

Review Article

Advance in the Role of Epigenetic Reprogramming in Somatic Cell Nuclear Transfer-Mediated Embryonic Development

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Somatic cell nuclear transfer (SCNT) enables terminally differentiated somatic cells to gain totipotency. Many species are successfully cloned up to date, including nonhuman primate. With this technology, not only the protection of endangered animals but also human therapeutics is going to be a reality. However, the low efficiency of the SCNT-mediated reprogramming and the defects of extraembryonic tissues as well as abnormalities of cloned individuals limit the application of reproductive cloning on animals. Also, due to the scarcity of human oocytes, low efficiency of blastocyst development and embryonic stem cell line derivation from nuclear transfer embryo (ntESCs), it is far away from the application of this technology on human therapeutics to date. In recent years, multiple epigenetic barriers are reported, which gives us clues to improve reprogramming efficiency. Here, we reviewed the reprogramming process and reprogramming defects of several important epigenetic marks and highlighted epigenetic barriers that may lead to the aberrant reprogramming. Finally, we give our insights into improving the efficiency and quality of SCNT-mediated reprogramming.

1. Introduction

Somatic cell nuclear transfer (SCNT), first demonstrated by Gurdon in 1962 [1], is a technology to form reconstructed embryos by injecting donor nucleus into enucleated oocytes and generate cloned animals. The success of SCNT makes the transition from terminally differentiated cells to totipotent cells a reality [2]. It has been about two decades that the first cloned mammal, “Dolly,” the sheep, was born [3]. Since then, investigations on SCNT and cloned animals boomed, and different species were successfully cloned by various donor cell types [4–6]. In 2018, the first nonhuman primate species has been cloned by using fetal fibroblasts as donor cells [7]. Besides animal cloning, SCNT technology is widely used to acquire nuclear transfer embryonic stem cells (ntESCs), which is called therapeutic cloning [8–10]. The derivation of human ntESCs, which was first achieved at

2013 [11] and further improved in the following years [12–14], implies SCNT technology holds great application prospects in human therapeutics.

Although successful, low efficiency (Table 1) as well as defects in extraembryonic tissues and cloned individuals in many species impedes the application of SCNT technology, which has been fully reviewed [4, 15, 16]. SCNT embryos are often arrested at the early stages of preimplantation development. For the most used animal model, mouse, SCNT embryos are usually arrested at 2-cell and 4-cell stages [17, 18]. Even if the embryos develop to blastocyst stage, postimplantation defects and abnormal placentas, like enlarged placenta, were still observed [19]. Only about 1–2% of reconstructed embryos enable to develop to term [4, 20]. For other species, the highest cloning efficiency was demonstrated in bovine, which is about 5–20%, still much lower than that of IVF (about 40–60%) [20]. Even after born,

TABLE 1: Cloning efficiency of inner-species SCNT-mediated reprogramming.

Species	Donor cell type	Total oocytes	Reconstructed oocytes	Cleaved embryo/rate	Blastocyst number/rate of cleaved embryo	Transferred embryo number	Birth pups	Birth rate of cleaved embryo	Birth rate of transferred embryo	References
Sheep	Adult mammary epithelium	—	277	247	29	29	1	0.40%	3.45%	[3]
Cow	Fetal fibroblasts (transgenic)	—	276	—	33/-	28	4	—	14.29%	[113]
Cow	Oviductal cells	150	88	77	20/25.97%	4	3	3.90%	75%	[114]
Cow	Adult cumulus	99	37	31	18/58.06%	6	5	16.13%	83.33%	[114]
Mouse	Adult cumulus cells	—	136	45	—	45 (transferred with 2-cell embryos)	7 (two died at day 6-7)	15.56%	15.56%	[115]
Goat	Fetal fibroblasts (transgenic)	—	138	48	—	47 (transferred with cleaved embryos)	1	2.13%	2.08%	[116]
Pig	Fetal fibroblasts	210	188	110	—	110 (2- and 8-cell stage embryos were transferred)	1	0.91%	0.91%	[117]
Pig	Granulosa cells	245	74	—	—	72	5	—	6.94%	[118]
Rabbit	Adult transgenic cumulus cells	—	775	—	—	371 (transferred with 4-cell stage embryos)	6	—	1.62%	[119]
Cat (<i>Felis domesticus</i>)	Adult cumulus cells	—	—	—	—	3	1	—	33.3%	[120]
Mule	Fetal fibroblasts	120	113	—	—	113 (transferred at different days)	1	—	0.88%	[121]
Horse	Adult skin fibroblasts	—	841	753	22/2.92%	22	1	0.13%	4.55%	[122]
Rat	Fetal fibroblasts	—	—	129	—	129 (transferred with 2-cell stage embryos)	2	1.55%	1.55%	[123]
Dog	Adult skin fibroblasts	—	—	1095	—	1095 (transferred with cleaved embryos)	2	0.18%	0.18%	[124]
Ferret	Adult cumulus cells	—	487	—	—	375 (transferred immediately after activation)	2	—	0.53%	[125]
Buffalo	Fetal fibroblasts & adult granulosa cells	—	—	—	42/11.04-31.39%	42	5 (one died 20 min after birth & 1 died on day 14 after birth)	—	11.9%	[126]
Camel	Adult cumulus cells	75	58	—	-(63.88 ± 8.66)	26	1	—	3.85%	[127]
Cynomolgus monkey	Fetal fibroblast	127	109	79	—	79 (transferred with 2-cell stage embryos)	2	2.53%	2.53%	[7]

abnormalities may still exist, for example, large offspring syndrome, failure of the immune system, and respiratory disorders [19, 20]. Although abnormal phenotypes exist, cloned animals are mostly fertile and the offspring show normal phenotypes [21–23]. Therefore, the abnormalities are largely caused by epigenetic reprogramming defects rather than genetic mutations. Indeed, it has been reported that aberrant reprogramming and epigenetic memories inherited from donor cells are barriers that impede reprogramming [17, 18, 24–27]. Therefore, understanding of epigenetic reprogramming process is essential for prompting the improvement of SCNT technology.

