Epigenetic abnormalities associated with somatic cell nuclear transfer

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Abstract

Twenty-five years have passed since the birth of Dolly the sheep, the first mammalian clone produced by adult somatic cell nuclear transfer (SCNT). During that time, the main thrust of SCNT-related research has been the elucidation of SCNT-associated epigenetic abnormalities and their correction, with the aim of improving the efficiency of cloned animal production. Through these studies, it has become clear that some epigenomic information can be reprogrammed by the oocyte, while some cannot. Now we know that the imprinting memories in the donor genome, whether canonical (DNA-methylation-dependent) or noncanonical (H3K27me3-dependent), are not reprogrammed by SCNT. Thus, SCNT-derived embryos have the normal canonical imprinting and the erased noncanonical imprinting, both being inherited from the donor cells. The latter can cause abnormal phenotypes in SCNT-derived placentas arising from biallelic expressions of noncanonically imprinted genes. By contrast, repressive epigenomic information, such as DNA methylation and histone modifications, might be more variably reprogrammed, leaving room for technical improvements. Low-input analytical technologies now enable us to analyze the genome of gametes and embryos in a high-throughput, genome-wide manner. These technologies are being applied rapidly to the SCNT field, providing evidence for incomplete reprogramming of the donor genome in cloned embryos or offspring. Insights from the study of epigenetic phenomena in SCNT are highly relevant for our understanding of the mechanisms of genomic reprogramming that can induce totipotency in the mammalian genome.

Introduction

Somatic cell nuclear transfer (SCNT) is the only reproductive technology that produces an animal individual from a single somatic cell nucleus and an enucleated oocyte. Unlike genomic reprogramming by transcription factor transduction for generating pluripotent stem cells, SCNT reprograms the somatic genome to generate a totipotent genome. Because SCNT-derived embryos (hereinafter SCNT embryos) are produced without fertilization – not using the germline genome – their development can be associated with abnormalities that are not common in normal embryonic development. Abnormalities found in SCNT embryos can be classified as either genetic or epigenetic according to their causes (Table 1). Genetic abnormalities can occur before or after SCNT; the former are of donor cell origin and the latter are induced by the nuclear transfer procedure. Typical genetic abnormalities that occur after nuclear transfer are chromosomal aberrations caused by cell cycle asynchrony between the recipient oocyte and the donor cell (Miki et al. 2004) or simply induced by handling in vitro. For example, in mice abnormal chromosome segregation (ASC) frequently occurs during the early cleavage stages of SCNT embryos although they can also be found in embryos produced by intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF) to a lesser extent (Mizutani et al. 2012). This SCNT-associated ASC is thought to be closely linked with misalignment of the donor chromosomes from MII oocytes, which is most frequently found in SCNT of mammals that are difficult to clone such as monkeys and rabbits (Yin et al. 2002, Simerly 2003). Genetic abnormalities in SCNT embryos might facilitate the birth of sex-reversed animals. Thus, in our
laboratory, one female mouse was born following a Sertoli cell SCNT experiment caused by an accidental XO chromosomal composition (Inoue et al. 2009). Although whether this could be attributed to the donor cell or to the SCNT procedure is unclear, this mouse was fertile and produced offspring after mating with a cloned male littermate.

Unlike genetic abnormalities, epigenetic abnormalities associated with SCNT are more diverse in their types, reflecting the complexity of the epigenetic regulatory mechanisms during embryonic development. However, similar to conventional genetic abnormalities, they can also be roughly divided into two groups: those derived from donor cells and those that arise after the procedure. The latter is likely to have much in common with assisted reproductive technologies (ARTs) such as ICSI and IVF, and in general abnormal phenotypes can be further reinforced following SCNT, as shown in cloned mouse and bovine embryos (Rhon-Calderon et al. 2019, Rivera 2019). By contrast, epigenetic abnormalities originating from the donor cell nucleus are SCNT-specific. With the exceptions of those accumulating in donor animals or induced during cell culture, SCNT-associated epigenetic abnormalities most likely reflect differences in epigenetic status between the donor somatic cells and germline cells. Most are thought to be somatic epigenomic markers that are resistant to genomic reprogramming by maternal factors and are thus carried over to the genome of the reconstructed SCNT embryos. Importantly, most of these donor cell-derived epigenetic abnormalities are nonrandom and occur in almost all SCNT embryos, placentas and offspring, whereas other genetic and post-nuclear transfer (NT) epigenetic abnormalities can occur randomly (Table 1). Such donor cell-derived epigenetic aberrations are a fundamental problem of SCNT and can provide important clues for understanding the mechanisms of genomic reprogramming. In this review, we describe and discuss the current knowledge on epigenetic abnormalities in embryos and placentas generated by SCNT, with special focus on those identified by recently developed low-input genome-wide sequencing technologies in mice. More detailed information on the technical aspects and practical applications of mammalian cloning by SCNT has been published elsewhere (Ogura et al. 2013, Loi et al. 2016, Czernik et al. 2019).

