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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.

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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

Table 1 Classification of abnormalities associated with SCNT.

Classification	Origin	Causes	Random or non-random	Examples
Genetic	Donor cell origin	<i>In vivo</i> -derived errors <i>In vitro</i> -derived errors during cell culture	Random Random or with a specific tendency	Cell aging Missing Y chromosome
	NT origin	<i>In vitro</i> handling* Physiological causes	Random Random	Mechanical damage by a small injection pipette Cell cycle asynchrony between the ooplasm and donor cell Instability of the spindle Cell aging
Epigenetic	Donor cell origin	<i>In vivo</i> -derived errors	Random	Cell aging
		<i>In vitro</i> -derived errors during cell culture	Random or with a specific tendency	Aberration of genomic imprinting
		Somatic cell-specific epigenome (excessive)	Non-random	Histone modifications (H3K9me3, H3K27me3)
	NT origin	Somatic cell-specific epigenome (absence)	Non-random	DNA methylation Histone modifications (H3K27me-dependent noncanonical imprinting)
		<i>In vitro</i> handling*	Random or with a specific tendency	Aberration of genomic imprinting
		Physiological causes	Random	Accidental reprogramming errors

*Many are common with assisted reproductive technologies (ARTs), for example, large offspring syndrome in ruminants.

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

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 methyltransferases (Matoba *et al.* 2014, Jafarpour *et al.* 2020), *Suv39h1* and *Suv39h2*, depletion 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 pre-implantation 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-dependently imprinted 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 *Símbt2* 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 bishydroxamide (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 Class I and IIa HDACs. Therefore, the targets for improving mouse SCNT most likely include Class I HDACs (HDAC-1, -2, -3, and -8) and/or Class IIa HDACs (HDAC-4, -5, -7, and -9). Based on their expressions in oocytes and embryos and their potency in causing histone deacetylation, we may assume that HDAC-1, -2, and/or -4 might be the ideal target HDACs for mouse SCNT.

By contrast, applications of HDAC inhibitors to large animal cloning have been hampered by the lack of solid evidence that they improve cloning efficiency. Although there are a number of reports on enhanced *in*

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 aberrant 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

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, *Mest*, *Grb10*, and *Gtl1* 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 trophoctoderm with fertilization-derived tetraploid trophoctoderm (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 (Itoh *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 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.

Figure 3 Epigenetic abnormalities and their phenotypic effects associated with mouse SCNT. A few of them have been corrected by genetic or epigenetic means as indicated by red arrows, but others remain unsolved. For those not described in this review, see *Hirasawa *et al.* 2013, **Wakisaka-Saito *et al.* 2006 and ***Inoue *et al.* 2010.

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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Author contribution statement

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