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Review

Somatic Cell Nuclear Transfer Reprogramming: Mechanisms and Applications

Shogo Matoba[1,2,*] and Yi Zhang[3,4,5,6,*]
[1]RIKEN Bioresource Research Center, Tsukuba, Ibaraki 305-0074, Japan
[2]Cooperative Division of Veterinary Sciences, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
[3]Howard Hughes Medical Institute
[4]Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA 02115, USA
[5]Department of Genetics, Harvard Medical School, Boston, MA 02115, USA
[6]Harvard Stem Cell Institute, Boston, MA 02115, USA
*Correspondence: shogo.matoba@riken.jp (S.M.), yzhang@genetics.med.harvard.edu (Y.Z.)
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Successful cloning of monkeys, the first non-human primate species, by somatic cell nuclear transfer (SCNT) attracted worldwide attention earlier this year. Remarkably, it has taken more than 20 years since the cloning of Dolly the sheep in 1997 to achieve this feat. This success was largely due to recent understanding of epigenetic barriers that impede SCNT-mediated reprogramming and the establishment of key methods to overcome these barriers, which also allowed efficient derivation of human pluripotent stem cells for cell therapy. Here, we summarize recent advances in SCNT technology and its potential applications for both reproductive and therapeutic cloning.

Introduction

Totipotency is defined as the ability of a cell to give rise to all cell types of an entire organism (Lu and Zhang, 2015). In normal mammalian development, totipotency is limited to zygotes or blastomeres of early-stage preimplantation embryos. After fertilization, oocytes are capable of reprograming terminally differentiated sperm into a totipotent state. Totipotency is then gradually lost during preimplantation development to give rise to inner cell mass (ICM) and trophectoderm (TE) cells.

Besides fertilization, an artificial method called somatic cell nuclear transfer (SCNT), or cloning, can also confer totipotency. Dr. John Gurdon was the first to demonstrate that animals could be cloned from differentiated frog somatic cells by SCNT (Gurdon, 1962; Figure 1). After three decades of efforts, Dolly the sheep, the first cloned mammal, was born (Wilmut et al., 1997). Since then, successful cloning of more than 20 mammalian species has been reported (Table 1).

Despite its success, several technical hurdles have limited the practical use of SCNT technology. First, the cloning efficiency is extremely low in essentially all species. Second, abnormalities are frequently observed in the extraembryonic tissues, such as placenta, of the cloned embryos (Ogura et al., 2013). Moreover, some abnormalities are observed in cloned animals even after their birth, including obesity, immunodeficiency, respiratory defects, and early death (Loi et al., 2016; Ogura et al., 2013), although these phenotypes are not transmitted to the offspring (Fulka et al., 2004; Tamashiro et al., 2002; Wakayama et al., 2013). These observations suggest the existence of barriers that prevent normal development of cloned embryos and animals.

In the past decade, great effort has been placed into improving cloning efficiency (Meissner and Jaenisch, 2006). However, the lack of understanding of reprogramming barriers has impeded improvements on cloning efficiency. Recent technical advances, particularly low-input sequencing techniques, have enabled analysis of transcriptome and epigenetic changes during SCNT reprogramming. These analyses have revealed molecular defects as well as provided clues about how to overcome the defects (Liu et al., 2016a; Matoba et al., 2014, 2018). Indeed, the recent success in monkey cloning (Liu et al., 2018b) has been largely attributed to this understanding and the establishment of approaches to overcome critical barriers of epigenetic reprogramming (Cibelli and Gurdon, 2018).

In addition to animal cloning, SCNT technology holds great potential for stem cell biology and human therapeutics. Similar to the derivation of embryonic stem cells (ESCs) from blastocysts of fertilized eggs, SCNT-generated blastocysts could be used to derive pluripotent stem cells (PSCs), also termed nuclear transfer ESCs (ntESCs). Because patient-derived ntESCs are isogenic to donor patients, they could be used for therapeutic purposes, including cell transplantation and disease modeling. Thus, the ntESC-derivation process is also called therapeutic cloning (Figure 1). Although derivation of human ntESCs was once very difficult because of the extremely low efficiency of cloned human embryos to reach the blastocyst stage, derivation of human ntESCs was finally achieved after decades of struggle in optimizing SCNT conditions (Tachibana et al., 2013) and further improved (Chung et al., 2014, 2015; Yamada et al., 2014), thus making therapeutic cloning a reality.

In this review, we summarize our current understanding of the cellular and molecular events associated with SCNT reprogramming. We discuss the epigenetic barriers and potential ways to overcome them for efficient cloning, with a focus on the mouse model. We also discuss the advantages and disadvantages of ntESCs compared with induced pluripotent stem cells (iPSCs) in human regenerative medicine. Finally, we discuss how this revamped technology could contribute to modern medicine when combined with CRISPR/Cas9-mediated genome editing.

Cellular Events following SCNT

The ooplasm has a remarkable ability to reprogram a differentiated cell nucleus. However, the cellular and molecular

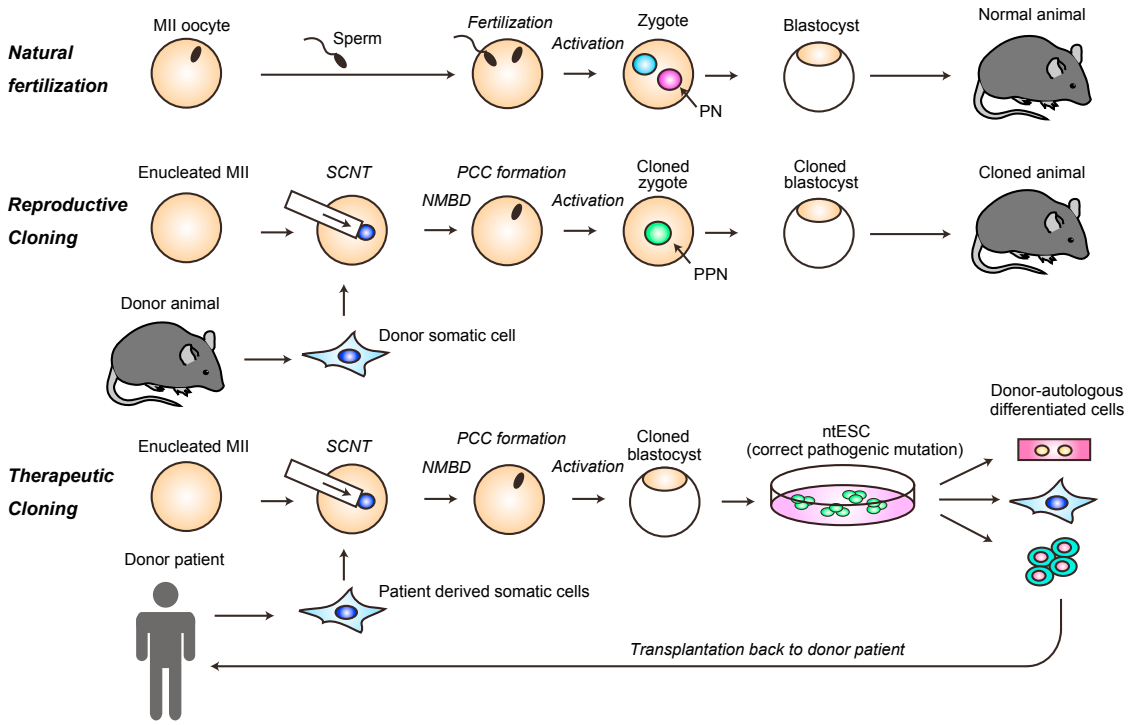

Figure 1. Diagram Showing the Major Steps of Reproductive and Therapeutic Cloning Compared to Natural Fertilization

Top: natural fertilization. Metaphase II (MII stage) oocytes are activated by fertilized sperm, form paternal and maternal pronuclei (PN), and continue preimplantation cleavages until they reach the blastocyst stage.

Middle: reproductive cloning. Donor somatic cell nuclei introduced into the enucleated oocytes quickly undergoes nuclear membrane breakdown (NMBD) to form metaphase chromosome in a process called premature chromosome condensation (PCC). The reconstructed SCNT oocytes are artificially activated to initiate developmental program to form blastocysts.

Bottom: therapeutic cloning. Patient-derived somatic cells are introduced to enucleated oocytes similar to reproductive cloning. Pluripotent ESCs can be derived from the blastocysts of nuclear transferred embryos (ntESCs), and the causative mutation can be corrected *in vitro* if desired.

events underlying the reprogramming process are poorly understood. Regardless of the species, the SCNT procedure involves three major steps: enucleation, injection/fusion, and activation. After removing the oocyte nucleus, the donor cell nucleus is injected or fused with the enucleated oocytes before the reconstructed embryos are activated. Below, we briefly summarize the cellular events following SCNT.

Nuclear Membrane Breakdown and PCC Formation

Following introduction into an enucleated oocyte cytoplasm, the donor nucleus quickly undergoes nuclear membrane breakdown to form condensed metaphase-like chromosomes. This process is called premature chromosome condensation (PCC) and is triggered by the M-phase-promoting factors (MPFs) present in the ooplasm (Campbell et al., 1996a). Although G0/G1-arrested quiescent cells are ideal donor cells, G2 or M phase cells can also be reprogrammed as long as the cell-cycle stage of donor cells and recipient oocytes are carefully coordinated (Ono et al., 2001). PCC appears to be required for reprogramming, as subsequent development of SCNT embryos is severely compromised without PCC (Kim et al., 2002). During PCC, most chromatin-bound proteins, including transcription factors (TFs), dissociate from the genome. PCC can be stably maintained for hours until reconstructed oocytes are activated.

Activation

Upon fertilization, sperm-borne phospholipase C zeta 1 (PLCZ1) induces oocyte activation through calcium oscillation and MPF breakdown, which triggers oocytes to exit M phase and initiate the developmental program (Saunders et al., 2002). However, since PLCZ1 is absent in somatic cells, SCNT reconstructed oocytes needs to be artificially activated to initiate the developmental program. The most popular method for activation of mouse SCNT reconstructed oocytes is strontium chloride ($SrCl_2$) treatment, as addition of $SrCl_2$ to the culture medium recapitulates the signals induced by fertilization. In some species, including human and monkey, that are less sensitive to $SrCl_2$ and easily restore MPF activity after activation (Liu et al., 2014), electropulse or calcium-ionophore treatment is widely used, while cycloheximide or 6-dimethylaminopurine (6-DMAP) is added to the medium during/after activation to inhibit the recovery of MPF activity for efficient metaphase exit.