### Histone modifications

#### Histone methylation

Histone methylation, which is regulated by histone methyltransferases and histone demethylases, plays critical roles during normal development at almost all stages (Jambhekar et al. 2019). One of the typical epigenetic abnormalities present in SCNT embryos is histone H3 lysine 9 trimethylation (H3K9me3) (Matoba et al. 2014, Chung et al. 2015). In differentiated cells, H3K9me3 is generally associated with constitutive heterochromatin where it is enriched for repetitive elements while being poor with genes (Becker et al. 2016). The genes in heterochromatin are transcriptionally silenced due to the condensed chromatin status. The nuclear H3K9me3 distribution in SCNT embryos is abnormal compared with fertilized zygotes in many species including bovine (Santos et al. 2003, Pichugin et al. 2010), rabbit (Yang et al. 2013), and mouse embryos (Ribeiro-Mason et al. 2012) (Fig. 1A). In 2014, by combining the transcriptomic and epigenomic analyses in mouse SCNT model, Matoba et al. showed that donor cell-derived H3K9me3 prevents zygotic genome activation (ZGA) from the injected donor somatic cell nucleus just after NT, thereby blocking the normal development of SCNT embryos from the two- to four-cell stages (Matoba et al. 2014). They termed

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**Table 1** Classification of abnormalities associated with SCNT.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Origin</th>
<th>Causes</th>
<th>Random or non-random</th>
<th>Examples</th>
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<tbody>
<tr>
<td>Genetic</td>
<td>Donor cell origin</td>
<td>In vivo-derived errors</td>
<td>Random</td>
<td>Cell aging</td>
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<td></td>
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<td>In vitro-derived errors during cell culture</td>
<td>Random or with a specific tendency</td>
<td>Missing Y chromosome</td>
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<tr>
<td>NT origin</td>
<td>In vitro handling*</td>
<td>Physiological causes</td>
<td>Random</td>
<td>Mechanical damage by a small injection pipette</td>
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<tr>
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<td>Donor cell origin</td>
<td>In vivo-derived errors</td>
<td>Random</td>
<td>Instability of the spindle</td>
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<td></td>
<td></td>
<td>In vitro-derived errors during cell culture</td>
<td>Random or with a specific tendency</td>
<td>Cell aging</td>
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<tr>
<td></td>
<td>Somatic cell-specific epigenome (excessive)</td>
<td></td>
<td>Non-random</td>
<td>Histone modifications (H3K9me3, H3K27me3)</td>
</tr>
<tr>
<td></td>
<td>Somatic cell-specific epigenome (absence)</td>
<td></td>
<td>Non-random</td>
<td>DNA methylation</td>
</tr>
<tr>
<td>NT origin</td>
<td>In vitro handling*</td>
<td>Physiological causes</td>
<td>Random or with a specific tendency</td>
<td>Aberration of genomic imprinting</td>
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<td></td>
<td></td>
<td></td>
<td>Random</td>
<td>Accidental reprogramming errors</td>
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*Many are common with assisted reproductive technologies (ARTs), for example, large offspring syndrome in ruminants.
Epigenetic abnormalities found in SCNT

Figure 1 Overview of the life cycle and epigenetic changes related to the phenotypes of somatic cell nuclear transfer (SCNT)-derived embryos and placentas. (A) Somatic cell-specific epigenetic marks (DNA methylation and H3K9me3). Somatic cell-specific marks are established in the embryonic lineage after implantation and are maintained in most adult tissues. Normal embryos do not have these marks because they are erased in the germline before gametogenesis. However, SCNT-derived embryos retain these marks from donor cells, leading to abnormal gene expressions. (B) Canonical (DNA methylation-dependent) genomic imprinting. When established in either the male or female germline, this is essentially maintained in the corresponding alleles of most somatic cells and is normally inherited by SCNT-derived embryos. (C) Noncanonical (H3K27me3-dependent) genomic imprinting. This is established during oogenesis, maintained until the peri-implantation period, and then erased in the embryonic lineage. Therefore, loss of imprinting (LOI) of these genes occurs in SCNT-derived embryos, leading to ectopic Xist expression in the maternal X chromosome in preimplantation embryos and biallelic expressions of placenta-specific genes. These figures are modified from Fig. 4B of Oikawa et al. (2014). The image of a mouse is from TogoTV (©2016 DBCLS TogoTV/CC-BY-4.0).