Up to date, great efforts have been made to improve cloning efficiency. However, due to the limitation of methodology and the scarcity of the required materials, especially 1-cell and 2-cell stage embryos, the progress went slowly. While with the development and improvement of low input high throughput sequencing technology, higher resolution of genome-wide epigenetic modification landscapes in SCNT embryos were detected, and our understanding of epigenetic reprogramming becomes clearer [18, 24, 26, 28, 29].

In this review, we will summarize our current knowledge on epigenetic reprogramming, mainly on DNA methylation, histone modifications, histone variants, X chromosome inactivation (XCI), chromatin accessibility, and 3D chromatin structures during SCNT embryo development and recent progress on elevating cloning efficiency and quality. Focusing on how to overcome reprogramming barriers to facilitate SCNT reprogramming and further improve reproductive as well as therapeutic cloning.

2. DNA Methylation

DNA methylation (5-methylcytosine, 5mC) is an epigenetic mark that occurs at cytosine residues in the CpG dinucleotide, generally regarded as associated with transcriptional silencing [30]. About 60–80% of the CpG sites in the mammalian genome are modified by 5mC [31]. DNMT3A and DNMT3B are two methyltransferases essential for *de novo* DNA methylation, and DNMT1 is responsible for its maintenance during embryogenesis [32–34]. DNA demethylation is triggered by ten-eleven translocation (TET) protein-mediated oxidation from 5mC to 5-hydroxymethylcytosine (5hmC) followed by thymine DNA glycosylase- (TDG-) mediated base excision repair [35–37]. In mouse, both maternal and paternal alleles undergo demethylation through active and/or passive manner after fertilization and finally reached the lowest level at the blastocyst stage [38, 39]. It has been reported that knockout of *Dnmt3a* and *Dnmt3b* leads to mouse infertility [32, 40], and deletion of *Tet3* causes an increased frequency of developmental failure in embryos [41], suggesting that optimized DNA methylation pattern is essential for normal development. Thus, a DNA methylation pattern that resembled that of fertilized embryos may be a permissive state for SCNT embryo development.

2.1. DNA Methylation Is Globally Reprogrammed during SCNT Embryo Development. Given that somatic donor cells

usually possess high DNA methylation levels [31], SCNT embryos must undergo global demethylation to reprogram the DNA methylation pattern of somatic cells to that of fertilized embryos. After activation, oocyte-stored TET3 immediately incorporated into pseudopronucleus (PPN) of the reconstructed embryo to catalyze conversion from 5mC to 5hmC, which implies active demethylation during SCNT embryo development [42], bearing resemblance with normal embryo development [42, 43]. Whole-genome bisulfite sequencing (WGBS) of mouse SCNT blastocysts revealed a very low DNA methylation level (15.6%) similar to that of IVF blastocysts (19.1%) [25]. Considering the high methylation level of the donor mouse embryonic fibroblast (MEF) cells (78%) used in the study, the result indicates successful global reprogramming of DNA methylation state. But this demethylation has not completed when the mouse SCNT embryos developed to the late 1 cell stage [28]. Our lab analyzed DNA methylation levels of SCNT embryos by using an embryo biopsy system along with single-cell reduced representation bisulfite sequencing (RRBS), and the results showed that at 2- and 4-cell stage, the SCNT samples possessed generally higher methylation level than the corresponding fertilized embryos [18], suggesting global demethylation in SCNT embryos may require several rounds of replication delusion.

2.2. Aberrant DNA Methylation Reprogramming in SCNT Embryos. Although successful global demethylation in blastocyst, aberrant DNA methylation patterns can be detected in SCNT embryos, even after implantation [5, 44, 45]. In mouse 4-cell stage SCNT embryos, especially arrested samples, the averaged methylation levels on gene body regions were significantly increased, resembling the trend of donor cells [18]. Similarly, cloned, but not fertilized, bovine morula possesses highly methylated nuclei in all blastomeres that resembled those of the fibroblast donor cells [44].

RRBS on 1 cell stage mouse SCNT embryos uncovered more than 20 genes, along with long interspersed elements (LINEs) and long terminal repeats (LTRs) defined as demethylation-resistant regions [28]. Nevertheless, by using ultralow-input WGBS, Gao et al. found that the persistently methylated differentially methylated regions (pDMRs) were moderately similar in arrest and normally developed NT embryos and were more frequently inherited from cleaved embryos to blastocyst stage, which reflects their functional irrelevance in the arrest of SCNT reprogramming [24]. Furthermore, they identified wide-spread regions that were aberrantly remethylated in SCNT embryos compared to the IVF counterparts, called remethylated differentially methylated regions (rDMRs), which are twice in arrested samples as many as in normally developed NT embryos (Figure 1). These rDMRs lead to misexpression of genes and retrotransposons important for zygotic genome activation (ZGA). Reduction of inappropriate DNA methylation rescued the developmental arrest at cleavage stages and facilitated proceeding to blastocyst development, increasing the blastocyst rate to 48.2% (compared to control of 39.5%) [24]. In conclusion, excessive DNA remethylation is a potent barrier that limits the full-term development of SCNT embryos, but the

