg1*, *Snrpn*, *Igf2r* and *H19*, was unstable during cell culture (P3-30), even in the same cell line, over time. Minoguchi and Iba reported that retroviral DNA that is introduced into mouse ES cells is progressively silenced by DNA methylation; however, a substantial amount of retroviral DNA is reversibly reactivated by DNA demethylation [23]. Such epigenetic drift has also been observed in human ES cells, depending on the method of establishment and the culture conditions [24].

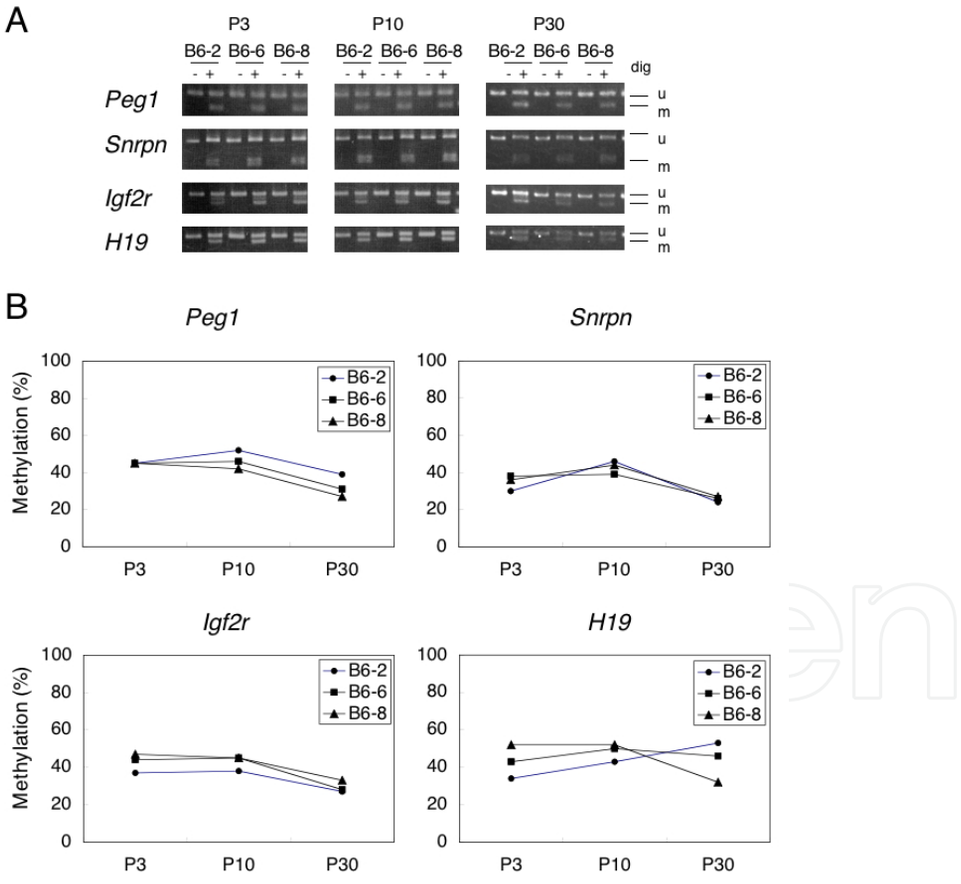

Figure 2. Epigenetic drift of imprinting methylations in fertilized embryo-derived ES cells. A. Combined bisulfite restriction analysis (COBRA) was conducted for three fertilized embryo-derived ES cell lines (B6-2, B6-6 and B6-8) during prolonged *in vitro* culture (P3, P10 and P30). The maternally methylated imprinted genes *Peg1*, *Snrpn* and *Igf2r*, and the paternally methylated imprinted gene, *H19*, were examined. B. Summary of imprinting methylations during prolonged culture of ES cells. dig, digestion by restriction enzymes; u, unmethylated PCR products; m, methylated PCR products.

5. Epigenetic differences between male and female ES cells

Large differences in epigenetic drift have been observed between male (XY) and female (XX) mouse ES cells. Global demethylation, including imprinted genes and satellite repeats, occurred more frequently in female ES cell lines compared to male ES cell lines [21, 25]. This global demethylation reflects the number and state of X chromosomes in ES cells. In general, both X chromosomes are active in female ES cells, whereas male ES cells have only one active X chromosome. The X chromosome state in female ES cells is thought to lead to downregulation of DNA methyltransferases (Dnmt3a and Dnmt3b) and, ultimately, to global hypomethylation [25]. Thus, DNA methylation of imprinted genes and repetitive sequences are gained or lost at high rates even in clonal populations of ES cells, and these alterations may have deleterious effects on phenotypes of ES cell-derived animals or tissues.