these H3K9me3-enriched regions ‘reprogramming resistant regions’ (RRRs) and found that these are highly condensed in many types of somatic cells, suggesting that they represent constitutive heterochromatin. Importantly, the H3K9me3 in these RRRs could be removed by overexpressing the H3K9me3-specific histone demethylase Kdm4d. This allowed the SCNT embryos to activate the genome normally at the two-cell stage and thereby greatly improved the full-term development rate of mouse SCNT embryos from 1 to 8% per transfer to pseudopregnant females. A combination of H3K9me3 removal by Kdm4d with correction of Xist gene expression in SCNT embryos further increased the cloning efficiency to 18.7% in cumulus cell cloning and 23.5% in Sertoli cell cloning (Matoba et al. 2018). Treatment of SCNT embryos with histone deacetylase inhibitors such as Trichostatin A (TSA) has also been shown to improve the developmental efficiency (see subsequently) (Kishigami et al. 2006, Van Thuan et al. 2009), but this improvement might be through activating RRRs based on a significant overlap between Kdm4d-activated genes and TSA-activated genes in SCNT embryos (Inoue et al. 2015). A recent study using low-input Hi-C analysis revealed that the action of Kdm4d involves the reorganization of chromatin architecture (Chen et al. 2020). In a ‘natural’ setting (i.e. normal SCNT reprogramming without any booster treatment), Kdm4b, which is also a H3K9me3 demethylase, appeared to play a predominant role in such H3K9me3-reprogramming, because knockdown of Kdm4b compromised the development of mouse SCNT embryos (Liu et al. 2016). Later, other types of Kdm4 family genes, such as KDM4A (Chung et al. 2015) or Kdm4b (Liu et al. 2016) were shown to be similarly effective in terms of improving the efficiency of SCNT. Importantly, this Kdm4-mediated boost in cloning efficiency has been shown to be effective in other mammals, including sheep (Zhang et al. 2018), bovines (Liu et al. 2018a), monkeys (Liu et al. 2018b), or even humans (Chung et al. 2015), although in the latter case, in vitro culture is ethically limited to the blastocyst stage, which can be used for NT embryonic stem (ES) cell derivation. It should be noted that H3K9me3 removal could sometimes cause other epigenetic abnormalities, including Xist derepression, which causes postimplantation embryo death, as observed in a porcine SCNT model (Ruan et al. 2018). Nonetheless, it is very likely that the types of H3K9me3 abnormalities seen in SCNT embryos are conserved across most mammalian species, and thus a Kdm4-mediated boost approach would be the first candidate approach to improve SCNT in most of them. Because H3K9me3 can be removed in donor somatic cells even before NT by knocking down the H3K9 methyltransferasen (Matoba et al. 2014, Jafarpour et al. 2020), Suv39h1 and Suv39h2, deletion or inhibition of these activities might also be another approach to improve SCNT.

In addition to H3K9me3, H3K4me3 also shows abnormalities in SCNT embryos. As described above, it is obvious that most SCNT embryos arrest development
at early developmental stages but some can survive even to term. This suggested the presence of embryo-to-embryo variation in developmental potential. To correlate the developmental potential of a single embryo with its transcriptional landscapes, Liu et al. performed biopsy-based single blastomere RNA sequencing and in vitro culture of biopsied SCNT embryos in mice (Liu et al. 2016). They found that the levels of Kdm5b—a H3K4me3-specific demethylase—at the 4-cell stage clearly correlated with the rate of development from the 4-cell to 8-cell stages. Importantly, when they injected Kdm5b mRNA into mouse SCNT embryos, the efficiency of this measure of development was improved significantly. Moreover, injection of Kdm4d and Kdm5b together synergistically improved the SCNT efficiency and over 95% of reconstructed embryos successfully reached the blastocyst stage. Consequently, the live pup formation rate of SCNT embryos was further improved up to 11% per transfer. Given that H3K4me3 is a marker for active genes, Kdm5b-mediated H3K4me3 removal would help in erasing the epigenetic memory of active transcription present in donor cells. Indeed, such transcriptional memory of donor cells has been documented not only in normal mouse SCNT (Gao et al. 2003) but also in a Xenopus egg NT system (Hörmanseder et al. 2017). Importantly, H3K4me3 removal by overexpression of Kdm5b also improved the development of bovine SCNT embryos (Zhou et al. 2020). These findings imply the importance of removing H3K4me3-mediated active transcriptional memory to improve SCNT efficiency in many mammalian species.

Another methylated histone, H3K27me3, can also be a target for improving SCNT efficiency. While H3K27me3 is normally located at gene promoter regions and serves to repress associated gene expression in differentiated cells, such promoter-associated H3K27me3 is mostly absent in preimplantation embryos; instead, H3K27me3 forms unique broad domains covering multiple genes in oocytes (Zheng et al. 2016) and subsequently represses the expression of associated genes after fertilization, which means that it functions as a marker of genomic imprinting (Inoue et al. 2017a). In SCNT embryos, although the promoter-associated H3K27me3 inherited from donor somatic cells might inhibit ZGA (Yang et al. 2018), it appeared to be successfully depleted before the morula stage, similar to normally fertilized embryos (Matoba et al. 2018). However, the unique broad domains of H3K27me3 were completely lost in SCNT embryos as well as in donor somatic cells; as a result, the H3K27me3-dependent imprinting genes were abnormally expressed from both maternal and paternal alleles after NT (Matoba et al. 2018). Recently, two groups, including ours, have reported independently that the loss of imprinting (LOI) of a set of H3K27me3-imprinted genes, such as Sfmmt2 or its associated microRNA cluster, was responsible for the poor development rate and large placentas in mouse SCNT models (Inoue et al. 2020, Wang et al. 2020) (see below).