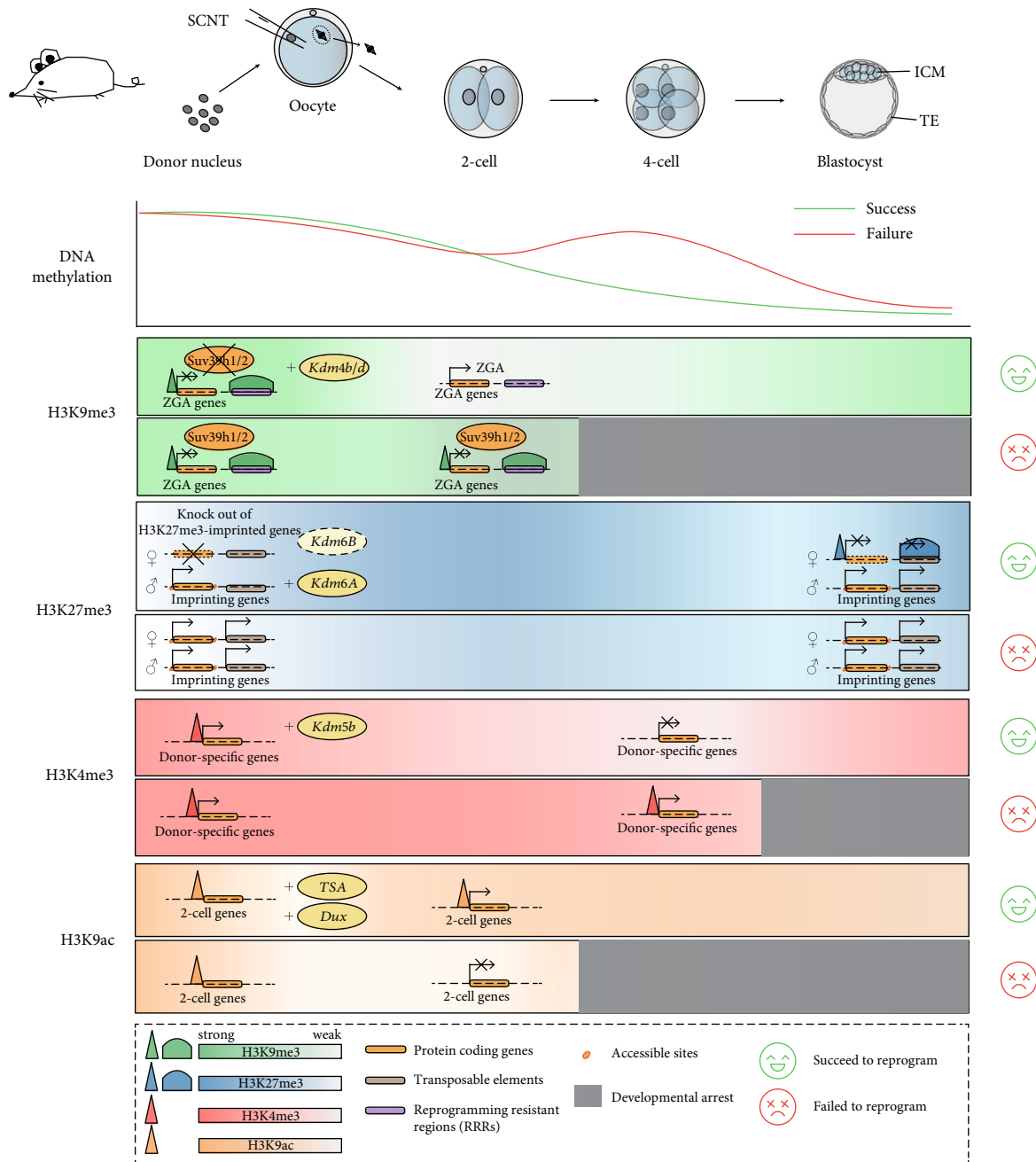


FIGURE 1: Epigenetic reprogramming of DNA methylation and histone modifications during mouse SCNT embryo development. DNA methylation: somatic donor cells usually possess high DNA methylation levels. After activation, the reconstructed embryos undergo global DNA demethylation although this demethylation has not been completed at the late 1 cell stage of SCNT embryos and requires several rounds of replication delusion. However, there is an aberrant remethylation in arrested 4 cell stage SCNT embryos and reduction of the inappropriate DNA methylation rescued the developmental arrest. *H3K9me3*: during SCNT embryo development, some zygotic genome activation (ZGA) genes and reprogramming resistant regions (RRRs) harbor donor cell-inherited H3K9me3 mark, which may be the cause of reprogramming failure. Removal of donor-inherited H3K9me3 either by ectopic expressing *Kdm4b/d* (H3K9me3-specific demethylases) or knockdown of *Suv39h1/2* (H3K9 methyltransferases) can help the embryo overcoming the reprogramming defects. *H3K27me3*: loss of H3K27me3-mediated imprinting leads to defects of extraembryonic tissues of SCNT embryos, such as large placenta phenotype. Although overexpression of H3K27me3-specific demethylase KDM6A elevated blastocyst developmental rate but not full-term development, both knock out of H3K27me3-imprinted genes and knockdown KDM6B can help SCNT embryos undergo successful reprogramming. *H3K4me3*: donor-inherited H3K4me3 is defined as an epigenetic barrier of SCNT reprogramming. H3K4me3 demethylation by *Kdm5b* overexpression is an important step to overcome reprogramming failure. *H3K9ac*: during SCNT development, aberrant H3K9ac regions impair ZGA. TSA treatment and *Dux* overexpression can correct the aberrant H3K9ac signal and help the embryos achieve successful reprogramming.

role of somatic-inherited DNA methylation still needs further proven, after all, an optimized DNA methylation pattern that resembled that of fertilized embryos is essential for SCNT reprogramming.