6. Epigenetic differences between *vivo* and *vitro* ES cells

6.1. Methylation state of *vivo* and *vitro* ES cells

In human ES cells, several studies have recently provided evidence for the efficient induction of endoderm, mesoderm, and ectoderm, and many of their downstream derivatives [26], and these reports offer broad possibilities for regenerative medicine. However, all human ES cell lines are established from *in vitro* manipulated embryos that often show abnormal genomic imprinting, which can lead to an increase in the frequency of diseases. Therefore, we have compared the methylation state of imprinted genes and the gene expression patterns of both Vivo and Vitro ES cell lines in mice [1].

Although the genomic imprinting is maintained during preimplantation development, normal imprinting can occasionally be disrupted in preimplantation embryos during IVC, resulting in biallelic expression of the *H19* gene [15,27]. To investigate whether Vitro ES cells take on abnormal imprinting from IVC blastocysts, we performed methylation analysis of the *H19* DMR for early passage (P2) cells (Fig. 3). COBRA analysis shows that the *H19* DMR is significantly demethylated in Vitro ES cells compared to Vivo ES cells. The *Igf2r* DMR2 also showed significant differences among Vitro vs. Vivo ES cells, but significant differences in the methylation of *Snrpn* and the major satellite repeats were not detected.

In additional experiments, both Vivo and Vitro ES cells were passaged several more times, and the methylation state of imprinted genes and satellite repeats was investigated at later passages (P5) (Fig. 3). Results from COBRA analysis at P5 showed no significant differences between Vivo and Vitro ES cells. Even Vivo ES cells exhibited highly demethylated alleles. In contrast, some Vitro ES cells had an almost normally methylated allele. This result indicates that the methylation state of ES cells at later passages depends more on the character of the individual cell lines than on the origin of the ES cells.

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6.2. Gene expression of *vivo* and *vitro* ES cells

We assessed gene expression patterns in ES cells at early and late passages by quantitative real-time RT-PCR. The expression of *Oct3/4* mRNA, a pluripotent cell marker, was significantly higher in early passage Vivo ES cells than in Vitro ES cells, whereas other pluripotent marker genes, *Nanog* and *Stella*, showed no significant differences in expression levels between the two types of ES cells. Among the methylation-related genes, mRNA expression of the *de novo* DNA methyltransferase, Dnmt3b, was significantly higher in Vivo ES cells. Expression of growth arrest and DNA damage-inducible protein 45 beta (Gadd45*b*), which is a putative demethylation factor [28,29], is higher in Vitro ES cells. Thus, mRNA expression patterns of several methylation-related genes tended to shift, resulting in the promotion of demethylation and the inhibition of methylation in Vitro ES cells. In contrast, at later passages, no significant differences between Vivo and Vitro ES cells were found with respect to the pluripotent marker genes and methylation-related genes that were examined.

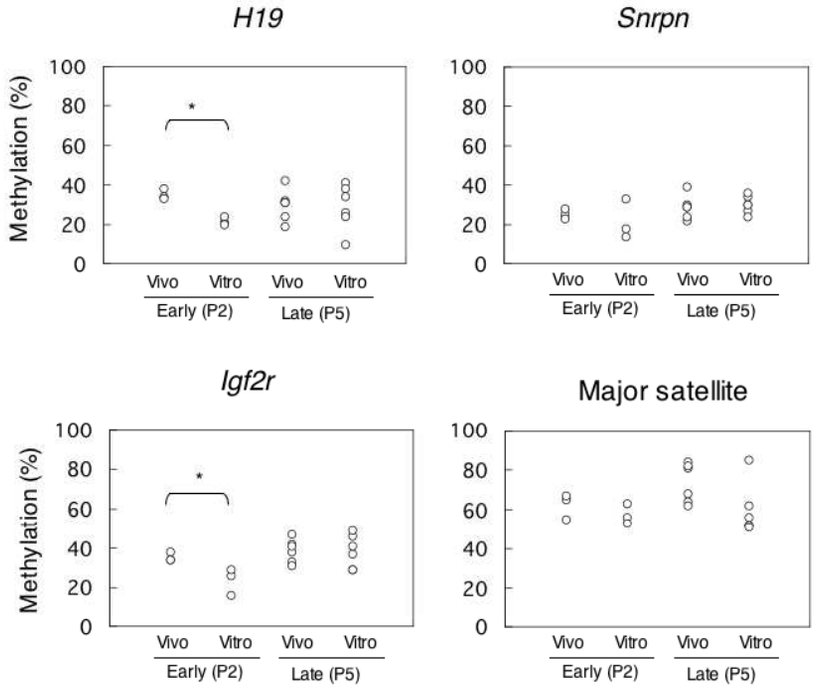

Figure 3. Epigenetic differences between Vivo and Vitro ES cells. DNA methylation status of imprinted genes, *H19*, *Snrpn* and *Igf2r*, and major satellite repeats were examined by COBRA in each ES cell line at an early passage (P2) and a later passage (P5). These graphs summarize previously reported data [1]. *, $P < 0.05$.