**Histone acetylation**

Histone acetylation is a fundamental regulatory mechanism for open chromatin and thus increases the accessibility of transcription factors and epigenetic modifiers to DNA. Therefore, it is likely that oocyte-derived reprogramming factors might exert stronger effects on the donor genome if the histones can gain more acetylation. To achieve this, two independent groups treated mouse reconstructed embryos with trichostatin A (TSA), a potent histone deacetylase (HDAC) inhibitor (Yoshida et al. 2003), and successfully increased the birth rates of cloned mice up to fivefold (Kishigami et al. 2006, Rybouchkin et al. 2006). These remarkable successes prompted many cloning researchers to seek more efficient HDAC inhibitors for SCNT experiments in mice and other mammalian species. Besides TSA, many HDAC inhibitors, including scriptaid (Thuan et al. 2009), suberoylanilide hydroxamic acid (SAHA), oxamflatin (Ono et al. 2010), m-carboxycinnamic acid bishydroxamid (Dai et al. 2010), and PXD101 (belinostat) (Qiu et al. 2017), have been proven to increase mouse cloning efficiency (Kamimura et al. personal communication). The relative efficacy of these inhibitors in terms of the birth rates of clones has been documented by our group and others (Ono et al. 2010, Thuan et al. 2010, Ogura et al. 2013). Despite such efforts to identify efficient HDAC inhibitors for SCNT experiments, all of these molecules are hydroxamates: typical pan-HDAC inhibitors. In mammals, there are 18 HDACs, which are classified according to functional and phylogenetic criteria as classes I, IIa, IIb, III, and IV. In general, most inhibitors have been developed to target class I, IIa, IIb, and IV (Zn²⁺-dependent) enzymes (Kovacs-Kasa et al. 2020). Therefore, we do not know yet which HDACs are the best targets for enhancing the development of SCNT embryos. To determine this, ideally it should be possible to inhibit specific HDAC(s) by more selective inhibitors so that unnecessary cytotoxicity can be avoided. Recently, we have found that chlamydocin analogues—a newly discovered family of HDAC inhibitors—significantly improved the development of mouse SCNT embryos as efficiently as TSA (Kamimura et al. under revision). These specifically inhibit HDAC inhibitors that are Zn²⁺-dependent enzymes. Based on their expressions in oocytes and embryos and their potency in causing histone deacetylation, we may assume that HDAC-1, -2, -3, and -8 and/or Class IIa HDACs (HDAC-4, -5, -7, and -9). Based on their expressions in oocytes and embryonic development of SCNT models via free access
vitro development of cloned embryos following the use of HDAC inhibitors in bovines (Akagi et al. 2011, Min et al. 2016), pigs (Hou et al. 2014, Jin et al. 2017, Wang et al. 2018) and sheep (Wen et al. 2014a), increased birth rates were only reported in pigs after scriptaid treatment (Zhao et al. 2010). The reasons for such discrepancies in the effects of HDAC inhibitors on in vitro and in vivo embryonic development are not clear, but the higher basal level of cloning efficiency in large animals compared with small animals such as mice is possible. The first births of cloned calves were achieved in 1998 with a birth rate as high as 80% (8 calves per 10 embryos transferred) (Kato et al. 1998). Although this high birth rate might be exceptional, bovine cloning has been generally efficient since the earliest studies, achieving calving rates of around 10–30% (Akagi et al. 2014, Liu et al. 2018a). By contrast, the first series of cloned mice were born with birth rates of only 2–3% and TSA treatment only increased the rates to as high as 5% (Wakayama et al. 1998, Kishigami et al. 2006). Unlike treatment with HDAC inhibitors, the removal of repressive histone methylation (see subsequently) is a potent epigenetic treatment for producing SCNT embryos because it has shown significant efficacies in the various animal species tested (Matoba & Zhang 2018). Although there is a commonality in the list of derepressed genes between TSA treatment and H3K9me3 demethylation, the latter has shown broader effects such as increased expressions of major satellite repeats and retrotransposons (Matoba et al. 2014, Inoue et al. 2015). This is consistent with a recent study using ultra-low-input native chromatin immunoprecipitation followed by sequencing (ULI-NChIP-seq) for the identification of H3K9ac-enriched regions. According to that study, TSA treatment could largely correct aberrantly acetylated regions in SCNT embryos, but some genic regions remained hypoacetylated (Yang et al. 2020a). Importantly, these regions that were not rescued by TSA treatment overlapped with H3K9me3-enriched regions in the donor cumulus cells, the so-called RRRs (see subsequently) defined by Matoba et al. (2014). It is noteworthy that injection of the mRNA for transcription factor double homeobox (Dux) into SCNT 1-cell embryos rescued H3K9ac in RRRs and increased their developmental efficiency, indicating that Dux may be a critical determinant for the outcome of mouse SCNT (Yang et al. 2020a). Conversely, Dux-deleted SCNT embryos arrested development at the two-cell stage (Yang et al. 2020b). Intriguingly, although Dux is known to be one of the important regulators of ZGA and can induce a two-cell-like state in ES cells (De Iaco et al. 2017, Fu et al. 2020), it is dispensable for normal embryonic development to term (Chen & Zhang 2019).

DNA methylation

DNA methylation (5mC) is a type of epigenetic mark of molecular memory that can interplay with other epigenetic marks during mammalian embryogenesis. After fertilization, both the maternal and paternal genomes undergo genome-wide active and passive DNA demethylation, except in ICRs and some repetitive elements (Lane et al. 2003, Hirasawa et al. 2008). The active demethylation process involves Tet3-mediated oxidation processes and a subsequent thymine DNA glycosylase (TDG)-mediated base-excision repair pathway (Kohli & Zhang 2013). After several cleavages of early embryos, their genome reaches the lowest DNA methylation level at the blastocyst stage. DNA methylation is reestablished after implantation in a lineage-specific manner, that is, with discrimination between the embryonic and extraembryonic lineages.