3. Histone Modifications

In eukaryotic cells, the basic functional unit of chromatin is the nucleosome, containing ~147 bp genomic DNA wrapped around a core histone octamer. Covalent histone modifications, such as acetylation, methylation, ubiquitination, and phosphorylation, are major epigenetic marks that regulate transcription [46–48]. Successful reprogramming of SCNT embryos should include reprogramming of histone modification patterns from somatic donor cells to those of normal embryos. Here, we will discuss the roles of several major histone modifications, including trimethylation at the 9th lysine residue of histone H3 (H3K9me3), trimethylation at the 27th lysine residue of histone H3 (H3K27me3), trimethylation at the 4th lysine residue of histone H3 (H3K4me3), and histone acetylation on SCNT reprogramming.

3.1. Aberrant H3K9me3 Reprogramming Impairs Preimplantation Development. H3K9me3 has been shown to play important roles in heterochromatin formation and repression of gene expression in various types of cells, including preimplantation embryos [7, 49]. In 2014, Matoba and colleagues identified 222 reprogramming resistant regions (RRRs) that failed to be activated in SCNT 2-cell embryos compared to IVF 2-cell embryos. Interestingly, these RRRs are enriched for H3K9me3 in somatic cells [17]. Removal of this epigenetic mark either through ectopic expression of *Kdm4d* (an H3K9me3-specific demethylase) in oocytes or knockdown of *Suv39h1* and *Suv39h2* (two H3K9 methyltransferases) in donor MEF cells not only attenuated the ZGA defect but also improved the reprogramming efficiency of SCNT embryos [17]. Further investigations by Liu et al. identified 7248 genes resisted donor-liked H3K9me3 signal at promoters in 2-cell stage SCNT embryos. Removal of the H3K9me3 mark inherited from donor cells by injecting *Kdm4b* helped the SCNT embryos go over 2-cell arrest and finally significantly elevated the potential of ntESC derivation, blastocyst rate, and even birth rate [18] (Figure 1). In bovine, KDM4D and KDM4E function as regulators that help SCNT embryos to break through H3K9me3 barriers [50]. Moreover, the expression of H3K9me3 demethylase *Kdm4d/4a* could reduce H3K9me3 level and significantly improve the efficiency of human SCNT blastocyst and ntESC cell line formation [13]. And the use of *Kdm4d* combined with histone deacetylase inhibitor (HDACi) trichostatin A (TSA) treatment successfully generated cloned cynomolgus (*Macaca fascicularis*) monkeys by using adult cumulus cells as donor cells [7], although the positive effect of TSA treatment might be functionally linked to H3K9me3 removal in mouse due to unchanged development potential by TSA treatment with *Kdm4d*-mRNA-injected mouse SCNT embryos [17]. The results above imply a conserved barrier of H3K9me3 inherited from donor cells during SCNT reprogramming in mammalian species.

Although the use of *Kdm4d* in SCNT results in an implantation rate comparable with that of IVF, only less than 15% of the implanted SCNT embryos develop to term, and abnormal large placentae are still observed in *Kdm4d*-injected SCNT embryos [17]. Additionally, *Kdm4A* addition was not able to enhance the in vivo long-term development capacity of porcine SCNT embryo [51], indicating H3K9me3 may mainly impede preimplantation development of SCNT embryos and other barriers may affect post-implantation development.

3.2. H3K27me3 Reprogramming Defects Are Obstacles in Pre- and Postimplantation SCNT Embryos. H3K27me3 is an epigenetic regulator widely known as a transcription repressor [52, 53]. During mouse preimplantation development, H3K27me3 is rapidly lost at both maternal and paternal alleles followed by dynamic especially when lineage specification of inner cell mass (ICM) and trophectoderm (TE) [54, 55]. Lots of studies have elucidated the critical role of H3K27me3 during both pre- and postimplantation embryo development [54, 56–59].

Aberrant H3K27me3 reprogramming may be a barrier of SCNT embryo development in various species [25, 60, 61]. Okae et al. identified three DNA methylation-independent imprinted genes *Gab1*, *Sfmbt2*, and *Slc38a4* showed loss of imprinting in all cloned mouse embryos [62], which might be involved in placentomegaly of cloned mouse when considering their important roles in placental development [63, 64]. Further studies found 76 genes with paternal allele-specific DNase I hypersensitive sites (DHSs) that are devoid of DNA methylation but harbor maternal allele-specific H3K27me3 [65]. Interestingly, all the three genes above are included in the 76 genes, which rise the suspect that the defect of H3K27me3 mediated imprinting may cause the abnormality of SCNT placentae. Indeed, many groups proved that loss of H3K27me3-imprinting in SCNT embryos disrupts mouse postimplantation development, and this defect can be detected earliest in blastocyst stage embryos up to now [25, 66, 67]. However, whether this defect exists more earlier in SCNT embryos requires further exploration [68]. A recent study found that the majority of H3K27me3-mediated imprinting regions are located to solo ERVK LTR repeats, which act as imprinted transcription initiation sites for noncoding RNAs and chimeric mRNA in extraembryonic tissues [69]. It is possible that the defects of H3K27me3 reprogramming are relevant to aberrant expression of transposable element during SCNT embryo development. Although restore the normal paternal expression of H3K27me3-imprinting genes (*Sfmbt2*, *Gab1*, and *Slc38a4*) in SCNT placentae by maternal knockout unchanged the enlarged placentae state [66], both correcting the expression of clustered miRNAs within the *Sfmbt2* gene and quadruple monoallelic deletion of *Sfmbt2*, *Jade1*, *Gab1*, and *Smoc1* ameliorates the placental phenotype, especially *Sfmbt2* [66, 67].