Nuclear Expansion

After activation, the donor cell genome enters G1 phase and forms the nuclear membrane. In a fertilized zygote, the two nuclei derived from sperm and oocyte are called paternal and maternal pronucleus (PN), respectively. The PN in SCNT embryos is called pseudo-pronucleus (PPN). The number of PPN varies between embryos depending on the random distribution of PCC

Table 1. List of Cloned Mammalian Species

Year of Publication	Donor Cell		Recipient Oocyte	References
	Species	Cell Type	Species	
Interspecies				
1996	sheep	differentiated embryonic cell line	sheep	(Campbell et al., 1996b)
1997	sheep	adult mammary epithelium	sheep	(Wilmut et al., 1997)
1998	cow	fetal fibroblasts (transgenic)	cow	(Cibelli et al., 1998)
1998	cow	adult cumulus and oviductal cells	cow	(Kato et al., 1998)
1998	mouse	adult cumulus cells	mouse	(Wakayama et al., 1998)
1999	goat	fetal fibroblasts (transgenic)	goat	(Baguisi et al., 1999)
2000	pig	fetal fibroblasts	pig	(Onishi et al., 2000)
2000	pig	cultured adult granulosa cells	pig	(Polejaeva et al., 2000)
2002	rabbit	adult transgenic cumulus cells	rabbit	(Chesné et al., 2002)
2002	cat	adult cumulus cells	cat	(Shin et al., 2002)
2003	mule	fetal fibroblasts	horse	(Woods et al., 2003)
2003	horse	adult skin fibroblasts	horse	(Galli et al., 2003)
2003	rat	fetal fibroblasts	rat	(Zhou et al., 2003)
2005	dog (Afghan hound)	adult skin fibroblasts	dog (golden retriever)	(Lee et al., 2005)
2006	ferret	adult cumulus cells	ferret	(Li et al., 2006)
2007	red deer	adult antlerogenic cells	deer	(Berg et al., 2007)
2007	buffalo	fetal fibroblasts and adult granulosa cells	buffalo	(Shi et al., 2007)
2010	camel	adult cumulus cells	camel	(Wani et al., 2010)
2018	cynomolgus monkey	fetal fibroblast	cynomolgus monkey	(Liu et al., 2018b)
Interspecies				
2000	gaur (*Bos gaur*)	cryopreserved adult skin cells	cow (*Bos taurus*)	(Lanza et al., 2000)
2001	mouflon (*Ovis orientalis musimon*)	adult granulosa cells	sheep (*Ovis aries*)	(Loi et al., 2001)
2001	zebu (*Bos indicus*)	morula stage blastomere	cow (*Bos taurus*)	(Meirelles et al., 2001)
2004	African wildcat (*Felis lybica*)	adult skin fibroblasts	cat (*Felis catus*)	(Gómez et al., 2004)
2004	banteng (*Bos javanicus*)	cryopreserved adult fibroblasts	cow (*Bos taurus*)	(Janssen et al., 2004)
2007	gray wolf (*Canis lupus*)	adult ear fibroblasts	dog (*Canis familiaris*)	(Kim et al., 2007)
2009	Pyrenean ibex (*Capra pyrenaica pyrenaica*)	cryopreserved skin fibroblasts	goat (*Capra aegagrus hircus*)	(Folch et al., 2009)
2013	coyote (*Canis latrans*)	neonatal/adult fibroblasts	dog (*Canis familiaris*)	(Hwang et al., 2013)
2017	Bactrian camel (*Camelus bactrianus*)	adult skin fibroblasts	dromedary camel (*Camelus dromedaries*)	(Wani et al., 2017)

chromosomes, but normally one or two PPNs are formed. One unique feature of PN in fertilized embryos is its large size. Like PN, SCNT PPN is also much larger than the original donor somatic cells (Prather et al., 1990). This is achieved through nuclear expansion, during which PPN incorporates a large amount of maternal proteins (Prather et al., 2000). Consequently, drastic changes in chromatin structure and protein association take place during this process.

DNA Replication

Mouse zygotes initiate DNA replication 5–6 hr after fertilization, and the replication continues for 6–7 hr (Yamauchi et al., 2009). Although SCNT embryos have similar dynamics, the timing of replication initiation appears to be variable among SCNT embryos. In naturally fertilized embryos, since proteins involved in DNA methylation maintenance, such as DNMT1 and UHRF1, are exported out of the nucleus at this stage (Hirasawa et al.,

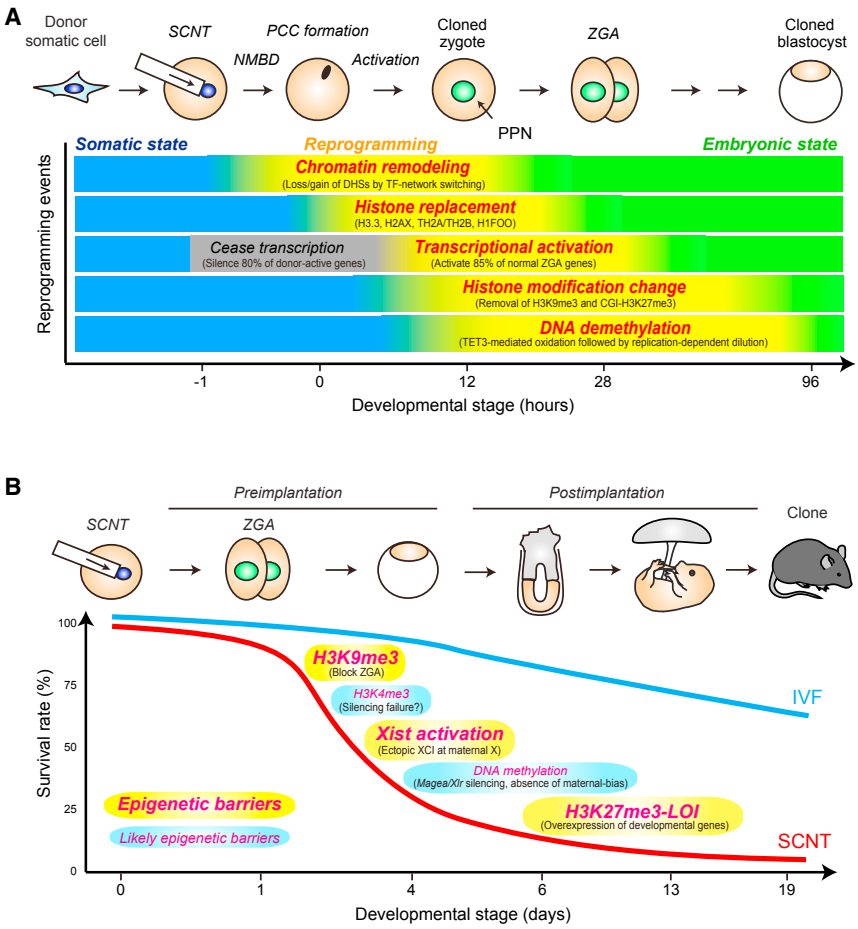

Figure 2. Molecular Mechanisms of SCNT Reprogramming and Its Associated Barriers in Reproductive Cloning

(A) Diagram showing the cellular and molecular events taking place in the SCNT reprogramming process. CGI, CpG island; DHSs, DNase I hypersensitive sites; PPN, pseudo pronuclei; TF, transcription factor; ZGA, zygotic genome activation.

(B) Diagram illustration of the various epigenetic barriers in reproductive cloning. LOI, loss of imprint; XCI, X chromosome inactivation.

2008; Maenohara et al., 2017), newly synthesized DNA, with the exception of imprinting genes, is mostly unmethylated, resulting in a passive dilution of DNA methylation and its oxidized derivatives in a replication-dependent manner (Inoue and Zhang, 2011; Inoue et al., 2011).

Zygotic Genome Activation

Mammalian oocytes and sperm are transcriptionally silent. Following fertilization, the zygote gradually restores transcription from its newly organized genome, which is called zygotic genome activation (ZGA). The timing of ZGA differs between species (at the 2-cell stage for mice and 8-cell stage for humans). As ZGA initiates, maternally stored RNAs are quickly degraded and replaced by newly synthesized zygotic RNAs. A similar mechanism is likely used in ZGA of SCNT embryos.

Chromatin, Epigenetics, and Transcriptional Reprogramming upon SCNT

Given that most cell types of an organism have the same genetic materials, SCNT reprogramming is likely achieved mainly through epigenetic reprogramming. Currently, there is no standard definition for SCNT reprogramming. Thus, we propose SCNT reprogramming as the cellular and molecular events taking place in the transferred somatic nucleus before the onset of the major developmental event, ZGA. Below, we summarize our current knowledge on the chromatin, epigenetics, and transcriptome changes (Figure 2A) during this short time window, with a focus on the best-characterized mouse model. Overall, SCNT can reprogram the epigenetic identity of donor somatic cells within a very short period of time, although some regions are resistant to this reprogramming in a genomic-context-dependent manner.

Chromatin Structure Reprogramming

In a eukaryotic cell, genomic DNA is packaged by histones to form chromatin in the nucleus. The nucleosome, the basic repeating unit of chromatin, is composed of ~147 bp DNA wrapped around a core histone octamer (2 copies of H2A, H2B, H3, and H4). The position of nucleosomes in the genome is dynamically regulated by chromatin remodeling factors and plays an important role in restricting the DNA access of TFs. Thus, nucleosome positioning and associated chromatin accessibility are expected to be globally reprogrammed to accommodate the transition from somatic cell to totipotent cell upon SCNT. Recent technological improvements have enabled the profiling of accessible chromatin sites of mouse and human preimplantation embryos using low-input DNase I hypersensitive sites sequencing (liDNase-seq) (Gao et al., 2018; Inoue et al., 2017a; Lu et al., 2016) or assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Wu et al., 2016).

Using liDNase-seq, chromatin accessibility profile of 1-cell SCNT embryos was recently analyzed, which revealed that global chromatin accessibility of donor somatic cells is quickly reprogramed to the pattern of a totipotent zygote within 12 hr post-activation (hpa) (Djekidel et al., 2018). The SCNT chromatin showed drastic loss of DNase I hypersensitive sites (DHSs) of the somatic donor cells concurrent with the appearance of some zygote-specific DHSs. Interestingly, the change in chromatin accessibility is independent of DNA replication. Based on the high correlation between each cell type's specific TFs and DHSs, SCNT reprogramming may involve global loss of somatic chromatin organization and gain of zygotic chromatin landscape through global TF network switching (Figure 2A).

Despite global chromatin landscape reprogramming, some regions are resistant to this reprogramming when compared to *in-vitro*-fertilized (IVF) counterparts. Interestingly, these regions are enriched for the heterochromatin marker H3K9me3 in both

donor somatic cells and 2-cell SCNT embryos (Djekidel et al., 2018). This observation is consistent with the fact that H3K9me3 in donor cells functions as an epigenetic barrier for SCNT reprogramming (Chung et al., 2015; Liu et al., 2016a; Matoba et al., 2014) (discussed below).

Incorporation of Histone Variants

In addition to canonical histones (H2A, H2B, H3, and H4), several histone variants can also be incorporated into nucleosomes. Upon fertilization, the sperm genome, originally packaged with protamine, undergoes global remodeling so that maternally stored histones, such as H3.3 (coded by *H3f3a* and *H3f3b*) (Akiyama et al., 2011; Inoue and Zhang, 2014) and H2AFX (Nashun et al., 2010), can repackage the sperm DNA. Although the chromatin of somatic cells is packaged with histones, similar drastic histone variant exchanges also occur in SCNT embryos (Nashun et al., 2011; Wen et al., 2014a, 2014b) (Figure 2A). Using ESCs that stably express FLAG-tagged histone variants as donor cells, Nashun et al. observed that most histone variants are eliminated within 5 hr after activation (Nashun et al., 2011). Similarly, macroH2A (coded by *H2afy* and *H2afy2*), which is enriched in repressive chromatin, is also quickly eliminated from the donor somatic nucleus after SCNT (Chang et al., 2010). Concomitant with global histone removal, all three H3 variants (H3.1, H3.2, and H3.3), as well as H2AFX, are efficiently incorporated into the donor nucleus upon SCNT (Nashun et al., 2011). These results indicate that donor cell histones are rapidly replaced by maternally stored histones upon SCNT (Figure 2A). This histone replacement appears to be critical for successful reprogramming, as knockdown (KD) of *H3.3* (*both of H3f3a and H3f3b*) prior to SCNT compromised pluripotent gene activation and SCNT embryo development (Wen et al., 2014a, 2014b). The role of other histone variants in SCNT reprogramming remains to be shown.

In addition to the histone variants mentioned above, oocytes have unique core histone variants, TH2A and TH2B (coded by *Hist1h2aa* and *Hist1h2ba*, respectively). These oocyte-specific histone variants are quickly incorporated into the PNs in IVF embryos and play critical roles in paternal genome activation and embryonic development (Shinagawa et al., 2014). Since overexpression of TH2A and TH2B in somatic cells induces chromatin opening and facilitates iPSC reprogramming, they may also contribute to SCNT reprogramming. Similarly, the canonical linker histone H1 in somatic cells is also globally replaced by the oocyte-specific H1FOO after SCNT (Gao et al., 2004; Teranishi et al., 2004). Addressing the functional importance of these variant histones in SCNT reprogramming and determining their genomic distribution before and after SCNT will contribute to our understanding of SCNT reprogramming.

Histone Modification Reprogramming

In addition to histone variants, covalent histone modifications, such as acetylation, methylation, ubiquitination, and phosphorylation, can also regulate gene transcription (Klose et al., 2006; Martin and Zhang, 2005). Thus, successful SCNT reprogramming should include reprogramming of histone modification patterns from the donor cell to those of the zygotes (Figure 2A). Earlier immunostaining studies revealed global differences in the acetylation and methylation patterns of SCNT embryos when compared to those of their IVF counterparts (Wang et al., 2007; Zhang et al., 2009). However, higher resolution of histone modification dynamics requires chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq).