DNA demethylation also occurs in SCNT embryos but might often present as an epigenetic abnormality specific for SCNT. Early studies on DNA methylation status in SCNT embryos were performed based on immunostaining for 5mC or methylation analysis of repetitive sequences. According to those studies, bovine SCNT embryos had aberrantly highly methylated genomes arising from remethylation during successive cleavages or from the persistence of methylated areas from the donor genome (Dean et al. 2001, Kang et al. 2001a) (Fig. 1A). However, porcine SCNT embryos showed typical demethylation processes in the repetitive sequences analyzed, indicating species specificity in DNA methylation status (Kang et al. 2001b). A recently developed genome-wide methylation sequencing technique has also provided essentially similar – if not identical – information on the methylation status of SCNT embryos. Concerning repetitive DNA elements, the demethylation efficiency in SCNT embryos depended on the class: long terminal repeat (LTR) elements were resistant to the demethylation process, although the effects on long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) seemed to vary with the analytical methods used (Chan et al. 2012, Gao et al. 2018, Matoba et al. 2018). For future improvements of SCNT technology, it will be necessary to identify the differences in DNA methylation patterns between developing and nondeveloping (arrested) embryos. Gao et al. identified aberrantly remethylated regions (rDMRs) including developmentally important genes and retrotransposons (Gao et al. 2018). Importantly, arrested embryos possessed almost twice as many rDMRs as developing embryos. To block the remethylation of rDMRs in SCNT embryos, they injected siRNAs targeting Dnmt3a and Dnmt3b into MII oocytes before NT. As a result, most rDMR genes and retrotransposons restored their expression levels and the birth rate of clones increased from 0.9 to 5.3%. The birth rate was further improved to 17.2% by coinjection with Kdm4b and Kdm5b mRNAs (Gao et al. 2018). As far as we know, this is the most effective treatment for correcting the aberrant DNA methylation status of SCNT embryos. Compared with the successful...
epigenetic treatments for histone modifications described above, those for DNA methylation, such as treatment with DNA methylation inhibitors (e.g. 5-aza-2'-deoxycytidine), often had only minor effects on improvements in SCNT efficacy (Whitworth & Prather 2010, Akagi et al. 2014).

Genomic imprinting

Genomic imprinting is a form of epigenetic regulation that ensures parental-specific gene expression that has evolved uniquely in eutherian mammals. Thus, genomic imprinting creates functional differences between paternal and maternal alleles. These differences help explain why maternally derived offspring (parthenotes) are never born in mammals, unlike some species in birds, reptiles, and fishes. The differences were demonstrated experimentally by pronuclear exchange experiments using mouse zygotes in 1984 (McGrath & Solter 1984, Surani et al. 1984). In those landmark experiments, the reconstructed maternal-only (gynogenetic) embryos and paternal-only (androgenetic) embryos could not survive to term. So far, the functions of many imprinted genes have been identified by gene knockout mouse experiments or in naturally occurring human chromosomal aberrations. Thus, many of the imprinted genes play essential roles in the development of fetuses, placentas or even the normal behavior of adults (Tucci et al. 2019). The parentally specific expression of imprinted genes is regulated by genomic elements called imprinting control regions (ICRs). One ICR controls the imprinting of a single gene or multiple genes within the same cluster. Most ICRs are primarily imposed as epigenetic memories (imprints) during germline development: oogenesis or spermatogenesis. The epigenetic basis of the ICRs of typical (canonical) imprinted genes is DNA methylation, leading to differentially methylated regions (DMRs). The germline-derived DMRs are resistant to two genome-wide waves of genomic reprogramming: fertilization and implantation. Consequently, the DMRs are maintained through that germline to embryos and neonates. Given this persistent nature of DMRs, genomic imprinting can be maintained normally in SCNT embryos and placentas (Inoue et al. 2002) (Fig. 1B). By contrast, a recently discovered noncanonical form of genomic imprinting that depends on regulation by histone methylation (H3K27me3) is placenta-specific and is largely eliminated in the embryonic lineage (i.e. in somatic cells) (Fig. 1C). These unique dynamics of noncanonical imprinting are important in the imprinting disorders associated with SCNT (see subsequently).

Canonical (DNA methylation-dependent) imprinting

As aforementioned, DMR-dependent canonical imprinting can be passed on normally to the genomes of SCNT embryos and their placentas. However, as known for ARTs in animals and humans, simple in vitro manipulation of gametes and embryos, such as embryo culture, often modifies the expression of specific imprinted genes. These are considered to be inherently vulnerable to changes in the external milieu. Indeed, in bovines, the developmental anomalies observed in SCNT embryos are similar to those observed during gestation from in vitro produced embryos (Smith et al. 2015). One of the most common ART-derived anomalies in ruminants is the large offspring syndrome (LOS), which correlates with imprinting disruption of the IGF2R gene (Young et al. 2001). In ruminants, other imprinted genes including SNRPN and H19/IGF2 are also known to be perturbed following conventional ART and SCNT (Smith et al. 2010, 2015). Importantly, the incidence of imprinting disorders and resultant phenotypes by ARTs can be further aggravated by SCNT (Smith et al. 2012). LOS is the most severe complication following bovine SCNT because it causes perinatal death of calves at a much higher frequency than IVF per se (Watanabe & Nagai 2009, Chavatte-Palmer et al. 2012).