Apart from the impact of loss of H3K27me3-imprinting on SCNT postimplantation, another group demonstrated H3K27me3 as an obstacle of SCNT preimplantation development. Overexpression of the H3K27me3-specific demethylase KDM6A significantly increased the SCNT blastocyst

formation rate but did not improve the rate of full-term development, implies lack of KDM6A may be not the reason for loss of H3K27me3-dependent imprinting, at least in mouse. Contrastingly, knockdown of KDM6B not only facilitated ZGA and improved the blastocyst formation rate but also increased birth rate and ntESC establishment efficiency [68] (Figure 1). Collectively, both deposition on specific regions (like H3K27me3-imprinting genes) and appropriate removal of H3K27me3 are important for successful SCNT reprogramming although underlaid mechanisms are still unknown.

3.3. Somatic Inherited H3K4me3 Is a Potent Barrier of SCNT-Mediated Reprogramming. H3K4me3 is usually associated with transcriptional activation. Many groups have depicted the pattern of H3K4me3 during preimplantation in mouse [54, 70, 71]. Both appropriate removal of non-canonical H3K4me3 by *Kdm5b* in oocyte and establishment of canonical and broad H3K4me3 in preimplantation embryos are essential for normal mouse development [54, 70]. Unlike the well-described H3K4me3 pattern in normal mouse preimplantation embryos, studies about the whole H3K4me3 pattern during SCNT reprogramming have not been reported until now.

In 2016, we found that *Kdm5b* failed to be activated in 4-cell-arrest SCNT embryos. Injection of si-*Kdm5b* in MII oocytes largely reduced the rate of high-quality blastocyst development, and overexpression of *Kdm5b* helped the SCNT embryos to pass 4-cell arrest and significantly increased blastocyst formation rate and quality. What is more, the gene expression levels of NT 4-cell embryos were largely rescued by the overexpression of *Kdm5b* [18]. Considering the role of *Kdm5b* as H3K4me3 demethylase and the function of H3K4me3 on transcription initiation, it is possible that H3K4me3 mark with donor-specific signature may be a barrier of SCNT reprogramming (Figure 1). This point has been proved in *Xenopus*, human, and bovine SCNT embryos that donor-inherited H3K4me3 acts as an epigenetic barrier impacts SCNT reprogramming [72, 73]. H3K4me3 demethylation by *Kdm5b* overexpression not only attenuated ON-memory genes (genes highly expressed in donor cells and SCNT embryos but not IVF embryos) but also improved cloning efficiency. The results indicate that removal of the donor-specific H3K4me3 mark may efficiently reprogram the SCNT embryos but much more further investigations about roles of H3K4me3 during SCNT-mediated reprogramming need to be performed.

3.4. Aberrant Histone Acetylation Impairs the SCNT Efficiency. Histone acetylation usually occurs on the lysine residues of core histones and marks both promoters and enhancers. Acetylation has the potential to loosen nucleosome configuration and increase chromatin accessibility for transcription factors [74]. During ZGA, the persistent accessible enhancers are marked by H3K27ac and characterized by distal H3K4me3 deposition in human early embryos, while the poised enhancers are likely to be activated in later development by remarked H3K27ac in a tissue-specific manner [75]. In early zebrafish embryos,

widespread H3K27ac deposition is found to be required for gene activation [76]. This indicates that histone acetylation reprogramming is another critical step for early embryo development.

When somatic cell nuclei are injected into the enucleated MII oocytes, the acetylated lysine residues are quickly deacetylated and then reacetylated after activation. The reestablishment of histone acetylation is essential for zygotic gene activation in cloned embryos [77]. However, several acetylation marks on histones, such as H4K8ac and H4K12ac, are persisted in the genome during SCNT, which may be responsible for the low cloning efficiency. On the other hand, histone deacetylase inhibitors (HDACi), which can improve histone acetylation and the success rate of cloning significantly, have been widely used during SCNT [78]. Recently, our group generated the genome-wide H3K9ac map during SCNT development and found the aberrant acetylated regions impair the zygotic gene activation. TSA treatment and Dux overexpression can correct the aberrant H3K9ac signal [79] (Figure 1). These suggest the reestablishment of histone acetylation is also a necessary part of epigenetic reprogramming. It should be noted that HDACi treatment can also improve nascent mRNA production [80] and gene expression [81] during SCNT embryo development, so the mechanism of HDACi treatment improves cloning efficiency still deserve further investigation.

4. Histone Variants

Aside from the canonical histones, histone variants endow chromatin critical functions, and their roles in oocyte-mediated reprogramming have been reviewed elsewhere [82–85]. The mammalian sperm genome is packaged into highly condensed chromatin consisting primarily of protamine but 5-15% residual histones. After fertilization, the paternal genome undergoes dramatic chromatin remodeling, and maternally stored histones, such as H3.3 (coded by *H3f3a* and *H3f3b*), are incorporated into the sperm nucleus as early as 1 h after fertilization [85]. And the incorporation is essential for the activation of the paternal genome and preimplantation development during embryogenesis [86].