Since it is difficult to obtain a sufficient number of SCNT embryos, particularly at the 1-cell and 2-cell stages, ChIP-seq analysis of SCNT embryos has been technically challenging. A recent study attempted to compare H3K9me3 distribution in donor cumulus cells and the resulting 2-cell SCNT embryos in mice (Liu et al., 2016a). The study revealed that the majority of H3K9me3-enriched promoters in donor cells become demethylated in 2-cell SCNT embryos, indicating a global H3K9me3 reprogramming. This demethylation might be mediated by an endogenous H3K9me3 demethylase *Kdm4b*, as its expression level correlates with the developmental potential of SCNT embryos (Liu et al., 2016a). Similarly, other H3K9me3 demethylases (*KDM4D* and *KDM4E*) are required for successful bovine SCNT reprogramming (Liu et al., 2018a). These observations suggest that H3K9me3 demethylation is generally required for successful SCNT reprogramming. Interestingly, some regions were not efficiently demethylated in the mouse SCNT 2-cell embryos (Liu et al., 2016a), suggesting that H3K9me3 demethylation might be a limiting factor for efficient SCNT reprogramming.

Very recently, our group successfully mapped H3K27me3 distribution in mouse SCNT morula embryos (Matoba et al., 2018). In contrast to the predominant enrichment of H3K27me3 in CpG island (CGI)-associated promoters in somatic cells, H3K27me3 is broadly distributed in the maternal genome but absent at promoter regions in IVF preimplantation embryos (Zheng et al., 2016). Interestingly, H3K27me3 is also absent at promoters in SCNT morulae, suggesting successful global H3K27me3 reprogramming upon SCNT. However, the maternal broad H3K27m3 domains that regulate a group of newly discovered imprinting genes (Inoue et al., 2017a, 2017b) are not established in SCNT embryos (Matoba et al., 2018), suggesting incomplete reprogramming of H3K27me3 in SCNT embryos.

DNA Methylation Reprogramming

In addition to histone modifications, DNA methylation at 5-methylcytosine (5mC) is another major epigenetic modification that plays a pivotal role in mammalian development. DNA methylation is established and maintained by DNA methyltransferases (DNMTs) and can be demethylated through Ten-eleven translocation (TET) protein-mediated oxidation followed by thymine DNA glycosylase (TDG)-mediated base excision repair (Wu and Zhang, 2017). During mouse preimplantation development, DNA undergoes extensive demethylation through a combination of active and passive processes to reach the lowest level at the blastocyst stage (Guo et al., 2014; Shen et al., 2014). Given that the somatic cell genome is heavily methylated at most CpGs, global demethylation might be a necessary step of SCNT reprogramming. Although several earlier studies using 5mC-antibody-based immunostaining suggested that the DNA methylation level is relatively high in bovine SCNT embryos throughout the preimplantation stages (Dean et al., 2001; Santos et al., 2003), whether global DNA demethylation takes place during SCNT reprogramming has been unknown for many years.

After identifying TET3 as a factor mediating active DNA demethylation in paternal PN of IVF embryos, several groups showed that oocyte-stored TET3 could localize to PPN in mouse SCNT embryos to induce 5mC to 5-hydroxymethylcytosine (5hmC) conversion (Gu et al., 2011; Iqbal et al., 2011; Wossidlo et al.,

2010, 2011), indicating active DNA demethylation indeed occurs in SCNT embryos. Gu et al. (2011) further showed that TET3 depletion in the recipient oocyte abolished 5hmC generation in mouse SCNT PPN, compromised DNA demethylation of the endogenous *Pou5f1* promoter, and delayed the activation of a silenced *Pou5f1-EGFP* allele in donor cells. However, the DNA methylation analysis was only limited to the *Pou5f1* locus, and more importantly, the effect of TET3 depletion on the developmental potential of SCNT embryos was not analyzed. Unexpectedly, we found that maternal TET3 is dispensable for embryonic development in mice (Inoue et al., 2015a). Thus, more analysis on the effect of TET3 depletion on SCNT reprogramming is warranted.

Alex Meissner and colleagues analyzed the DNA methylome of late-stage 1-cell mouse SCNT embryos using reduced-representation bisulfite sequencing (RRBS) (Chan et al., 2012). At this stage (12–14 hpa), global DNA methylation patterns of SCNT embryos were still more similar to the donor somatic cells than to IVF embryos, suggesting that reprogramming of DNA methylation had not yet completed at this time point. Interestingly, they found that genes related to germline development were subjected to significant demethylation and that certain types of repetitive elements, including long interspersed nuclear elements (LINEs) and long terminal repeats (LTRs), maintained a high methylation level even after 1-round of replication. Another study led by Shaorong Gao found that high DNA methylation levels similar to those of donor cumulus cells were maintained until the 4-cell stage in SCNT embryos (Liu et al., 2016a). These observations suggest that similar to IVF embryos, global demethylation in SCNT embryos may require several rounds of replication.

Our recent study confirmed this notion. By performing whole-genome bisulfite sequencing (WGBS), we provided the first comprehensive DNA methylome of mouse SCNT blastocysts, which revealed very low DNA methylation levels (~20% on average) similar to those of IVF blastocysts (Matoba et al., 2018). Given that replication-dependent dilution is a driving force for DNA demethylation during preimplantation development (Shen et al., 2014), this result indicates that the same mechanism also works in mouse SCNT embryos. Although previous RRBS analysis in 1-cell SCNT embryos identified demethylation-resistant CpGs at LINEs and LTRs (Chan et al., 2012), WGBS analysis revealed that the methylation levels of these regions have become similar to that of the IVF at the blastocyst stage (Matoba et al., 2018), indicating replication-dependent dilution may be able to compensate for the initial DNA methylation differences in the 1-cell embryo, at least in mice. Thus, DNA methylation reprogramming in SCNT occurs mainly through replication-dependent dilution during preimplantation development of mouse SCNT embryos (Figure 2A). Given that DNA demethylation through replication-dependent dilution at the preimplantation stages is likely conserved among mammals, this mechanism may be generally applicable to SCNT reprogramming in different mammalian species.

Transcriptome Reprogramming

Since transcription is tightly regulated by chromatin structure and epigenetic state, the chromatin and epigenetic reprogramming described above is expected to have a transcriptional consequence (Figure 2A). Using RNA sequencing (RNA-seq), several groups analyzed the transcriptional activities of SCNT embryos in mouse (Liu et al., 2016a; Matoba et al., 2014), human (Chung et al., 2015), and bovine (Liu et al., 2018a). In mouse, the transcriptome differences between SCNT and IVF embryos become obvious at the 2-cell stage, as over 1,000 genes failed to activate properly in SCNT embryos (Matoba et al., 2014). Based on whether their transcriptional activities are successfully reprogrammed in 2-cell embryos, genes can be categorized into different groups. Among the genes highly expressed in donor cumulus cells, ~80% were quickly downregulated in both SCNT and IVF 2-cell embryos, indicating that the transcriptional state of this group of genes were properly reprogrammed. However, the remaining 20% of genes maintained their high expression level in 2-cell SCNT embryos, but they were silenced in the IVF counterpart. This group of genes maintained their donor somatic transcriptional "memory," which was not reprogramed immediately after SCNT. Another gene group is embryonic specific; they are silenced in somatic donor cells, but they are activated in 2-cell IVF embryos. Approximately 85% of this gene group is activated in 2-cell SCNT embryos, suggesting global transcriptional reprogramming. However, the remaining 15% were resistant to reprogramming in SCNT embryos. Further studies revealed that the reprogramming-resistant regions or genes (resistant to silencing or activation) possess specific epigenetic features that will be discussed below (Chung et al., 2015; Hörmanseder et al., 2017; Matoba et al., 2014).

Epigenetic Barriers that Impede SCNT Reprogramming

Despite success in cloning more than 20 mammalian species, the rate of cloned animals reaching term is extremely low. For example, in mice, ~70% of SCNT embryos arrest development before reaching the blastocyst stage, and only 1%–2% of embryos transferred to surrogate mothers can reach term (Ogura et al., 2013). The problem appears to be more severe in primates. Although Mitalipov's group successfully generated SCNT blastocysts and derived ntESCs in rhesus monkeys more than 10 years ago (Byrne et al., 2007), the blastocyst rate remained ~16%, and attempts to clone a monkey were unsuccessful until recently. Moreover, abnormalities were frequently observed in extraembryonic tissues such as placenta in almost all cloned mammalian species (Ogura et al., 2013), indicating the existence of barriers preventing normal development of cloned animals (Figure 2B).

In the 20 years following the birth of Dolly the sheep, great efforts have been made into identifying conditions and parameters that affect cloning efficiency, including donor cell types and embryo culture conditions, but progress has been limited. One important finding was the use of a histone deacetylase inhibitor (HDACi), trichostatin A (TSA), which led to the increase of overall cloning efficiency from 1% to 6% (Kishigami et al., 2006; Rybouchkin et al., 2006). Although the same approach appears to have also improved the cloning efficiency of pig (Zhao et al., 2010) and cow (Akagi et al., 2011), the effect was limited, and more importantly, how the HDACi treatment improved SCNT reprogramming remained unclear. Nevertheless, these studies suggest that epigenetic changes might be an important aspect of SCNT reprogramming. This notion is further supported by recent transcriptome and epigenomic studies revealing that a significant proportion of genomic regions are resistant to

reprogramming. By characterizing the epigenetic features of these reprogramming-resistant regions, epigenetic barriers to SCNT reprogramming have been identified. Below, we summarize the recent progress in identifying and overcoming these epigenetic barriers, with the goal of increasing SCNT cloning efficiency (Figure 2B).

Aberrant Xist Activation Impedes Postimplantation Development

One of the most critical developmental steps where SCNT embryos exhibit developmental defects is implantation. To identify the molecular cause of implantation defects, Inoue et al. compared the transcriptome of single SCNT blastocysts to those of sex-matched IVF counterparts and discovered that many X-linked genes were specifically and consistently repressed in SCNT embryos regardless of sex (Inoue et al., 2010). This observation established a link between SCNT and X chromosome inactivation (XCI), a female-specific dosage-compensation mechanism. XCI occurs in an imprinted manner at the paternal X chromosome in preimplantation embryos and extra-embryonic tissues, but it occurs randomly in embryonic epiblast cells (Lee and Bartolomei, 2013). Imprinted XCI is initiated by expression of the X-linked non-coding RNA *Xist* from the paternal allele. *Xist* RNA then coats the entire X chromosome in *cis* and recruits Polycomb repressive complex 2 (PRC2) to deposit the repressive histone mark H3K27me3 (Cao et al., 2002), leading to heterochromatinization of the entire X chromosome (Plath et al., 2003). In contrast, the maternally derived X chromosome remains active in both males and females. As such, female (XX) and male (XY) cells achieve similar gene dosage for X-linked genes.

Inoue et al. (2010) postulated that X-chromosome-wide gene repression observed in SCNT blastocysts might be caused by ectopic XCI. Indeed, they found that *Xist* RNA is transcribed from both maternal and paternal alleles and thereby induced abnormal silencing of both X chromosomes in SCNT preimplantation embryos. Importantly, they could overcome this abnormal XCI by using *Xist* heterozygous knockout donor cells, which resulted in an 8- to 9-fold increase in SCNT cloning pup rate. A similar effect was observed when small interfering RNA (siRNA) against *Xist* was injected into the male 1-cell SCNT embryos (Matoba et al., 2011), suggesting that abnormal *Xist* activation at the preimplantation stage has a long-term effect on the developmental capacity of SCNT embryos. Although aberrant *Xist* activation following SCNT has been observed in bovine (Inoue et al., 2010) and pig (Ruan et al., 2018), it is not clear whether this mechanism is conserved in human and monkey due to the diverse XCI initiation mechanism in these species (Okamoto et al., 2011). The molecular mechanism underlying ectopic *Xist* activation in mouse SCNT embryos is likely due to the lack of the recently discovered H3K27me3 imprinting in donor somatic cells (Inoue et al., 2017a) (see below).