In mice, it has also been reported that ARTs can alter the methylation levels and expression patterns of imprinted genes. Among the ART procedures in mice, superovulation is the first common process before embryo manipulation, and thus it is well documented in terms of its effects on the imprinting status of specific imprinted genes such as H19, Snrpn, Peg3, and Kcnq1ot1 (Fauque 2013, Duranthon & Chavatte-Palmer 2018). However, as superovulation involves the use of systemic hormone treatment, it is difficult to understand how it affects imprinting status. Furthermore, it is known that there are substantial mouse strain-dependent differences, according to the studies so far published (Duranthon & Chavatte-Palmer 2018). By contrast, IVF, ICSI and embryo culture solely constitute oocyte/embryo handling in vitro. A simple and reliable experimental setting is the use of different media for embryo culture. Among the five culture media tested, potassium simplex optimized medium with amino acids (KSOM+AA) gave the best results in terms of imprinted DNA methylation and expression at three well-studied genes (H19, Peg3, and Snrpn) in mouse embryos cultured from the 2-cell to the blastocyst stage (Market-Velker et al. 2010). Importantly, all culture media resulted in loss of H19 imprinting accompanying ectopic paternal expression, consistent with the prevailing view that H19 is unstable under artificial conditions including cell culture (Dean et al. 1998). Consistent with this, in mouse SCNT embryos derived from immature Sertoli cells, H19 was overexpressed at the blastocyst stage compared with IVF-generated embryos, although its imprinting status was unknown (Cao et al. 2013). We have also confirmed that H19 is upregulated in cumulus cell-derived SCNT blastocysts (unpublished results). As mentioned above, DNA-methylation-dependent

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canonically imprinted genes in the donor cell genome maintain their correct imprinting statuses in cloned embryos (Fig. 1B). However, their expression levels can fluctuate according to the NT procedures used. In mice, these aberrant gene expressions seem to appear more typically in the extraembryonic lineage (placentas) rather than the embryonic lineage. Indeed, mouse ESCs are known to be easily established from SCNT embryos while maintaining the correct expressions of imprinted genes (Brambrink et al. 2006). In mouse SCNT placentas at term, Grb10, and Gab1 were significantly downregulated despite their correct imprinting status, while embryonic tissues did not show any aberrated expressions of imprinted genes (Inoue et al. 2002). That nonimprinted genes – Igfbp2, Igfbp6, and Flk1 – were also consistently repressed in SCNT placentas suggests that a subset of placenta-related genes are downregulated specifically by nonimprinting mechanisms.

**Noncanonical (H3K27me3-dependent) imprinting**

One of the major purposes of analyzing gene expression patterns in SCNT placentas is to elucidate the mechanisms underlying placental anomalies associated with the procedure. Defective placental development is at least one of the major causes of low efficiency of SCNT, as demonstrated by improved development of SCNT mouse embryos following exchange of SCNT trophoderm with fertilization-derived tetraploid trophoderm (Lin et al. 2011). It is well known that abnormalities of extraembryonic tissues occur frequently in mammalian SCNT, although their phenotypes depend on the species (Yang et al. 2007). In ovine and bovine SCNT, reduced numbers or altered shapes of placentomes or reduced vascularization have been observed (Palmieri et al. 2008). In mouse SCNT, placental enlargement (hyperplasia) was first reported in early mouse cloning experiments (Wakayama & Yanagimachi 1999). Since then, this has been an insoluble question for 20 years, even with protocols for overcoming the epigenetic barriers inherited from the donor genome (Kishigami et al. 2006, Miyamoto et al. 2017, Matoba et al. 2018). Placental hyperplasia following mouse SCNT is characterized by an extreme increase in the spongiotrophoblast layer caused by a proliferation of glycogen and trophoblast cells and by distortion of the boundary between the spongiotrophoblast and labyrinthine layers: two placental constituents on the fetal side (Tanaka et al. 2001, Wakisaka et al. 2008). Although previous studies had identified some dysregulated genes in SCNT placentas by analyzing gene expression or DNA methylation levels in SCNT placentas (Inoue et al. 2002, Ohgane et al. 2004, Singh et al. 2004), their direct causal relationships with placental hyperplasia remained unclear. There was a possibility that these abnormalities found in specific gene expression levels might be downstream effects of placental morphological abnormalities.