7. Epigenetic instability in SCNT and uniparental ES cells

7.1. SCNT ES cells

Maintenance of the normal epigenetic state in SCNT-ES cells is crucial for their use in therapeutic applications. We established two SCNT-ES cell lines from embryos that were produced by introducing mouse embryonic fibroblast (MEF) donor cells into enucleated oocytes. Only two ES cell lines were generated by SCNT, which give a small sample size to examine, but the DNA methylation state of imprinted genes seems to be more severely altered compared to normal ES cell lines at early passages (Fig. 2 and Fig. 4). The abnormal DNA methylation in SCNT-ES cells undergoes further changes during prolonged culture (P10 and P30). For example, the imprinting methylation of the *Snrpn* gene has been completely lost in both the Nt-1 and Nt-2 lines, and that of the *H19* gene has been completely lost in the Nt-1 line (Fig. 4). Chang *et al.* reported that the *H19* imprinted gene displays distinct abnormalities both in SCNT-ES and fertilized embryo-derived ES cell lines after long-term culture *in vitro*, and both exhibit indistinguishable DNA methylation patterns of the imprinted gene [30]. Nevertheless, methylation imprints vary widely in cultured donor cells and their derivative cloned mice, even across the same subclone of donor cells [22]. In fact, results from previous studies indicate that the methylation state of imprinted genes is frequently disrupted in SCNT embryos and their derivative cloned animals [31,32]. In addition, the process of nuclear transfer itself could alter the DNA methylation and gene expression [33]. Thus, the epigenetic marks in SCNT-ES cells may potentially be varied and altered compared to normal ES cells, at least in early passages.

7.2. Uniparental (parthenogenetic) ES cells

We and other groups have suggested that parthenogenetic ES (PgES) cells may be a pluripotent stem cell that could serve as a source of tissue for transplantation [34-36]. PgES cells do not require the destruction of viable biparental embryos as do normal ES cells. In addition, PgES cells do not need viruses or expression plasmids for the establishment of iPS cells. These are very powerful advantages for therapeutic applications. However, the biased epigenetic status and poor pluripotency of parthenogenetic cells are major issues to be overcome. PgES cells are established from parthenogenetic embryos that are produced by the artificial activation of the oocyte. Therefore, PgES cells that possess only maternal genomes could exhibit biallelic or silenced expression of imprinted genes, which causes poor pluripotency. Indeed, parthenogenetic embryos show poor growth and restricted tissue contribution in chimeras [37,38]. However, established PgES cells exhibit an improved contribution to chimeras, compared to chimeras derived from parthenogenetic embryos [39]. Recent reports have shown that loss of imprinting occurred in PgES cells and derivative somatic cells in chimeras and led to changes in the gene expression of imprinted genes and improved pluripotency [2,40]. For example, *Peg1* and *Snrpn* genes are originally silenced in parthenogenetic cells, whereas expression of these genes is elevated in PgES cells by demethylation of the DMR of each gene. PgES cell lines that were reprogrammed by loss of imprinting are closest to normal ES cell lines in

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terms of gene expression pattern and pluripotency. Thus, reprogrammed PgES cells will provide a good tool for therapeutic applications. This is a case in which epigenetic instability in ES cells resulted in a desirable outcome. However, epigenetic instability in ES cells most often leads to undesirable results.