Although the somatic cell genome is packaged by histones rather than protamine, global chromatin remodeling was still observed [85, 87]. After activation, donor cell-derived histone H3 variants H3.1, H3.2, and H3.3, as well as H2A, H2A.Z, and microH2A, were rapidly eliminated from the chromatin [87, 88]. All the three oocyte-stored H3 variants, H2A.X, and oocyte-specific H1 variant, H1FOO, were incorporated into the donor genome within minutes of nuclear transfer [87, 89, 90]. Knockdown of histone variant H3.3 in mouse oocytes results in compromised reprogramming and downregulation of key pluripotent genes, and this compromised reprogramming was rescued by injecting exogenous H3.3 mRNA, but not H3.2 mRNA into oocytes [85], revealing a critical role of optimized chromatin variants incorporation in normal SCNT reprogramming.

5. X Chromosome Inactivation (XCI)

XCI is a remarkable event during normal embryogenesis [62, 91]. X chromosome is inactivated during spermatogenesis. During mouse embryogenesis, the paternal X chromosome is reactivated at the 2-cell stage. After that, the paternal X chromosome will be silenced again through an imprinted manner and persisted in extraembryonic lineages. In contrast, the paternal X chromosome is reactivated in the epiblast in the late blastocyst, then, the X chromosome from maternal or paternal genomes is randomly inactivated during embryo development [92–94]. The precise regulation of dynamic activity of the X chromosome is crucial for the epigenetic reprogramming during early embryo development [95].

XCI ensures a similar dosage of X-linked genes between male and female cells. However, this event in SCNT embryos is largely abnormal among species [27, 51]. In mouse SCNT embryos, X-linked genes were largely downregulated, which is caused by ectopic expression of *Xist* from the active X chromosome regardless of sex, leading to abnormal inactivation of both X chromosomes [27]. Similarly, *Xist* is also known to be aberrantly expressed in bovine and pig SCNT embryos and proven to be associated with prenatal death [96, 97], suggesting excessive *Xist* expression may be a barrier of SCNT-mediated reprogramming. Deletion of *XIST* on the active X chromosome rescued global gene expression and resulted in about an 8- to 9-fold increase in cloning efficiency [27]. Concordantly, prior injection of *Xist*-siRNA into reconstructed oocytes normalized global gene expression of mouse SCNT embryos at the morula stage and further improved cloning efficiency 10-folds higher than control [98]. Moreover, correction of the abnormal XCI has a synergistic effect with TSA but ectopic activation of *Xist* is reprogramming barrier independent of H3K9me3 inherited from donor cells [25, 98]. Differently in pig, abnormal XCI seems linked with H3K9me3 for that increased quality of *XIST*-deficient SCNT embryos was associated with the global H3K9me3 reduction and vice versa; *Kdm4a* addition also induced *XIST* derepression in the active X chromosome [51]. This discrepancy may be a result of different XCI processes among different species, and the underlaid mechanisms require further understanding.

6. Chromatin Accessibility

Chromatin accessibility is a good indicator of transcriptional regulatory elements and can serve as a predictor of gene transcription activity. In recent years, with the development and improvement of low-input DNase I hypersensitive sequencing (liDNase-seq) and assay for transposase-accessible chromatin using sequencing (ATAC-seq), accessible chromatin sites of mouse and human preimplantation embryos enabled to be profiled [29, 65, 99, 100]. By using liDNase-seq, Lu et al. uncovered that DNase I-hypersensitive site (DHS) landscape is progressively established with a drastic increase at the 8-cell stage of mouse preimplantation embryos [29]. The global chromatin de- and recondensation is likely promoted by cis-regulating of LINE-1 transcriptional activity [101]. Tran-

scription factors *Nfya* and *Oct4* were responsible for DHS formation at 2- and 8-cell stage embryos, respectively [29].

Full-pattern of chromatin accessibility during mouse and human SCNT embryo development has not been elucidated, but a recent research profiled DHSs in donor cells and late-1-cell stage mouse SCNT embryos. They found SCNT-mediated reprogramming of chromatin accessibility is largely completed by 12 h after activation because DHSs of the donor cells are drastically changed to recapitulate that of the IVF zygotes within 12 h. Surprisingly, this change is DNA replication-independent, which is conserved in *Xenopus* SCNT embryos [102], and the switch from donor-specific TF network to that of zygotic may be the critical factor responsible for the DHS profile reprogramming [103].

Despite global reprogramming, some regions are resistant to reprogram [103]. Failure to close accessible somatic promoters or to open distal regulatory regions required for differentiation program may be the major reprogramming barriers. It is interesting that these regions are enriched for H3K9me3, a robust reprogramming barrier discussed above, in both donor cells and 2-cell SCNT embryos [103]. Considering the change of the TF network which accompanies with this reprogramming, failure of specific somatic cell TFs to dissociate from chromatin can also be a barrier in SCNT reprogramming. ATAC-seq on *Xenopus* SCNT embryos revealed great loss of chromatin accessible sites before first cleavage compared to that of donor cells, which is concordant with the pattern in mouse. The researchers found genes that are silenced but have preexisting open transcription start sites (TSSs) in donor cells are prone to be activated after SCNT, while genes resistant to reprogramming are associated with closed chromatin configurations [102]. It is possible that preexisted open accessibility of donor-specific genes and closed accessibility of zygotic-essential genes inherited from donor cells may be barriers during SCNT reprogramming, but it needs further proven.

7. Higher-Order Chromatin Structure

Chromatin in the nucleus of eukaryotic cells is packaged in a hierarchical structure, which is associated with many biological processes [104, 105]. The role of the 3D genome organization during mammalian embryogenesis has been investigated benefit from the advance of the low-input Hi-C (genome-wide chromosome conformation capture) technology in recent years [106–108], which reveals the removal and reestablishment of chromatin higher-order structure are essential for both mouse [106, 107] and human [108] embryogenesis.