H3K9me3 Impedes ZGA and Preimplantation Development

The earliest time when SCNT embryos exhibit developmental arrest phenotype is highly correlated with ZGA in different species. Given that successful ZGA is required for embryonic development, the correlation between ZGA and SCNT embryo arrest suggests that ZGA failure might be a problem in SCNT embryos. In mice, as discussed above, ~1,000 genomic regions or genes failed to activate at ZGA in SCNT embryos (Matoba et al., 2014). Interestingly, these reprogramming-resistant regions (RRRs) are enriched for the transcription repressive marker H3K9me3, which raises the possibility that H3K9me3 in somatic donor cells may serve as a barrier preventing ZGA of SCNT embryos. This notion turned out to be true, as injecting mRNAs encoding the H3K9me3-specific demethylase *Kdm4d* rescued not only the ZGA defect but also the preimplantation developmental arrest phenotype (Matoba et al., 2014), resulting in a pup rate increase from less than 1% to more than 8%. Importantly, the H3K9me3 reprogramming barrier appears to be conserved in humans, as injection of mRNAs encoding a human H3K9me3 demethylase, *KDM4A*, not only facilitated ZGA of human SCNT embryos but also improved the development of human SCNT embryos to reach the expanded blastocyst stage from which human ntESCs were successfully derived (Chung et al., 2015). It is worth noting that the positive effect of TSA treatment might be functionally linked to H3K9me3 removal, as TSA treatment did not further improve the development of *Kdm4d*-mRNA-injected SCNT embryos (Matoba et al., 2014). In addition, genes activated by *Kdm4d* mRNA injection and TSA treatment overlap significantly (Inoue et al., 2015b). Besides mouse and human, recent studies indicated that *KDM4* mRNA injection can also increase the cloning efficiency in pig (Ruan et al., 2018), bovine (Liu et al., 2018a), and monkey (Liu et al., 2018b). Thus, H3K9me3 in somatic cells appears to be a general barrier in mammalian SCNT reprogramming.

H3K27me3 Imprinting Defects Impede Postimplantation Development

Another SCNT reprogramming barrier comes from RNA-seq analysis of cloned placentae (Okae et al., 2014). A comprehensive allelic transcriptome analysis of mouse embryonic day 13.5 (E13.5) placentae identified three genes, *Sfmbt2*, *Gab1*, and *Slc38a4*, that exhibit paternal allele-specific expression in normal placenta but biallelic expression in SCNT placentae (Okae et al., 2014). Given that these three genes play important roles in placental development (Itoh et al., 2000; Miri et al., 2013; S.M., unpublished data), loss of imprint (LOI) of these genes may contribute to the enlarged placental phenotype commonly observed in SCNT embryos in a wide range of species. Interestingly, the imprinting state of these three genes is independent of maternal DNA methylation (Okae et al., 2014).

In an effort to characterize allelic differences in chromatin accessibility and expression during early embryonic development, we recently uncovered a novel genomic imprinting mechanism by which the maternally deposited H3K27me3 represses maternal allele expression of at least 76 genes during preimplantation development (Inoue et al., 2017a). Surprisingly, all three imprinted genes dysregulated in SCNT placenta belong to the newly identified H3K27me3-dependent imprinted genes. Moreover, allelic expression analysis of mouse SCNT blastocysts revealed that essentially all the H3K27me3-dependent imprinted genes detectable at that blastocyst stage lost their imprinting state to become biallelically expressed (Matoba et al., 2018). This LOI is likely due to the absence of H3K27me3 imprinting mark in the donor somatic cells, because it is intrinsically lost in the embryonic lineage from which the donor cells are derived. Indeed, ChIP-seq analysis revealed lack of maternal-specific domain-like H3K27me3 in SCNT morula embryos (Matoba

et al., 2018). Since at least some of the H3K27me3-imprinted genes are functionally important for postimplantation development, complete LOI in these genes likely contributes to the postimplantation developmental arrest phenotypes observed in mouse SCNT embryos. Because *Xist* is also regulated by maternal H3K27me3 (Inoue et al., 2017b), ectopic activation of *Xist* in SCNT embryos (described above) is also likely due to the lack of the H3K27m3 mark at the *Xist* locus in donor somatic cells. Future studies should address whether the H3K27me3-imprinting system as well as its LOI in SCNT embryos is conserved in other mammalian species.

Other Epigenetic Barriers, DNA Methylation, and H3K4me3

In addition to H3K9me3, *Xist* activation, and LOI on H3K27me3, other epigenetic barriers may exist. For example, in the case of X-linked genes, although the use of *Xist* heterozygous knockout (KO) cells as donors resulted in the derepression of most X-linked genes in mouse SCNT blastocysts, genes at the *Magea* and *Xlr* clusters of the X chromosomes still failed to be activated (Inoue et al., 2010), suggesting that an additional silencing mechanism independent of *Xist* is in play, at least in mice. Our recent study analyzing the DNA methylome of mouse SCNT blastocysts revealed high levels of promoter DNA methylation at most of these genes (Matoba et al., 2018), suggesting DNA methylation as a possible barrier. Moreover, this DNA methylome study also indicated that oocyte-derived DNA methylation marks that are inherited by the blastocysts via fertilization are absent in SCNT embryos. Since some of the maternally biased DNA methylation passed down to embryos plays an important role in trophoblast development (Branco et al., 2016), the absence of oocyte-like DNA methylation might also serve as a barrier.

In addition to H3K9me3, H3K4me3 might also affect transcriptional reprogramming and thus impair the developmental potential of SCNT embryos. By performing single-cell RNA-seq of mouse preimplantation SCNT embryos, Liu et al. revealed a correlation between the level of *Kdm5b*, an H3K4me3 demethylase, and the 4-cell to 8-cell SCNT embryo rate (Liu et al., 2016a). Importantly, KD of *Kdm5b* caused a 4-cell arrest while overexpression of *Kdm5b* rescued 4-cell arrest of SCNT embryos, indicating that *Kdm5b* level is critical for SCNT embryos to pass the 4-cell stage. Since *Kdm5b* is an H3K4me3-specific demethylase involved in gene repression, it is possible that H3K4me3 in donor cells serves as a barrier preventing silencing of somatic cell signature genes, which leads to developmental arrest of SCNT embryos at the 4-cell stage. This is consistent with a previous report demonstrating donor cell transcriptional memory in SCNT embryos (Gao et al., 2003). A similar observation was also reported using a *Xenopus* oocyte transcriptional reprogramming system (Hörmanseder et al., 2017). Thus, efficient removal of a donor cell-specific H3K4me3 mark might contribute to SCNT reprogramming, although the mechanism is currently unclear.

Therapeutic Cloning

Successful cloning of the Dolly the sheep in 1997 not only made reproductive cloning possible but also raised the possibility of therapeutic cloning, or the generation of pluripotent human ESCs from cloned blastocysts. This possibility became a realistic hope when the first human ESC line was derived in 1998 (Thomson et al., 1998). The notion of deriving human ESCs by nuclear transfer (ntESCs) is exciting, as the pluripotent ntESC has the same nuclear genetic material as the donor and therefore could be used for regenerative medicine. The first proof-of-concept experiment was performed in a mouse model, where ntESCs were derived from cloned blastocysts (Munsie et al., 2000) and had a differentiation capacity similar to those derived from a blastocyst by normal fertilization (Wakayama et al., 2001). Genetic fixation of a mutant allele in ntESCs was achieved by homologous recombination, and the resultant ntESCs were used as a cell source for treating immunodeficient mice (Rideout et al., 2002). Despite success in mice, derivation of ntESCs in other animals, including primates, remained difficult for many years.

Derivation of Human ntESCs

A key breakthrough was made in 2007 by Mitalipov's group, when they successfully derived rhesus monkey ntESCs by inhibiting premature activation of recipient oocytes (Byrne et al., 2007). Nevertheless, the efficiency of ntESC derivation was only 0.7% per oocyte used for SCNT. Using a similar approach, coupled with TSA treatment, the same group also established the first human ntESC lines using fetal or infant fibroblast as donor cells (Tachibana et al., 2013). The next year, using the same or slightly modified conditions, two groups succeeded in generating ntESCs using adult donor cells, including patients with type 1 diabetes (T1D) (Chung et al., 2014; Yamada et al., 2014). Importantly, one group showed that the ntESC lines derived from T1D patients could differentiate into insulin-secreting beta cells (Yamada et al., 2014), demonstrating a potential use of ntESCs in cell replacement therapy. Despite the success in ntESC generation, all three groups observed great variation among the egg donors in the capacity of the SCNT embryos to reach the blastocyst stage. Thus, it is necessary to establish a method to mitigate the variation for consistent ntESC generation.

Since injection of *Kdm4d* mRNA can improve preimplantation development of mouse SCNT embryos, allowing them to reach the blastocyst stage with a rate comparable to that of IVF (Matoba et al., 2014), we asked whether a similar approach could improve the ability of human SCNT embryos to reach the blastocyst stage so that ntESCs can be derived. Injection of human *KDM4A* mRNA, which encodes a H3K9me3 demethylase, greatly improved the developmental potential of human SCNT embryos and allowed production of at least one expanded blastocyst from each oocyte donor and subsequent establishment of multiple ntESC lines (Chung et al., 2015). In contrast, none of the SCNT embryos reached the blastocyst stage in the non-injected control groups. Thus, *KDM4A* mRNA injection can greatly facilitate patient-specific ntESC derivation.

SCNT and iPSC Reprogramming Use Different Mechanisms

In addition to SCNT, PSCs can also be generated by cell fusion or transcription-factor-induced reprogramming (iPSCs) (Yamanaka and Blau, 2010). However, SCNT and iPSC reprogramming may use different mechanisms. In a landmark study, Takahashi and Yamanaka (2006) demonstrated that pluripotency can be induced by simply overexpressing a set of TFs (*Pou5f1*, *Sox2*, *Klf4*, and *Myc*) in mouse somatic cells. One year later, two groups demonstrated that the same approach could also be

Table 2. Comparison of SCNT and iPSC Reprogramming

Reprogramming Method	Endpoint	Speed	Reprogramming Factors
SCNT	totipotency	fast (hours)	*Tet3, Kdm4b*, *Kdm4d/KDM4D*, *KDM4E*, and *Kdm5b (Pou5f1* is dispensable)
iPSCs	pluripotency	slow (days/weeks)	*Tet1*, *Tet2, Kdm2a*, *Kdm2b*, *Kdm6a, Pou5f1*, *Sox2*, *Klf4*, *Myc*, *Lin28a, Chd1* and *Ino80 etc.*

used to generate human iPSCs from adult human somatic cells (Takahashi et al., 2007; Yu et al., 2007). Although both the SCNT and the iPSC technologies can reprogram differentiated somatic cells into cells of embryonic state, there are fundamental differences between these two reprogramming technologies (Table 2). First, iPSC technology reprograms cells into a pluripotent state similar to ESCs, while SCNT technology reprograms cells into a totipotent state similar to zygotes. Thus, although the final destination of a pluripotent state is the same in SCNT and iPSCs, the paths to achieve pluripotency are likely different. Second, the speed of reprogramming is very different. SCNT reprogramming is very fast, as evidenced by chromatin accessibility (Djekidel et al., 2018) and transcriptome (Egli et al., 2011) reprogramming within hours, probably due to the rapid histone exchanges driven by ooplasmic histone chaperones. In contrast, establishment of stable iPSCs takes several days to weeks (Takahashi and Yamanaka, 2006). Furthermore, SCNT reprogramming, at least for chromatin accessibility and transcriptome reprogramming, is much more efficient than that of iPSCs. These differences likely reflect their diverging reprogramming mechanisms. For example, *Pou5f1* is a core pluripotent TF for iPSC generation but is dispensable for SCNT reprogramming (Wu et al., 2013). Additionally, although H3K9me3 is a key reprogramming barrier in both systems (Chen et al., 2013; Matoba et al., 2014; Soufi et al., 2012), the critical genes impeded by this barrier are different; while it prevents activation of *Sox2* or *Nanog* in iPSC generation, it inhibits ZGA in SCNT. Collectively, these results strongly suggest that iPSC and SCNT reprogramming are mechanistically different.

Advantages and Disadvantages of ntESCs Compared with iPSCs

So far, three types of PSCs (ESCs, iPSCs, and ntESCs) have been generated. With regenerative medicine in mind, traditional human ESCs generation that involves normal fertilization is unlikely suitable not only because of ethical concerns but also because they generally have genomes different from those of the patient (allogenic) and therefore could cause severe immune rejection after transplantation. The two reprogrammed stem cell types, ntESCs and iPSCs, possess an advantage for regenerative medicine, because isogenic cells can be generated directly from the patient somatic cells. Which cell type is better? Clearly, iPSCs have technical and ethical advantages, especially during the derivation steps, as ntESC derivation involves technically difficult SCNT procedures and requires oocytes, while iPSCs can easily be derived even with commercial kits and without ethical concerns. However, recent studies on the molecular characteristics of iPSC and ntESCs revealed critical differences between these two cell types that could impact their use for therapeutic purposes. Below, we compare the molecular features of these two cell types for their respective advantages and disadvantages.