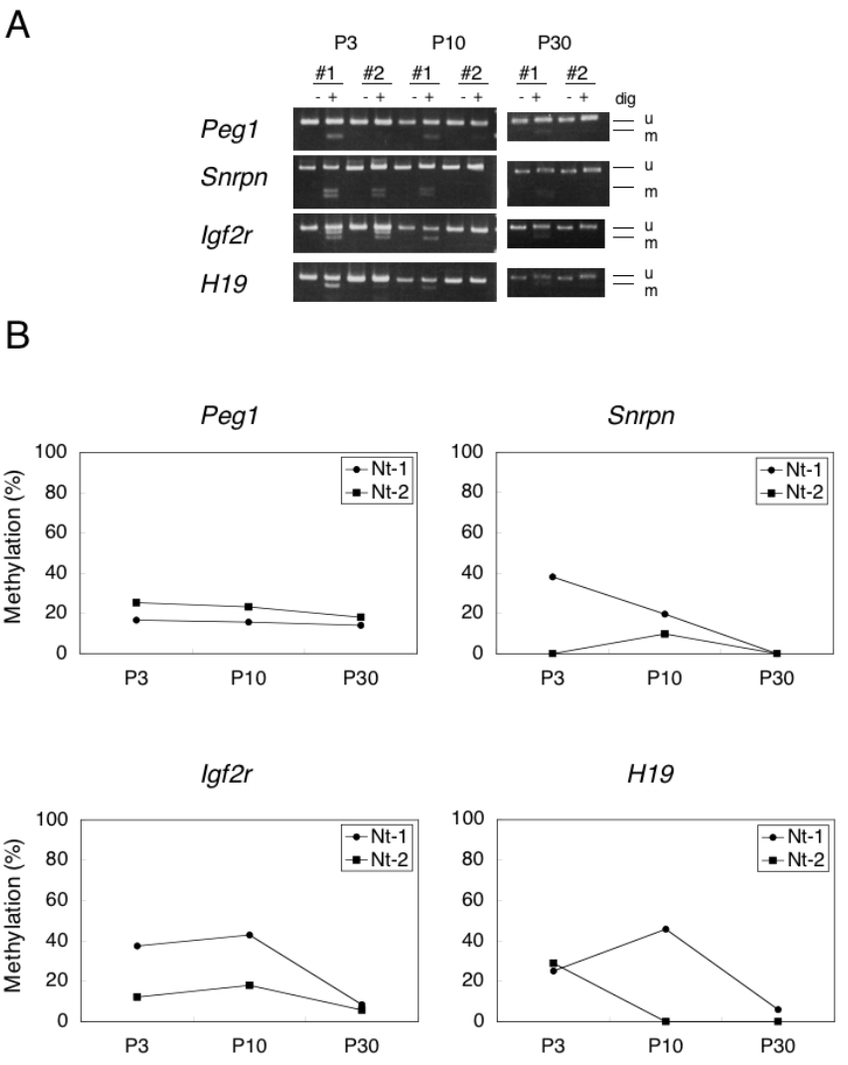

Figure 4. Epigenetic instability in SCNT-ES cells during prolonged culture. Methylation in two SCNT ES cell lines (Nt-1 and Nt-2) was examined by COBRA during prolonged *in vitro* culture (P3, P10 and P30).

8. Effect of altered DNA methylation on pluripotency and disease

In humans, a growing number of reports suggest that children born following ART have an increased risk of developing epigenetic diseases such as Beckwith-Wiedemann syndrome [41,42] and Angelman Syndrome [43], which are caused by epigenetic modifications of im-

printed genes. In sheep, epigenetic changes in the *Igf2r* imprinted gene are associated with fetal overgrowth after IVC [44]. Genome-wide altered DNA methylation also causes epigenetic diseases. For example, genome-wide DNA hypomethylation is commonly observed in human cancers and schizophrenia, and occasionally induces tumors in mice [45-47]. Moreover, hypomethylation in the classical DNA satellites II and III, which are major components of constitutive heterochromatin, is found in ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome in humans [48].

How do these abnormalities in ES cells affect chimeric mice or ES cell-derived tissues? Several studies have indicated that the accumulation of epigenetic alterations during prolonged culture causes a loss of pluripotency in ES cells [21,49]. In chimeras, prolonged culture of ES cells gives rise to abnormalities and frequently results in postnatal death of chimeras possessing a high ES cell contribution [20]. One reason for these problems could be that a loss of imprinting enhances tumorigenesis. In fact, mice derived from ES cells that had a global loss of DNA methylation display widespread cancer formation [50].