One clue emerged from a comprehensive analysis of imprinted gene expression abnormalities of mouse SCNT placentas, which demonstrated that some of the placenta-specific imprinted genes (Gab1, Slc38a4, and Sfmbt2) were biallelically expressed by LOI in all SCNT placentas (Okae et al. 2014). This was the first identification of LOI in SCNT placentas. The LOI of these genes was also confirmed in trophoblast stem cells generated by SCNT using cumulus or Sertoli cells (Hirose et al. 2018). We have assumed that specific epigenetic abnormalities that always accompany SCNT, rather than unexplained dysregulation of certain genes, might be the most probable cause of placental hyperplasia and that LOI applied to this scenario. Later, Inoue et al. identified a set of imprinted genes under a DNA methylation-independent mechanism and, intriguingly, the genes showing LOI (Gab1, Slc38a4, and Sfmbt2) in SCNT placentas were included among them (Inoue et al. 2017a). These newly identified imprinted genes were regulated by histone H3K27me3 and are referred to as noncanonical imprinted genes to distinguish them from conventional (canonical) DNA methylation-dependent imprinted genes. The repressive H3K27me3 marks of the noncanonically imprinted genes are imposed during oogenesis, maintained in preimplantation embryos, and passed on to the extraembryonic lineage as secondary imprinted DNA methylation, but not to the embryonic lineage (Inoue et al. 2017a, Chen et al. 2019, Hanna et al. 2019) (Fig. 1C). This means that they normally show paternal expression patterns exclusively in extraembryonic tissues, including placentas. Therefore, their biallelic expression in SCNT placentas can be explained by erasure of H3K27me3 imprinting in the genomes of somatic cell donors, the descendants of the embryonic lineage (Fig. 1C). Functional analyses of Gab1, Sfmbt2, and Slc38a4 using gene knockout mice indicated that all these genes positively regulate placental morphogenesis (Ioh et al. 2000, Miri et al. 2013, Matoba et al. 2019), although the diversity of protein structures they encode implies that there are different underlying mechanisms. Thus, it was expected that placental hyperplasia in SCNT might be explained by the overexpression of these genes in a biallelic manner. To test this possibility, we performed SCNT experiments using donor cells carrying maternal gene knockouts for Gab1, Sfmbt2, and Slc38a4, ensuring their normal paternal expression levels in the placentas. However, none of the maternal allelic deletions in these genes produced improvements in placental weight or morphology, suggesting that they were not the primary causes of SCNT-related placental hyperplasia.

Although Sfmbt2 is widely conserved in mammals, a large micro (mi)RNA cluster (Sfmbt2 miRNA cluster/C2MC) exists specifically in rodents (Wang et al. 2011). This is predicted to contain 72 miRNA genes in mice, and it is one of the largest miRNA clusters in the mouse genome. The Sfmbt2 miRNA cluster is paternally
expressed as the host gene Sfmbt2 and has an essential role in proliferation of the spongiotrophoblast layer during placental development (Inoue et al. 2017b). Because of LOI of Sfmbt2 in SCNT placentas, Sfmbt2 miRNAs also lost imprinting regulation and showed upregulated expressions. When the normal paternal expression of Sfmbt2 miRNAs was recovered in SCNT placentas by use of donor cells lacking the maternal allele, the placental size was significantly reduced because of the decreased spongiotrophoblast layer (Inoue et al. 2020) (Fig. 2). This effect was further manifested when combined with deletion of the maternal Gab1 allele. Importantly, the amelioration of hyperplasia of SCNT placentas by normal Sfmbt2 miRNA expression was associated with modest but significant improvements in the birth rates of cloned mice (Inoue et al. 2020). Thus, the Sfmbt2 miRNA cluster appears to be the primary cause of placental hyperplasia in mouse SCNT, and Gab1 might also be involved in this process to some extent. Shortly after this report, Wang et al. achieved corrections of multiple noncanonical genes in SCNT placentas by using genetically manipulated haploid ES cells. The best result was obtained when the maternal alleles of four genes (Gab1, Sfmbt2, Jade1/Phf17, and Smoc1) were deleted; the resulting placental morphologies were nearly normal, and the birth rate of clones was increased to about 14% (Wang et al. 2020). Although Sfmbt2 miRNAs were not depleted in their Sfmbt2 knockout mice, this result was another clear demonstration of the involvement of noncanonical imprinted genes in SCNT-specific placental anomalies and poor embryo development rates to term. Thus, the currently available approaches for restoring the paternal expression of these imprinted genes require genetic modification of the donor somatic cells. It would be ideal to restore the maternal H3K27me3 marks in SCNT embryos using epigenetic means. Such an approach remains challenging because of the difficulty in targeting maternal alleles exclusively. However, recent epigenome editing technology with the dCas9-fusion system (Brocken et al. 2018) might make it possible to introduce maternal H3K27me3 in donor somatic cells or reconstructed SCNT embryos, which might contribute to improve SCNT efficiency. It should be noted that, although the H3K27me3 imprinting system appears to be conserved in the human genome (Zhang et al. 2019), it is not clear whether this is further conserved across a wide range of mammalian species.