Mitochondrial Replacement. One of the most critical differences between ntESCs and iPSCs is the composition of mtDNA. Although the nuclear DNA of ntESCs is from donor somatic cells (the patient), mtDNA is from the recipient oocytes. In contrast, both the nuclear DNA and mtDNA of iPSCs are from the starting somatic cells (the patient). Mitochondria play a major role in energy production by oxidative phosphorylation, and mutation of mtDNA can cause metabolic disorders (Gorman et al., 2016). Therefore, derivation of ntESCs followed by differentiation to the desired cell or tissue types and transplantation back to patients can cure mitochondrial diseases. Mitalipov's group indeed demonstrated that the mitochondrial composition of ntESCs derived from a patient with Leigh syndrome (a mitochondrial disease) was almost completely replaced with oocyte-derived mitochondria (over 99%) (Ma et al., 2015). Moreover, the disorder-related defects observed in the donor fibroblasts and fibroblast-derived iPSCs were functionally rescued in ntESCs (Ma et al., 2015). Although there were concerns regarding the potential incompatibility between nuclear DNA and different haplotypes of mitochondria from oocytes, the ntESCs derived from relatively different haplotypes (47 SNPs in mtDNA) did not show any functional abnormalities, suggesting normal nuclear-mitochondrial interactions. However, another study reported that transplantation of ntESCs derived from two different mouse strains with polymorphisms in mtDNA-coded proteins to the nuclear allogenic host induced weak immune responses, although the weak immune response could be tolerated (Deuse et al., 2015). This indicates that careful studies are needed before clinical trials can be initiated. Nonetheless, the unique potential of ntESC in mitochondria replacement holds great potential for the treatment of mitochondrial diseases.

Genetic and Epigenetic Mutations. For therapeutic purposes, genetic and epigenetic stability is an important consideration for the different PSCs. Detailed genetic and epigenetic analyses have been performed comparing iPSCs to ESCs (Bock et al., 2011; Lister et al., 2011). Bock et al. (2011) analyzed DNA methylomes and transcriptomes of 12 human iPSC lines and 20 ESC lines and found that both iPSCs and ESCs contained inherent variations. Although they failed to identify common epigenetic defects in iPSC lines, they observed greater variation in iPSC lines than ESC lines, suggesting that the iPSC generation process could introduce more variables than that of ESCs. One factor contributing to the variation is the so-called epigenetic memory of the donor cells, which has been shown to require a long culture time to remove (Nishino et al., 2011). Similarly, Lister et al. (2011) performed DNA methylome and transcriptome analyses of 5 iPSC lines derived using different methods or different starting cell types and compared the results with ESC lines. Although the global DNA methylome of iPSCs resembled that of ESCs, hundreds of differentially methylated regions (DMRs) were identified. Some of the DMRs were common to all 5 iPSC lines, suggesting the existence of common failed epigenetic reprogramming in iPSC lines.

Following the success in deriving human ntESCs (Tachibana et al., 2013), comparison of the genetic and epigenetic mutations of isogenic iPSC and ntESCs became possible. Ma et al. (2014) compared 4 ntESC lines and 7 iPSC lines derived from the same human fetal fibroblasts, with 2 IVF lines from the same egg donor as that of ntESCs. While they did not detect any statistically significant difference in the frequency of copy-number variation (CNV) among the samples, they observed that the DNA methylome of ntESCs was more similar to IVF than iPSCs. Although both ntESC and iPSC lines possess residual DNA methylation memory of donor somatic cells, iPSCs had 8 times more such memory than ntESCs. Moreover, iPSCs had 60 times more aberrantly methylated loci than ntESCs, and the great majority of these (90%) were likely induced during reprogramming processes. Consistently, transcriptome analysis revealed that more differentially expressed genes are present in iPSCs than in ntESCs (Ma et al., 2014). While these results show that ntESC is advantageous to iPSCs in terms of the epigenetic errors introduced during reprogramming, another study reported no significant differences on the levels of genetic and epigenetic mutations when ntESCs and iPSCs were compared (Johannesson et al., 2014). Although the reasons for the discrepancy of the two studies are unknown, the passage numbers of the cells used for the analysis and the technical differences in iPSCs generation between the two studies may account for the differences. While the first study used viral expression in fetal fibroblasts for iPSC generation, the latter used modified mRNA expression in neonatal or adult fibroblasts. Regardless of the source of discrepancy, functional characterization of isogenic iPSCs and ntESCs showed no significant difference, indicating both methods could generate pluripotent cells capable of differentiating to functional cells (Zhao et al., 2017).

A recent study revealed that long-term culture of PSCs is a source of oncogenic mutation in the *TP53* gene (Merkle et al., 2017). Kevin Eggan and colleagues performed extensive genomic and transcriptomic sequencing analyses on 140 human ESC lines, including 26 lines prepared for potential clinical use, and found that *TP53*, encoding a well-known tumor suppressor P53, is frequently mutated during *in vitro* culture and cells harboring a mutant allele have growth advantages during ESC passage (Merkle et al., 2017). Given that the establishment of epigenetically stable iPSC lines requires long-term culture to get rid of somatic cell epigenetic memory, the genetic mutations introduced during such long-term culture has raised a potential safety concern. Indeed, a comprehensive analysis of 711 human iPSC lines derived from 301 healthy individuals revealed that many iPSC lines possess recurrent copy-number alterations (CNAs) at certain genomic regions on chromosomes X, 17, and 20 (Kilpinen et al., 2017). These observations suggest that iPSCs may have genetic mutations at specific genomic regions due to the long-term derivation and maintenance processes. The fact that the ntESC derivation process is relatively shorter may give ntESC an advantage, at least in terms of culturing-induced mutations. To demonstrate this is indeed the case, careful comparison of the frequency of genetic mutations in ESC, iPSC, and ntESC should be performed using a large number of lines under comparable experimental settings, including donor somatic cell source, recipient eggs, culturing conditions, and passage numbers. However, the number of currently existing ntESC lines is limited due to the extremely low efficiency in ntESC derivation. In this regard, more efficient human ntESC derivation involving *KDM4A* mRNA injection (Chung et al., 2015) using healthy donor somatic cells will facilitate such comparative study.

Future Directions in SCNT Research

With technological advancement, particularly the development of more sensitive sequencing-related methods, great progress has made in understanding SCNT reprogramming barriers that lead to improved cloning efficiency. In addition, work focusing on the molecular events immediately after SCNT is beginning to reveal reprogramming mechanisms. Below, we discuss the main remaining questions and where we will likely see great progress in the years to come.

Further Understanding of Reprogramming Mechanisms

Five decades have lapsed since the first successful frog cloning, yet our understanding of SCNT reprogramming at the molecular level is still limited. Toward this goal, a systematic and detailed analysis of the chromatin and epigenomic changes during the reprogramming process is needed. Although it is still technically challenging to obtain sufficient SCNT samples for such analyses, recent studies have demonstrated the feasibility of performing such studies using preimplantation embryos (Ke et al., 2017; Lu et al., 2016; Zheng et al., 2016). Thus, further improvement of the current techniques may make the analysis of SCNT samples possible. Such analysis may reveal new epigenetic abnormalities in SCNT embryos, which can serve as the basis for improving SCNT cloning efficiency. In addition, comparative analysis of SCNT reprogramming with other reprogramming systems, such as iPSCs and cell fusion, may reveal novel insights as different reprogramming systems may share some common features. Indeed, H3K9me3 has also been shown to be a barrier to iPSC reprogramming (Soufi et al., 2012; Sridharan et al., 2013). Similarly, chromatin assembly factor 1 (CAF1) complex and CBX5 (also known as HP1) can serve as barriers to iPSC reprogramming (Cheloufi et al., 2015; Sridharan et al., 2013). Testing whether these barriers also function in SCNT reprogramming would be of potential interest.

Improving Efficiency and Quality of Cloning by Targeted Epigenetic Modification

As a natural extension to the previous findings that ectopic activation of *Xist* and H3K9me3 in somatic donor cells serve as barriers impeding SCNT reprogramming, we applied a combined approach using *Xist* KO donor cells coupled with *Kdm4d* mRNA injection and achieved 24% pup rate, the highest cloning rate reported in mouse when Sertoli cells are used as donors (Matoba et al., 2018). However, this rate is still lower than that of IVF (more than 50%), and the resultant SCNT embryos still had abnormally enlarged placentae. Detailed allelic transcriptome, ChIP-seq, and DNA methylome analyses revealed that embryos generated in this manner exhibit LOI on H3K27me3-imprinted genes as well as aberrant DNA methylation at many loci (Matoba et al., 2018). These results suggest both aberrant DNA methylation and H3K27me3-mediated genomic imprinting defects are likely responsible for the observed phenotypes (Matoba et al., 2018). Since these epigenetic modifications are reversible, targeted epigenetic changes might be a good strategy to overcome the defects. In this regard, the dCas9-guided epigenetic modulation system is particularly powerful, as it has been

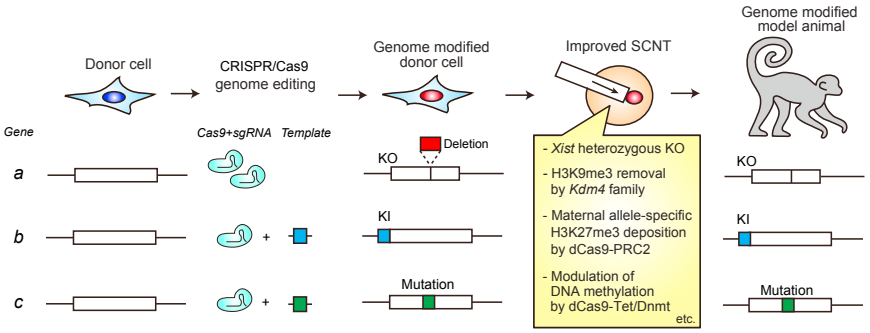

Figure 3. Application of SCNT Technology in Human Disease Model Generation
CRISPR/Cas9-mediated genome editing coupled with improved SCNT cloning enables rapid generation of human disease animal models. CRISPR/Cas9-mediated genome editing can induce deletion to produce gene knockout (KO) alleles, insertion to produce knockin (KI) alleles, or replacement of specific pathogenic mutation via HDR.

recently used to achieve targeted DNA methylation and histone modification changes (Hilton et al., 2015; Kungulovski and Jeltsch, 2016; Liu et al., 2016b). However, a special consideration for epigenetic editing of imprinted genes is its allele-specific targeting. For example, in the case of H3K27me3-dependent imprinted genes, LOI in SCNT embryos is likely due to the absence of the H3K27me3 mark at the maternal allele of the donor somatic cells (Matoba et al., 2018). Therefore, to fix the LOI problem, targeted deposition of H3K27me3 in the maternal allele in the donor cells would be necessary.

Generation of Novel Human Disease Models for Drug Development

The recent progress in improving cloning efficiency has made the various applications of SCNT technology possible. In addition to mitochondrial replacement therapy for therapeutic cloning, reproductive cloning has tremendous potential for expanding agriculturally and economically important animal traits and rescuing near-extinct animals (Beyhan et al., 2007; Loi et al., 2001) without sacrificing donor animals (Kamimura et al., 2013; Wakayama et al., 2008). To realize this potential, the first thing that needs to be done is to test whether the reprogramming barriers identified in mice are conserved in other relevant animal species. Thus, we are likely to see more studies on improving cloning efficiency in agriculturally and economically important animal species, including pig, bovine, and sheep. Similarly, improved cloning techniques will likely be used for pets, like dogs and cats, to make this service more affordable.

Another potential application of SCNT technology that we would like to emphasize is the generation of novel animal models for human diseases (Figure 3). Large-scale sequencing efforts have established a correlation between genetic variations and pathogenic phenotypes in human (Landrum et al., 2016). However, the causal relationships between genetic variations and pathogenic phenotypes need to be addressed using *in vitro* and *in vivo* models. Indeed, many such *in vivo* models have been produced in mice (Birling et al., 2017), but due to the physiological differences between rodent and human, many human diseases, including psychiatric and immune diseases, cannot be modeled in rodents. Rather, primate species that are physiologically more relevant to human might be needed (Izpisua Belmonte et al., 2015).