A recent study of our group profiles the spatiotemporal dynamic of 3D chromatin structure in SCNT early embryos and reveals 3D chromatin structure can be rapidly reorganized to an embryo-like state after nuclear transfer. However, the aberrant TADs and compartment A/B organization can be observed and remain throughout preimplantation SCNT embryo development. Overexpression of KDM4B, a H3K9me3 demethylase, can partially improve the abnormal 3D chromatin structures [26] (Figure 2). This

3D chromatin structure

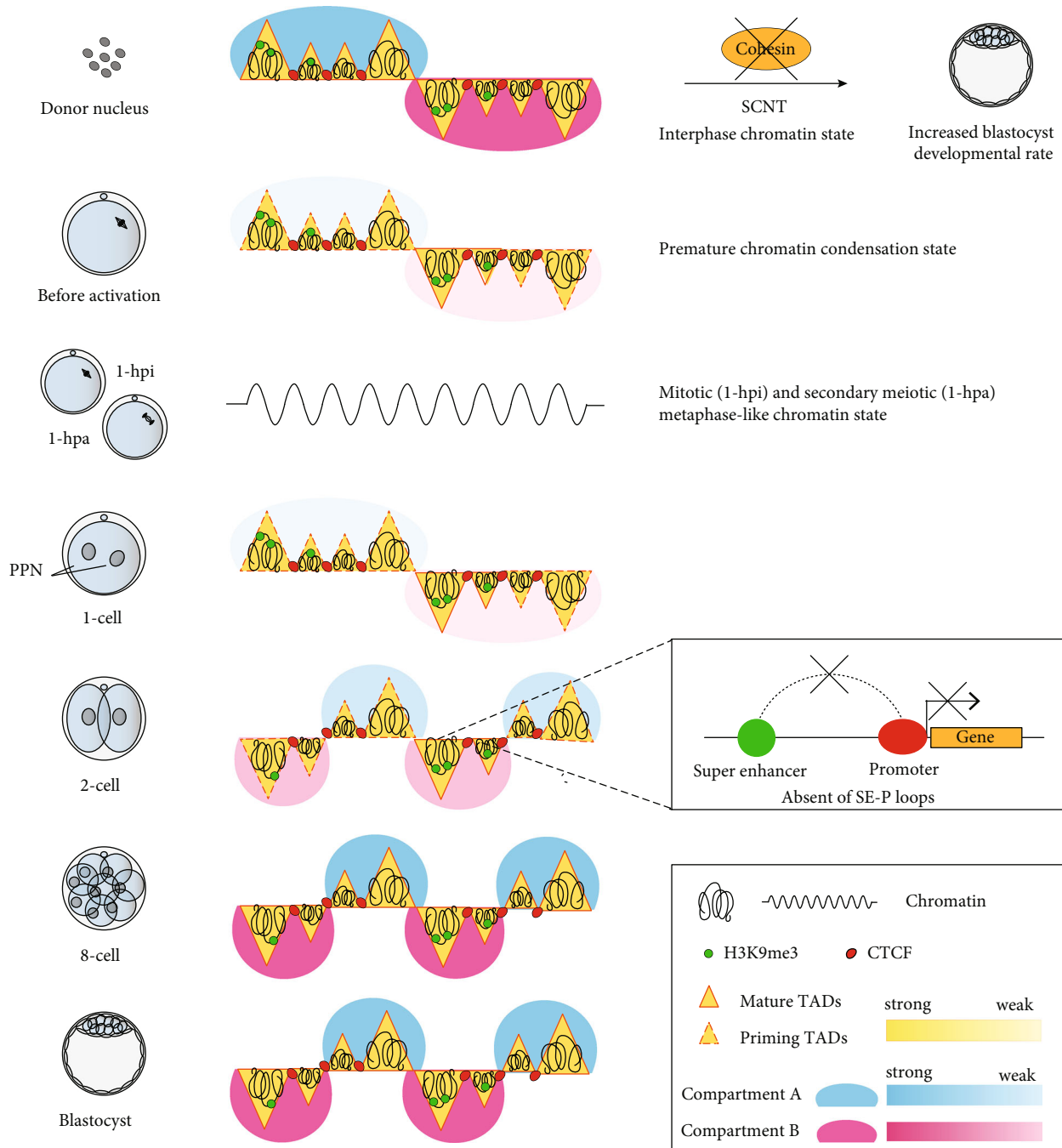


FIGURE 2: The higher-order chromatin organization in mouse SCNT embryos. Somatic donor cells exhibit interphase-state chromatin characterized by mature compartments and topologically associating domains (TADs). Before activated, the transferred nucleus first enters a mitotic-like state (premature chromatin condensation) followed by exhibiting mitotic and secondary meiotic metaphase-like chromatin states lacking compartments and TADs 1 hour postinjection (1-hpi) and 1-hour postactivation (1-hpa), respectively. TADs are stronger in SCNT 1-cell stage embryos and then become weaker at the 2-cell stage and gradually consolidating. Super enhancer-promoter (SE-P) loops that exist in fertilized 2-cell embryos are absent in SCNT 2-cell embryos, which is correlated with aberrant H3K9me3 and TAD persistence. Compartments A/B are markedly weak in 1-cell SCNT embryos and become increasingly strengthened afterward. By the 8-cell stage, somatic chromatin architecture is largely reset to embryonic patterns until the blastocyst stage. Predepleting cohesin in donor cells increases SCNT reprogramming efficiency.

indicates a correlation between the organization of 3D chromatin structure and histone modifications during epigenetic reprogramming.