9. Candidate genes that cause altered DNA methylation

9.1. DNA methyltransferases

The most important factors for the maintenance of DNA methylation are the DNA methyltransferases. Three CpG DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, coordinately regulate CpG methylation in the genome [12-14]. Deletion of Dnmt1, Dnmt3a or Dnmt3b induces hypomethylation of genomic DNA [14,51], and forced expression of Dnmts causes genomic hypermethylation [52-54]. One of the Dnmt family members, Dnmt3L, is not expressed in differentiated somatic cells but is expressed in ES cells. Although Dnmt3L lacks the functional domains required for catalytic activity, overexpression or downregulation of Dnmt3L results in changes in DNA methylation in ES cells [55]. Thus, the upregulation or downregulation of Dnmts could cause epigenetic instability in ES cells. Indeed, hypomethylation in XX ES cells is associated with reduced levels of Dnmt3a and Dnmt3b, which is the result of both X chromosomes being in the active state [25]. Among Dnmts, a number of alternative splicing variants that lack the regulatory and/or catalytic regions have been reported. In particular, Dnmt3b has nearly 40 different isoforms generated by alternative splicing and/or alternative promoter usage. We recently reported that murine Dnmt3b lacking exon 6 (exon 5 in human) is highly expressed in *in vitro* manipulated embryos and their derivative ES cells that exhibit CpG hypomethylation [17]. Gopalakrishnan *et al*. reported that this isoform is expressed in tumor and iPS cells, and that ectopic overexpression resulted in repetitive element hypomethylation [56]. Similarly, forced expression of human specific DNMT3B4, which lacks a catalytic domain, induced DNA demethylation on satellite 2 in pericentromeric DNA [57]. These reports indicate that Dnmts have complex roles in the maintenance of the DNA methylation state. If this balance collapses, epigenetic instability will result.

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9.2. Other methylation factors

Other new methylation factors are Stella (PGC7) and Zfp57. Stella (PGC7), a primordial germ cell and ES cell marker, protects against DNA demethylation in early embryogenesis [58]. Zfp57, a putative KRAB zinc finger protein, is also required for the post-fertilization maintenance of maternal and paternal methylation at multiple imprinted domains [59]. Reductions of the levels of these factors could induce hypomethylation of DNA in ES cells.

9.3. Active demethylation factors

Active DNA demethylation via the base excision repair pathway has recently been proposed in mammals. In zebrafish, the coupling of a deaminase (activation-induced cytidine deaminase, AID), a glycosylase (methyl-CpG binding domain protein 4, MBD4), and Gadd45 is involved in DNA demethylation [60]. In mammals, AID is indeed required for reprogramming of the somatic cell genome by demethylation of pluripotency genes in ES-somatic cell fusion [61]. Gadd45 also promotes epigenetic gene activation by repair-mediated demethylation in mammals [28,29]. A *Gadd45b* gene is activated in Vitro ES cells that possess hypomethylated imprinted genes and repetitive sequences [1]. Another recently proposed demethylation pathway is the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) mediated by the Ten-eleven translocation (TET) proteins, which ultimately results in DNA demethylation [62-63]. In fact, the TET proteins (Tet1 and Tet2) that regulate 5-hmC production [64] are abundantly expressed in ES cells and may be a cause of epigenetic instability in ES cells.

9.4. Chromatin structure specific to ES cells

In ES cells, bivalent domains of chromatin, that regulate several key developmental genes, contain both repressive (histone H3 lysine 27 methylation) and activating (histone H3 lysine 4 methylation) histone modifications that are usually mutually exclusive [65]. Bivalent domains silence developmental genes in ES cells while preserving their potential to become activated upon initiation of specific differentiation programs. DNA methylation was thought to determine the chromatin structure; however, recent reports suggest that chromatin can affect DNA methylation and demethylation [66-67]. Therefore, bivalent chromatin modifications specific to ES cells could be associated with DNA methylation instability.

10. Conclusion

ES cells exhibit instabilities in DNA methylation that are correlated with the origin of the blastocysts from which they were derived (*in vivo*, *in vitro*, SCNT and uniparental), the culture conditions, sex, and prolonged culture. Epigenotyping of ES cells should be adopted as a prerequisite safety evaluation before their use in chimera production or therapeutic applications. Furthermore, genes associated with aberrant DNA methylation should be monitored in ES cell lines to ensure that the cells do not accumulate epigenetic instabilities.

Nomenclature

5-hmC, 5-hydroxymethylcytosine; 5-mC, 5-methylcytosine; AID, activation-induced cytidine deaminase; COBRA, Combined bisulfite restriction analysis; DMR, differentially methylated region; Dnmt, DNA methyltransferase; ES, embryonic stem; Gadd45, Growth arrest and DNA damage-inducible protein 45; ICM, inner cell mass; IVC, *in vitro* culture; IVF, *in vitro* fertilization; MBD4, methyl-CpG binding domain protein 4; PgES, parthenogenetic ES; SCNT, somatic cell nuclear transfer; TET, Ten-eleven translocation; Vitro ES, ES cells derived from in vitro manipulated embryos; Vivo ES, ES cells derived from embryos developed in vivo.

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