One unique feature of SCNT is that it enables direct generation of organisms from single donor cells. This feature allows quick and efficient generation of human disease models in large animals, including primates, particularly when combined with genome-editing techniques such as CRISPR/Cas9 (Doudna and Charpentier, 2014; Hsu et al., 2014). Although direct injection of CRSPR/Cas9 genome-editing components into zygotes allows generation of such human disease models in various species, several important problems exist in such an approach, including frequent mosaicism (Yen et al., 2014), random mutations, and relatively low editing efficiency for homology-dependent repair (HDR)-mediated knockin (KI) or KO. This is especially problematic when trying to establish disease models involving multiple edited genes, as the chance to obtain desired multiple gene mutations is extremely low. In small animal models with relatively short generation time and large litter sizes, these issues are not detrimental as the desired whole-body mutant line can be established by crossing offspring of the founder line. However, such an approach is not feasible for large animals with longer gestation periods, sexual maturation time, and uniparous features. For example, in the case of the cynomolgus monkey, gestation takes 164 days to produce a single offspring per pregnancy, and sexual maturation takes 3–4 years. Thus, it would take more than 10 years to establish a disease model in such primate species using this approach. On the other hand, if CRISPR/Cas9 genome editing in somatic cells and screening for the desired mutations are done *in vitro* prior to SCNT, the desired disease model can be generated within 1–2 years.

Indeed, a similar approach has been employed in goat (Ni et al., 2014) and pig (Yan et al., 2018; Zhou et al., 2015), and it has been summarized in a review (Tan et al., 2016). Nevertheless, the limiting factor of such an approach is the extremely low efficiency of animal cloning. For example, based on the summary, the cloning efficiency of genome-edited somatic cells is only 0.5%–1.0% in livestock animals (Tan et al., 2016). If the cloning efficiency is improved, such an approach will be the most efficient way for generating large animal models of human diseases, including non-human primate models.

Concluding Remarks

Here, we summarize our current understanding of the cellular and molecular mechanisms of reprogramming by SCNT. Recent technology advancements have revealed reprogramming barriers and prompted the development of methods to overcome such barriers leading to increased cloning efficiency. Increased cloning efficiency has made both therapeutic cloning and reproductive cloning, including cloning of primates, possible. The generation of patient-specific ntESCs has provided a complementary strategy to iPSCs in pluripotent cell generation. Improved cloning technology, when combined with state-of-art genome editing technology, will expand the potential applications, including generation of novel human disease models for drug screening and evaluation. We believe that further

refinement of SCNT technology will turn many of these applications from dreams of the past into reality.

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DECLARATION OF INTERESTS

Y.Z. is a scientific founder of NewStem Biotechnology. Y.Z. and S.M. are inventors of a patent on the role of Kdm4 in improving cloning efficiency.

REFERENCES

Akagi, S., Matsukawa, K., Mizutani, E., Fukunari, K., Kaneda, M., Watanabe, S., and Takahashi, S. (2011). Treatment with a histone deacetylase inhibitor after nuclear transfer improves the preimplantation development of cloned bovine embryos. J. Reprod. Dev. *57*, 120–126.

Akiyama, T., Suzuki, O., Matsuda, J., and Aoki, F. (2011). Dynamic replacement of histone H3 variants reprograms epigenetic marks in early mouse embryos. PLoS Genet. *7*, e1002279.

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., et al. (1999). Production of goats by somatic cell nuclear transfer. Nat. Biotechnol. *17*, 456–461.

Berg, D.K., Li, C., Asher, G., Wells, D.N., and Oback, B. (2007). Red deer cloned from antler stem cells and their differentiated progeny. Biol. Reprod. *77*, 384–394.

Beyhan, Z., Iager, A.E., and Cibelli, J.B. (2007). Interspecies nuclear transfer: implications for embryonic stem cell biology. Cell Stem Cell *1*, 502–512.

Birling, M.-C., Herault, Y., and Pavlovic, G. (2017). Modeling human disease in rodents by CRISPR/Cas9 genome editing. Mamm. Genome *28*, 291–301.

Bock, C., Kiskinis, E., Verstappen, G., Gu, H., Boulting, G., Smith, Z.D., Ziller, M., Croft, G.F., Amoroso, M.W., Oakley, D.H., et al. (2011). Reference maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. Cell *144*, 439–452.

Branco, M.R., King, M., Perez-Garcia, V., Bogutz, A.B., Caley, M., Fineberg, E., Lefebvre, L., Cook, S.J., Dean, W., Hemberger, M., and Reik, W. (2016). Maternal DNA methylation regulates early trophoblast development. Dev. Cell *36*, 152–163.

Byrne, J.A., Pedersen, D.A., Clepper, L.L., Nelson, M., Sanger, W.G., Gokhale, S., Wolf, D.P., and Mitalipov, S.M. (2007). Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature *450*, 497–502.

Campbell, K.H., Loi, P., Otaegui, P.J., and Wilmut, I. (1996a). Cell cycle coordination in embryo cloning by nuclear transfer. Rev. Reprod. *1*, 40–46.

Campbell, K.H.S., McWhir, J., Ritchie, W.A., and Wilmut, I. (1996b). Sheep cloned by nuclear transfer from a cultured cell line. Nature *380*, 64–66.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043.

Chan, M.M., Smith, Z.D., Egli, D., Regev, A., and Meissner, A. (2012). Mouse ooplasm confers context-specific reprogramming capacity. Nat. Genet. *44*, 978–980.

Chang, C.-C., Gao, S., Sung, L.-Y., Corry, G.N., Ma, Y., Nagy, Z.P., Tian, X.C., and Rasmussen, T.P. (2010). Rapid elimination of the histone variant MacroH2A from somatic cell heterochromatin after nuclear transfer. Cell. Reprogram. *12*, 43–53.

Cheloufi, S., Elling, U., Hopfgartner, B., Jung, Y.L., Murn, J., Ninova, M., Hubmann, M., Badeaux, A.I., Euong Ang, C., Tenen, D., et al. (2015). The histone chaperone CAF-1 safeguards somatic cell identity. Nature *528*, 218–224.

Chen, J., Liu, H., Liu, J., Qi, J., Wei, B., Yang, J., Liang, H., Chen, Y., Chen, J., Wu, Y., et al. (2013). H3K9 methylation is a barrier during somatic cell reprogramming into iPSCs. Nat. Genet. *45*, 34–42.

Chesné, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L., and Renard, J.-P. (2002). Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat. Biotechnol. *20*, 366–369.

Chung, Y.G., Eum, J.H., Lee, J.E., Shim, S.H., Sepilian, V., Hong, S.W., Lee, Y., Treff, N.R., Choi, Y.H., Kimbrel, E.A., et al. (2014). Human somatic cell nuclear transfer using adult cells. Cell Stem Cell *14*, 777–780.

Chung, Y.G., Matoba, S., Liu, Y., Eum, J.H., Lu, F., Jiang, W., Lee, J.E., Sepilian, V., Cha, K.Y., Lee, D.R., and Zhang, Y. (2015). Histone demethylase expression enhances human somatic cell nuclear transfer efficiency and promotes derivation of pluripotent stem cells. Cell Stem Cell *17*, 758–766.

Cibelli, J.B., and Gurdon, J.B. (2018). Custom-made oocytes to clone non-human primates. Cell *172*, 647–649.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de León, F.A., and Robl, J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science *280*, 1256–1258.

Dean, W., Santos, F., Stojkovic, M., Zakhartchenko, V., Walter, J., Wolf, E., and Reik, W. (2001). Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc. Natl. Acad. Sci. USA *98*, 13734–13738.

Deuse, T., Wang, D., Stubbendorff, M., Itagaki, R., Grabosch, A., Greaves, L.C., Alawi, M., Grünewald, A., Hu, X., Hua, X., et al. (2015). SCNT-derived ESCs with mismatched mitochondria trigger an immune response in allogeneic hosts. Cell Stem Cell *16*, 33–38.

Djekidel, M.N., Inoue, A., Matoba, S., Suzuki, T., Zhang, C., Lu, F., Jiang, L., and Zhang, Y. (2018). Reprogramming of chromatin accessibility in somatic cell nuclear transfer is DNA replication independent. Cell Rep. *23*, 1939–1947.

Doudna, J.A., and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science *346*, 1258096.

Egli, D., Chen, A.E., Saphier, G., Ichida, J., Fitzgerald, C., Go, K.J., Acevedo, N., Patel, J., Baetscher, M., Kearns, W.G., et al. (2011). Reprogramming within hours following nuclear transfer into mouse but not human zygotes. Nat. Commun. *2*, 488.

Folch, J., Cocero, M.J., Chesné, P., Alabart, J.L., Domínguez, V., Cognié, Y., Roche, A., Fernández-Arias, A., Martí, J.I., Sánchez, P., et al. (2009). First birth of an animal from an extinct subspecies (Capra pyrenaica pyrenaica) by cloning. Theriogenology *71*, 1026–1034.

Fulka, J., Jr., Miyashita, N., Nagai, T., and Ogura, A. (2004). Do cloned mammals skip a reprogramming step? Nat. Biotechnol. *22*, 25–26.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R., and Lazzari, G. (2003). Pregnancy: a cloned horse born to its dam twin. Nature *424*, 635.

Gao, S., Chung, Y.G., Williams, J.W., Riley, J., Moley, K., and Latham, K.E. (2003). Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. Biol. Reprod. *69*, 48–56.

Gao, S., Chung, Y.G., Parseghian, M.H., King, G.J., Adashi, E.Y., and Latham, K.E. (2004). Rapid H1 linker histone transitions following fertilization or somatic cell nuclear transfer: evidence for a uniform developmental program in mice. Dev. Biol. *266*, 62–75.

Gao, L., Wu, K., Liu, Z., Yao, X., Yuan, S., Tao, W., Yi, L., Yu, G., Hou, Z., Fan, D., et al. (2018). Chromatin accessibility landscape in human early embryos and its association with evolution. Cell *173*, 248–259.e15.

Gómez, M.C., Pope, C.E., Giraldo, A., Lyons, L.A., Harris, R.F., King, A.L., Cole, A., Godke, R.A., and Dresser, B.L. (2004). Birth of African Wildcat cloned kittens born from domestic cats. Cloning Stem Cells *6*, 247–258.

Gorman, G.S., Chinnery, P.F., DiMauro, S., Hirano, M., Koga, Y., McFarland, R., Suomalainen, A., Thorburn, D.R., Zeviani, M., and Turnbull, D.M. (2016). Mitochondrial diseases. Nat. Rev. Dis. Primers *2*, 16080.

Gu, T.-P., Guo, F., Yang, H., Wu, H.-P., Xu, G.-F., Liu, W., Xie, Z.-G., Shi, L., He, X., Jin, S.G., et al. (2011). The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature *477*, 606–610.

Guo, F., Li, X., Liang, D., Li, T., Zhu, P., Guo, H., Wu, X., Wen, L., Gu, T.-P., Hu, B., et al. (2014). Active and passive demethylation of male and female pronuclear DNA in the mammalian zygote. Cell Stem Cell *15*, 447–459.

Gurdon, J.B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J. Embryol. Exp. Morphol. *10*, 622–640.

Hilton, I.B., D'Ippolito, A.M., Vockley, C.M., Thakore, P.I., Crawford, G.E., Reddy, T.E., and Gersbach, C.A. (2015). Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat. Biotechnol. *33*, 510–517.

Hirasawa, R., Chiba, H., Kaneda, M., Tajima, S., Li, E., Jaenisch, R., and Sasaki, H. (2008). Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. Genes Dev. *22*, 1607–1616.

Hörmanseder, E., Simeone, A., Allen, G.E., Bradshaw, C.R., Figlmüller, M., Gurdon, J., and Jullien, J. (2017). H3K4 methylation-dependent memory of somatic cell identity inhibits reprogramming and development of nuclear transfer embryos. Cell Stem Cell *21*, 135–143.e6.

Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell *157*, 1262–1278.

Hwang, I., Jeong, Y.W., Kim, J.J., Lee, H.J., Kang, M., Park, K.B., Park, J.H., Kim, Y.W., Kim, W.T., Shin, T., et al. (2013). Successful cloning of coyotes through interspecies somatic cell nuclear transfer using domestic dog oocytes. Reprod. Fertil. Dev. *25*, 1142–1148.