8. Removal of Multiple Barriers Is a Promising Approach to Improve SCNT Reprogramming

It has been over two decades that the first mammalian species has been successfully cloned, but low efficiency was still observed until recently. Numerous efforts have been made to increase reprogramming efficiency by removing epigenetic barriers. Matoba et al. found H3K9me3 inherited from donor cells act as a barrier that impede mouse SCNT-mediated preimplantation development. Removal of H3K9me3 in donor cells by injecting *Kdm4d* mRNA into reconstructed embryos 5 hours postactivation (hpa) significantly increased the blastocyst rate up to 81.2% (% blastocyst of cleaved embryos) regardless of donor cell types and elevated birth rate from only 1% up to 8.7%. Besides, the rate of ntESC line derivation was increased from 10.1% to 50% after *Kdm4d* injection. Moreover, to prevent the establishment of H3K9me3 in donor cells, they knock down *Suv39h1/2* (H3K9me3 transferases) in donor cells prior SCNT and improved blastocyst rate from 6.7% to 49.9% [17]. Accordingly, our lab found another H3K9me3 demethylase, *Kdm4b*, efficiently removed the H3K9me3 barrier to increase blastocyst rate from about 30% to over 80%. Simultaneously, we found H3K4me3 may be a candidate epigenetic barrier that impedes SCNT-mediated reprogramming. Injection of *Kdm5b* mRNA into enucleated oocyte significantly improves mouse blastocyst rate from about 30% to over 50%. It is worth noting that coinjection of *Kdm4b* and *Kdm5b* successfully elevated blastocyst rate over 95% and led to over 11% of cloned embryos developing to live animals, moreover, 60% ntESC derivation efficiency based on the total number of MII oocytes rise the possibility that removing multibarriers may be a more efficient way to improve cloning efficiency [18].

We found excessive remethylation is a potent epigenetic barrier in another study. Optimized DNA methylation level by injecting siRNAs of *Dnmt3a* and *Dnmt3b* into enucleated oocytes, 48.2% blastocysts were generated from cleaved embryos (39.5% blastocyst rate of control group). Furthermore, of enucleated oocytes that subjected to *Kdm4b+5b* mRNA and siDnmt3a+3b co-injection, 92.3% cleaved embryos developed to blastocyst stage [24]. Another study of Matoba et al. demonstrated that using a combination of *Xist* knockout donor cells and overexpression of *Kdm4b*, more than 20% birth rate of mouse SCNT embryos were achieved [25], which is coincided with the idea that removing multiple epigenetic barriers is a more efficient method for SCNT reprogramming.

9. Concluding Remarks

SCNT provides the only way to reprogram somatic cells into totipotent embryos and generate viable animals [9, 109, 110]. After injected into enucleated oocytes, the donor nucleus quickly undergoes nuclear membrane breakdown followed by premature chromosome condensation (PCC), which is

triggered by the M-phase-prompting factors (MPFs) stored at ooplasm [111]. After activation, the nuclear membrane is reformed to envelop PPN and incorporates amounts of maternal factors [112]. Then, the reconstructed embryos undergo SCNT-mediated embryogenesis. However, only few of reconstructed embryos can develop to the blastocyst stage, let alone develop to term. ZGA failure and disrupted transcriptome were detected in SCNT embryos very often, and this is largely affected by aberrant epigenetic reprogramming [4].

In this review, we concluded our understanding on epigenetic barriers of SCNT-mediated reprogramming and methods to overcome these epigenetic berries. Given that removal of multiple barriers that impede SCNT-mediated reprogramming gives a blastocyst rate over 95% of cleaved embryos and ntESC derivation efficiency of 60% based on the total number of MII oocytes [18], and over 20% birth rate of mouse [25], we demonstrate removing multiple barriers may be a more efficient approach to achieve complete reprogramming compared to single barrier removal. However, low birth rate compared with IVF counterpart and large placentae were still observed. Therefore, further studies need to focus on exploring more about reprogramming barriers and emphasizing on removing multiple barriers to achieve nearly complete SCNT reprogramming.

Abbreviations

SCNT:	Somatic cell nuclear transfer
ntESCs:	Nuclear transfer embryonic stem cells
XCI:	X chromosome inactivation
5mC:	5-Methylcytosine
DNMT3A:	DNA methyltransferase 3A
DNMT3B:	DNA methyltransferase 3B
DNMT1:	DNA methyltransferase 1
TET:	Ten-eleven translocation
5hmC:	5-Hydroxymethylcytosine
TDG:	Thymine DNA glycosylase
PPN:	Pseudopronucleus
WGBS:	Whole-genome bisulfite sequencing
MEF:	Mouse embryonic fibroblast
RRBS:	Reduced representation bisulfite sequencing
LINEs:	Long interspersed elements
LTRs:	Long terminal repeats
pDMRs:	Persistently methylated differentially methylated regions
rDMRs:	Remethylated differentially methylated regions
ZGA:	Zygotic genome activation
H3K9me3:	Trimethylation at the 9 th lysine residue of histone H3
H3K27me3:	Trimethylation at the 27 th lysine residue of histone H3
H3K4me3:	Trimethylation at the 4 th lysine residue of histone H3
HDACi:	Histone deacetylase inhibitor
TSA:	Trichostatin A
PRC2:	Polycomb repressive complex 2
liDNase-seq:	DNase I hypersensitive sequencing

ATAC-seq: Transposase-accessible chromatin using sequencing
 DHS: DNase I-hypersensitive site
 TSSs: Transcription start sites
 hpa: Hour postactivation
 PCC: Premature chromosome condensation
 MPFs: M-phase-prompting factors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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