There is another imprinted gene that shows LOI in SCNT embryos: Xist (X-inactive specific transcript), a noncoding RNA gene responsible for X chromosome inactivation (XCI) in eutherian mammals. Xist expression leads to imprinted XCI in female preimplantation embryos, in which the paternal X chromosome is inactivated while the maternal one is activated by Xist repression induced by maternal imprinting (Augui et al. 2011). Besides XCI, the activated maternal X chromosome is critically important for the survival of preimplantation embryos of both sexes, because the maternal X is present in both female and male cells. However, in mouse SCNT embryos, the maternal X is aberrantly inactivated by ectopic expression of Xist, leading to postimplantation death in most SCNT embryos of both sexes. Indeed, the

![Figure 2](https://rep.bioscientifica.com)

**Figure 2** Correction of hyperplasia (enlargement) of SCNT-derived placentas by maternal deletion of Sfmbt2 micro (mi)RNAs. Maternal deletion of Sfmbt2 miRNAs restores their exclusive paternal expressions in SCNT-derived placentas from the biallelic expression of WT placentas.
use of donor cells with maternal Xist deletion or temporal suppression of Xist by specific short interfering (si)RNA sequences (for only male embryos) improved the birth rate of SCNT embryos eight- to tenfold (Inoue et al. 2010, Matoba et al. 2011, Oikawa et al. 2013). The most probable cause of this ectopic maternal Xist expression is LOI of Xist in the maternal allele. It was assumed that the repressive imprinting of Xist was established during oogenesis, based on the analysis of embryos generated from non-growing and fully grown oocytes (Tada et al. 2000). Later, our large-scale NT study using different somatic cells and germ cells also demonstrated that the Xist-repressing imprint is established at the final stage of oogenesis (Oikawa et al. 2014). Importantly, this imprint is erased after implantation in both embryonic and extraembryonic lineages (Oikawa et al. 2014), which explains the LOI of Xist in SCNT embryos (Fig. 1C). Thus, this mechanism might resemble the LOI of H3K27me3-dependent noncanonical imprinting in SCNT placentas described above. Indeed, Xist was proven to be one of the noncanonical imprinted genes by its dependency on the broad H3K27me3 domain established during oogenesis (Inoue et al. 2017c). Taken together, we conclude that the LOI of noncanonical imprinted genes largely affects the development of mouse SCNT embryos in two ways: abnormal placental development by biallelic expressions of placenta-specific genes including Sfmbt2 miRNAs; and massive repression of X-linked genes in SCNT embryos by ectopic maternal expression of Xist (Fig. 1C).

Search for the totipotent genome signature

SCNT is a technology to make somatic cell genomes totipotent and to have the same ability as the normally fertilized oocyte genome. With the recent development of low-input sequencing analysis technology, research on the unique characters of genomes from the zygote to ZGA stages – with totipotent nuclei – has progressed greatly. In particular, great efforts have been made to clarify how the sperm genome, which lacks most of the histones, and the oocyte genome at MII can form a totipotent nuclear architecture. Studies using low-input Hi-C that defines topologically associating domains (TADs) and A/B compartments have contributed significantly to the accumulation of knowledge on the totipotent nucleus. In addition, low-input ChIP-seq analysis of histone variants, especially H3.3 that plays an essential role in genomic reprogramming (Wen et al. 2014b,c), for zygotes and two-cell embryos has provided invaluable information on the totipotent state. As a result, we now know that the mouse chromatin immediately after fertilization is in a highly dispersed and relaxed state, as the formation of TADs is weak.
and the distribution of H3.3 is uniform (Du et al. 2017, Ke et al. 2017, Ishiuchi et al. 2020). These findings are consistent with a report that the transcription pattern in zygotes is characterized by extensive expression of intergenic regions and low-level expressions of thousands of genes (Abe et al. 2015). In 2020, two research groups independently reported the dynamics of genomic architecture following SCNT in the mouse (Chen et al. 2020, Zhang et al. 2020). Both studies showed that the donor genome was extensively reprogrammed after premature chromosome condensation within enucleated MII oocytes, but TADs remained stronger than those of zygotes and were then reestablished gradually. Therefore, reprogramming of the donor genome by NT was incomplete or slower than that following normal fertilization, although the global trend of TADs became identical to those of normal embryos by the 8-cell stage. Interestingly, the two groups rescued SCNT embryos in different ways. Zhang et al. depleted cohesins from the donor genome to improve minor ZGA, which is necessary for the major wave of ZGA, and successfully increased the blastocyst formation rate (Zhang et al. 2020). Chen et al. identified that the defective TADs in the early SCNT genome were caused by donor-inherited H3K9me3, which could be removed by the injection of Kdm4d mRNA (Chen et al. 2020). It will also be interesting to determine how H3.3 is reorganized within the donor genome after SCNT. With such studies using cutting-edge technology being conducted widely using SCNT embryos, it may not be long before we understand the mechanisms of genomic reprogramming that induce totipotency in the mammalian genome.

Conclusions

For 25 years, cloning researchers have made great efforts to elucidate SCNT-associated epigenetic abnormalities and correct them with the aim of improving the efficiency of cloned animal production. These epigenetic abnormalities and related phenotypes following SCNT in mice are summarized in Fig. 3. Especially in these several years, low-input analytical technologies have provided evidence for incomplete or erroneous reprogramming of the donor genome in reconstructed embryos and cloned offspring. Insights from the study of epigenetic phenomena in SCNT are highly relevant for our understanding of the mechanisms of genomic reprogramming that can induce totipotency in the mammalian genome.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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