Inoue, A., and Zhang, Y. (2011). Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. Science *334*, 194.

Inoue, A., and Zhang, Y. (2014). Nucleosome assembly is required for nuclear pore complex assembly in mouse zygotes. Nat. Struct. Mol. Biol. *21*, 609–616.

Inoue, K., Kohda, T., Sugimoto, M., Sado, T., Ogonuki, N., Matoba, S., Shiura, H., Ikeda, R., Mochida, K., Fujii, T., et al. (2010). Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. Science *330*, 496–499.

Inoue, A., Shen, L., Dai, Q., He, C., and Zhang, Y. (2011). Generation and replication-dependent dilution of 5fC and 5caC during mouse preimplantation development. Cell Res. *21*, 1670–1676.

Inoue, A., Shen, L., Matoba, S., and Zhang, Y. (2015a). Haploinsufficiency, but not defective paternal 5mC oxidation, accounts for the developmental defects of maternal Tet3 knockouts. Cell Rep. *10*, 463–470.

Inoue, K., Oikawa, M., Kamimura, S., Ogonuki, N., Nakamura, T., Nakano, T., Abe, K., and Ogura, A. (2015b). Trichostatin A specifically improves the aberrant expression of transcription factor genes in embryos produced by somatic cell nuclear transfer. Sci. Rep. *5*, 10127.

Inoue, A., Jiang, L., Lu, F., Suzuki, T., and Zhang, Y. (2017a). Maternal H3K27me3 controls DNA methylation-independent imprinting. Nature *547*, 419–424.

Inoue, A., Jiang, L., Lu, F., and Zhang, Y. (2017b). Genomic imprinting of *Xist* by maternal H3K27me3. Genes Dev. *31*, 1927–1932.

Iqbal, K., Jin, S.-G., Pfeifer, G.P., and Szabó, P.E. (2011). Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. Proc. Natl. Acad. Sci. USA *108*, 3642–3647.

Itoh, M., Yoshida, Y., Nishida, K., Narimatsu, M., Hibi, M., and Hirano, T. (2000). Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. Mol. Cell. Biol. *20*, 3695–3704.

Izpisua Belmonte, J.C., Callaway, E.M., Caddick, S.J., Churchland, P., Feng, G., Homanics, G.E., Lee, K.-F., Leopold, D.A., Miller, C.T., Mitchell, J.F., et al. (2015). Brains, genes, and primates. Neuron *86*, 617–631.

Janssen, D.L., Edwards, M.L., Koster, J.A., Lanza, R.P., and Ryder, O.A. (2004). Postnatal management of chryptorchid Banteng calves cloned by nuclear transfer utilizing frozen fibroblast cultures and enucleated cow ova. Reprod. Fertil. Dev. *16*, 224.

Johannesson, B., Sagi, I., Gore, A., Paull, D., Yamada, M., Golan-Lev, T., Li, Z., LeDuc, C., Shen, Y., Stern, S., et al. (2014). Comparable frequencies of coding mutations and loss of imprinting in human pluripotent cells derived by nuclear transfer and defined factors. Cell Stem Cell *15*, 634–642.

Kamimura, S., Inoue, K., Ogonuki, N., Hirose, M., Oikawa, M., Yo, M., Ohara, O., Miyoshi, H., and Ogura, A. (2013). Mouse cloning using a drop of peripheral blood. Biol. Reprod. *89*, 24.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H., and Tsunoda, Y. (1998). Eight calves cloned from somatic cells of a single adult. Science *282*, 2095–2098.

Ke, Y., Xu, Y., Chen, X., Feng, S., Liu, Z., Sun, Y., Yao, X., Li, F., Zhu, W., Gao, L., et al. (2017). 3D chromatin structures of mature gametes and structural reprogramming during mammalian embryogenesis. Cell *170*, 367–381.e20.

Kilpinen, H., Goncalves, A., Leha, A., Afzal, V., Alasoo, K., Ashford, S., Bala, S., Bensaddek, D., Casale, F.P., Culley, O.J., et al. (2017). Common genetic variation drives molecular heterogeneity in human iPSCs. Nature *546*, 370–375.

Kim, J.-M., Ogura, A., Nagata, M., and Aoki, F. (2002). Analysis of the mechanism for chromatin remodeling in embryos reconstructed by somatic nuclear transfer. Biol. Reprod. *67*, 760–766.

Kim, M.K., Jang, G., Oh, H.J., Yuda, F., Kim, H.J., Hwang, W.S., Hossein, M.S., Kim, J.J., Shin, N.S., Kang, S.K., and Lee, B.C. (2007). Endangered wolves cloned from adult somatic cells. Cloning Stem Cells *9*, 130–137.

Kishigami, S., Mizutani, E., Ohta, H., Hikichi, T., Thuan, N.V., Wakayama, S., Bui, H.-T., and Wakayama, T. (2006). Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem. Biophys. Res. Commun. *340*, 183–189.

Klose, R.J., Kallin, E.M., and Zhang, Y. (2006). JmjC-domain-containing proteins and histone demethylation. Nat. Rev. Genet. *7*, 715–727.

Kungulovski, G., and Jeltsch, A. (2016). Epigenome editing: state of the art, concepts, and perspectives. Trends Genet. *32*, 101–113.

Landrum, M.J., Lee, J.M., Benson, M., Brown, G., Chao, C., Chitipiralla, S., Gu, B., Hart, J., Hoffman, D., Hoover, J., et al. (2016). ClinVar: public archive of interpretations of clinically relevant variants. Nucleic Acids Res. *44* (D1), D862–D868.

Lanza, R.P., Cibelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin, C.E., Hammer, C.J., West, M.D., and Damiani, P. (2000). Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning *2*, 79–90.

Lee, J.T., and Bartolomei, M.S. (2013). X-inactivation, imprinting, and long noncoding RNAs in health and disease. Cell *152*, 1308–1323.

Lee, B.C., Kim, M.K., Jang, G., Oh, H.J., Yuda, F., Kim, H.J., Hossein, M.S., Kim, J.J., Kang, S.K., Schatten, G., and Hwang, W.S. (2005). Dogs cloned from adult somatic cells. Nature *436*, 641.

Li, Z., Sun, X., Chen, J., Liu, X., Wisely, S.M., Zhou, Q., Renard, J.-P., Leno, G.H., and Engelhardt, J.F. (2006). Cloned ferrets produced by somatic cell nuclear transfer. Dev. Biol. *293*, 439–448.

Lister, R., Pelizzola, M., Kida, Y.S., Hawkins, R.D., Nery, J.R., Hon, G., Antosiewicz-Bourget, J., O'Malley, R., Castanon, R., Klugman, S., et al. (2011). Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature *471*, 68–73.

Liu, Y., Han, X.-J., Liu, M.-H., Wang, S.-Y., Jia, C.-W., Yu, L., Ren, G., Wang, L., and Li, W. (2014). Three-day-old human unfertilized oocytes after in vitro fertilization/intracytoplasmic sperm injection can be activated by calcium ionophore a23187 or strontium chloride and develop to blastocysts. Cell. Reprogram. *16*, 276–280.

Liu, W., Liu, X., Wang, C., Gao, Y., Gao, R., Kou, X., Zhao, Y., Li, J., Wu, Y., Xiu, W., et al. (2016a). Identification of key factors conquering developmental arrest of somatic cell cloned embryos by combining embryo biopsy and single-cell sequencing. Cell Discov. *2*, 16010.

Liu, X.S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R.A., and Jaenisch, R. (2016b). Editing DNA methylation in the mammalian genome. Cell *167*, 233–247.e17.

Liu, X., Wang, Y., Gao, Y., Su, J., Zhang, J., Xing, X., Zhou, C., Yao, K., An, Q., and Zhang, Y. (2018a). H3K9 demethylase KDM4E is an epigenetic regulator for bovine embryonic development and a defective factor for nuclear reprogramming. Development *145*, dev158261.

Liu, Z., Cai, Y., Wang, Y., Nie, Y., Zhang, C., Xu, Y., Zhang, X., Lu, Y., Wang, Z., Poo, M., and Sun, Q. (2018b). Cloning of Macaque monkeys by somatic cell nuclear transfer. Cell *172*, 881–887.e7.

Loi, P., Ptak, G., Barboni, B., Fulka, J., Jr., Cappai, P., and Clinton, M. (2001). Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat. Biotechnol. *19*, 962–964.

Loi, P., Iuso, D., Czernik, M., and Ogura, A. (2016). A new, dynamic era for somatic cell nuclear transfer? Trends Biotechnol. *34*, 791–797.

Lu, F., and Zhang, Y. (2015). Cell totipotency: molecular features, induction, and maintenance. Natl. Sci. Rev. *2*, 217–225.

Lu, F., Liu, Y., Inoue, A., Suzuki, T., Zhao, K., and Zhang, Y. (2016). Establishing chromatin regulatory landscape during mouse preimplantation development. Cell *165*, 1375–1388.

Ma, H., Morey, R., O'Neil, R.C., He, Y., Daughtry, B., Schultz, M.D., Hariharan, M., Nery, J.R., Castanon, R., Sabatini, K., et al. (2014). Abnormalities in human pluripotent cells due to reprogramming mechanisms. Nature *511*, 177–183.

Ma, H., Folmes, C.D.L., Wu, J., Morey, R., Mora-Castilla, S., Ocampo, A., Ma, L., Poulton, J., Wang, X., Ahmed, R., et al. (2015). Metabolic rescue in pluripotent cells from patients with mtDNA disease. Nature *524*, 234–238.

Maenohara, S., Unoki, M., Toh, H., Ohishi, H., Sharif, J., Koseki, H., and Sasaki, H. (2017). Role of UHRF1 in de novo DNA methylation in oocytes and maintenance methylation in preimplantation embryos. PLoS Genet. *13*, e1007042.

Martin, C., and Zhang, Y. (2005). The diverse functions of histone lysine methylation. Nat. Rev. Mol. Cell Biol. *6*, 838–849.

Matoba, S., Inoue, K., Kohda, T., Sugimoto, M., Mizutani, E., Ogonuki, N., Nakamura, T., Abe, K., Nakano, T., Ishino, F., and Ogura, A. (2011). RNAi-mediated knockdown of Xist can rescue the impaired postimplantation development of cloned mouse embryos. Proc. Natl. Acad. Sci. USA *108*, 20621–20626.

Matoba, S., Liu, Y., Lu, F., Iwabuchi, K.A., Shen, L., Inoue, A., and Zhang, Y. (2014). Embryonic development following somatic cell nuclear transfer impeded by persisting histone methylation. Cell *159*, 884–895.

Matoba, S., Wang, H., Jiang, L., Lu, F., Iwabuchi, K.A., Wu, X., Inoue, K., Yang, L., Press, W., Lee, J.T., et al. (2018). Loss of H3K27me3 imprinting in somatic cell nuclear transfer embryos disrupts post-implantation development. Cell Stem Cell *23*, 343–354.

Meirelles, F.V., Bordignon, V., Watanabe, Y., Watanabe, M., Dayan, A., Lôbo, R.B., Garcia, J.M., and Smith, L.C. (2001). Complete replacement of the mitochondrial genotype in a Bos indicus calf reconstructed by nuclear transfer to a Bos taurus oocyte. Genetics *158*, 351–356.

Meissner, A., and Jaenisch, R. (2006). Mammalian nuclear transfer. Dev. Dyn. *235*, 2460–2469.

Merkle, F.T., Ghosh, S., Kamitaki, N., Mitchell, J., Avior, Y., Mello, C., Kashin, S., Mekhoubad, S., Ilic, D., Charlton, M., et al. (2017). Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. Nature *545*, 229–233.

Miri, K., Latham, K., Panning, B., Zhong, Z., Andersen, A., and Varmuza, S. (2013). The imprinted polycomb group gene Sfmbt2 is required for trophoblast maintenance and placenta development. Development *140*, 4480–4489.

Munsie, M.J., Michalska, A.E., O'Brien, C.M., Trounson, A.O., Pera, M.F., and Mountford, P.S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. Curr. Biol. *10*, 989–992.

Nashun, B., Yukawa, M., Liu, H., Akiyama, T., and Aoki, F. (2010). Changes in the nuclear deposition of histone H2A variants during pre-implantation development in mice. Development *137*, 3785–3794.

Nashun, B., Akiyama, T., Suzuki, M.G., and Aoki, F. (2011). Dramatic replacement of histone variants during genome remodeling in nuclear-transferred embryos. Epigenetics *6*, 1489–1497.

Ni, W., Qiao, J., Hu, S., Zhao, X., Regouski, M., Yang, M., Polejaeva, I.A., and Chen, C. (2014). Efficient gene knockout in goats using CRISPR/Cas9 system. PLoS ONE *9*, e106718.

Nishino, K., Toyoda, M., Yamazaki-Inoue, M., Fukawatase, Y., Chikazawa, E., Sakaguchi, H., Akutsu, H., and Umezawa, A. (2011). DNA methylation dynamics in human induced pluripotent stem cells over time. PLoS Genet. *7*, e1002085.

Ogura, A., Inoue, K., and Wakayama, T. (2013). Recent advancements in cloning by somatic cell nuclear transfer. Philos. Trans. R. Soc. Lond. B Biol. Sci. *368*, 20110329.

Okae, H., Matoba, S., Nagashima, T., Mizutani, E., Inoue, K., Ogonuki, N., Chiba, H., Funayama, R., Tanaka, S., Yaegashi, N., et al. (2014). RNA sequencing-based identification of aberrant imprinting in cloned mice. Hum. Mol. Genet. *23*, 992–1001.

Okamoto, I., Patrat, C., Thépot, D., Peynot, N., Fauque, P., Daniel, N., Diabangouaya, P., Wolf, J.-P., Renard, J.-P., Duranthon, V., and Heard, E. (2011). Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development. Nature *472*, 370–374.

Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H., and Perry, A.C. (2000). Pig cloning by microinjection of fetal fibroblast nuclei. Science *289*, 1188–1190.

Ono, Y., Shimozawa, N., Ito, M., and Kono, T. (2001). Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. Biol. Reprod. *64*, 44–50.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131–135.

Polejaeva, I.A., Chen, S.-H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., et al. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. Nature *407*, 86–90.

Prather, R.S., Sims, M.M., and First, N.L. (1990). Nuclear transplantation in the pig embryo: nuclear swelling. J. Exp. Zool. *255*, 355–358.

Prather, R.S., Kühholzer, B., Lai, L., and Park, K.-W. (2000). Changes in the structure of nuclei after transfer to oocytes. Cloning *2*, 117–122.

Rideout, W.M., 3rd, Hochedlinger, K., Kyba, M., Daley, G.Q., and Jaenisch, R. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell *109*, 17–27.

Ruan, D., Peng, J., Wang, X., Ouyang, Z., Zou, Q., Yang, Y., Chen, F., Ge, W., Wu, H., Liu, Z., et al. (2018). XIST derepression in active X chromosome hinders pig somatic cell nuclear transfer. Stem Cell Reports *10*, 494–508.

Rybouchkin, A., Kato, Y., and Tsunoda, Y. (2006). Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. Biol. Reprod. *74*, 1083–1089.

Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W., and Dean, W. (2003). Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr. Biol. *13*, 1116–1121.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., and Lai, F.A. (2002). PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development *129*, 3533–3544.

Shen, L., Inoue, A., He, J., Liu, Y., Lu, F., and Zhang, Y. (2014). Tet3 and DNA replication mediate demethylation of both the maternal and paternal genomes in mouse zygotes. Cell Stem Cell *15*, 459–471.

Shi, D., Lu, F., Wei, Y., Cui, K., Yang, S., Wei, J., and Liu, Q. (2007). Buffalos (Bubalus bubalis) cloned by nuclear transfer of somatic cells. Biol. Reprod. *77*, 285–291.

Shin, T., Kraemer, D., Pryor, J., Liu, L., Rugila, J., Howe, L., Buck, S., Murphy, K., Lyons, L., and Westhusin, M. (2002). A cat cloned by nuclear transplantation. Nature *415*, 859.

Shinagawa, T., Takagi, T., Tsukamoto, D., Tomaru, C., Huynh, L.M., Sivaraman, P., Kumarevel, T., Inoue, K., Nakato, R., Katou, Y., et al. (2014). Histone variants enriched in oocytes enhance reprogramming to induced pluripotent stem cells. Cell Stem Cell *14*, 217–227.

Soufi, A., Donahue, G., and Zaret, K.S. (2012). Facilitators and impediments of the pluripotency reprogramming factors' initial engagement with the genome. Cell *151*, 994–1004.

Sridharan, R., Gonzales-Cope, M., Chronis, C., Bonora, G., McKee, R., Huang, C., Patel, S., Lopez, D., Mishra, N., Pellegrini, M., et al. (2013). Proteomic and genomic approaches reveal critical functions of H3K9 methylation and heterochromatin protein-1γ in reprogramming to pluripotency. Nat. Cell Biol. *15*, 872–882.

Tachibana, M., Amato, P., Sparman, M., Gutierrez, N.M., Tippner-Hedges, R., Ma, H., Kang, E., Fulati, A., Lee, H.-S., Sritanaudomchai, H., et al. (2013). Human embryonic stem cells derived by somatic cell nuclear transfer. Cell *153*, 1228–1238.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

Tamashiro, K.L.K., Wakayama, T., Akutsu, H., Yamazaki, Y., Lachey, J.L., Wortman, M.D., Seeley, R.J., D'Alessio, D.A., Woods, S.C., Yanagimachi, R., and Sakai, R.R. (2002). Cloned mice have an obese phenotype not transmitted to their offspring. Nat. Med. *8*, 262–267.

Tan, W., Proudfoot, C., Lillico, S.G., and Whitelaw, C.B.A. (2016). Gene targeting, genome editing: from Dolly to. Transgenic Res. *25*, 273–287.

Teranishi, T., Tanaka, M., Kimoto, S., Ono, Y., Miyakoshi, K., Kono, T., and Yoshimura, Y. (2004). Rapid replacement of somatic linker histones with the oocyte-specific linker histone H1foo in nuclear transfer. Dev. Biol. *266*, 76–86.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145–1147.

Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R., and Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature *394*, 369–374.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A.C., Studer, L., and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science *292*, 740–743.

Wakayama, S., Ohta, H., Hikichi, T., Mizutani, E., Iwaki, T., Kanagawa, O., and Wakayama, T. (2008). Production of healthy cloned mice from bodies frozen at -20 degrees C for 16 years. Proc. Natl. Acad. Sci. USA *105*, 17318–17322.

Wakayama, S., Kohda, T., Obokata, H., Tokoro, M., Li, C., Terashita, Y., Mizutani, E., Nguyen, V.T., Kishigami, S., Ishino, F., and Wakayama, T. (2013). Successful serial recloning in the mouse over multiple generations. Cell Stem Cell *12*, 293–297.

Wang, F., Kou, Z., Zhang, Y., and Gao, S. (2007). Dynamic reprogramming of histone acetylation and methylation in the first cell cycle of cloned mouse embryos. Biol. Reprod. *77*, 1007–1016.

Wani, N.A., Wernery, U., Hassan, F.A.H., Wernery, R., and Skidmore, J.A. (2010). Production of the first cloned camel by somatic cell nuclear transfer. Biol. Reprod. *82*, 373–379.

Wani, N.A., Vettical, B.S., and Hong, S.B. (2017). First cloned Bactrian camel (Camelus bactrianus) calf produced by interspecies somatic cell nuclear transfer: A step towards preserving the critically endangered wild Bactrian camels. PLoS ONE *12*, e0177800.

Wen, D., Banaszynski, L.A., Liu, Y., Geng, F., Noh, K.-M., Xiang, J., Elemento, O., Rosenwaks, Z., Allis, C.D., and Rafii, S. (2014a). Histone variant H3.3 is an essential maternal factor for oocyte reprogramming. Proc. Natl. Acad. Sci. USA *111*, 7325–7330.

Wen, D., Banaszynski, L.A., Rosenwaks, Z., Allis, C.D., and Rafii, S. (2014b). H3.3 replacement facilitates epigenetic reprogramming of donor nuclei in somatic cell nuclear transfer embryos. Nucleus *5*, 369–375.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature *385*, 810–813.

Woods, G.L., White, K.L., Vanderwall, D.K., Li, G.-P., Aston, K.I., Bunch, T.D., Meerdo, L.N., and Pate, B.J. (2003). A mule cloned from fetal cells by nuclear transfer. Science *301*, 1063.

Wossidlo, M., Arand, J., Sebastiano, V., Lepikhov, K., Boiani, M., Reinhardt, R., Schöler, H., and Walter, J. (2010). Dynamic link of DNA demethylation, DNA strand breaks and repair in mouse zygotes. EMBO J. *29*, 1877–1888.

Wossidlo, M., Nakamura, T., Lepikhov, K., Marques, C.J., Zakhartchenko, V., Boiani, M., Arand, J., Nakano, T., Reik, W., and Walter, J. (2011). 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat. Commun. *2*, 241.

Wu, X., and Zhang, Y. (2017). TET-mediated active DNA demethylation: mechanism, function and beyond. Nat. Rev. Genet. *18*, 517–534.

Wu, G., Han, D., Gong, Y., Sebastiano, V., Gentile, L., Singhal, N., Adachi, K., Fischedick, G., Ortmeier, C., Sinn, M., et al. (2013). Establishment of totipotency does not depend on Oct4A. Nat. Cell Biol. *15*, 1089–1097.

Wu, J., Huang, B., Chen, H., Yin, Q., Liu, Y., Xiang, Y., Zhang, B., Liu, B., Wang, Q., Xia, W., et al. (2016). The landscape of accessible chromatin in mammalian preimplantation embryos. Nature *534*, 652–657.

Yamada, M., Johannesson, B., Sagi, I., Burnett, L.C., Kort, D.H., Prosser, R.W., Paull, D., Nestor, M.W., Freeby, M., Greenberg, E., et al. (2014). Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells. Nature *510*, 533–536.

Yamanaka, S., and Blau, H.M. (2010). Nuclear reprogramming to a pluripotent state by three approaches. Nature *465*, 704–712.

Yamauchi, Y., Ward, M.A., and Ward, W.S. (2009). Asynchronous DNA replication and origin licensing in the mouse one-cell embryo. J. Cell. Biochem. *107*, 214–223.

Yan, S., Tu, Z., Liu, Z., Fan, N., Yang, H., Yang, S., Yang, W., Zhao, Y., Ouyang, Z., Lai, C., et al. (2018). A Huntingtin knockin pig model recapitulates features of selective neurodegeneration in Huntington's disease. Cell *173*, 989–1002.

Yen, S.-T., Zhang, M., Deng, J.M., Usman, S.J., Smith, C.N., Parker-Thornburg, J., Swinton, P.G., Martin, J.F., and Behringer, R.R. (2014). Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev. Biol. *393*, 3–9.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917–1920.

Zhang, M., Wang, F., Kou, Z., Zhang, Y., and Gao, S. (2009). Defective chromatin structure in somatic cell cloned mouse embryos. J. Biol. Chem. *284*, 24981–24987.

Zhao, J., Hao, Y., Ross, J.W., Spate, L.D., Walters, E.M., Samuel, M.S., Rieke, A., Murphy, C.N., and Prather, R.S. (2010). Histone deacetylase inhibitors improve in vitro and in vivo developmental competence of somatic cell nuclear transfer porcine embryos. Cell. Reprogram. *12*, 75–83.

Zhao, M.-T., Chen, H., Liu, Q., Shao, N.-Y., Sayed, N., Wo, H.-T., Zhang, J.Z., Ong, S.-G., Liu, C., Kim, Y., et al. (2017). Molecular and functional resemblance of differentiated cells derived from isogenic human iPSCs and SCNT-derived ESCs. Proc. Natl. Acad. Sci. USA *114*, E11111–E11120.

Zheng, H., Huang, B., Zhang, B., Xiang, Y., Du, Z., Xu, Q., Li, Y., Wang, Q., Ma, J., Peng, X., et al. (2016). Resetting epigenetic memory by reprogramming of histone modifications in mammals. Mol. Cell *63*, 1066–1079.

Zhou, Q., Renard, J.-P., Le Friec, G., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A., and Cozzi, J. (2003). Generation of fertile cloned rats by regulating oocyte activation. Science *302*, 1179.

Zhou, X., Xin, J., Fan, N., Zou, Q., Huang, J., Ouyang, Z., Zhao, Y., Zhao, B., Liu, Z., Lai, S., et al. (2015). Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer. Cell. Mol. Life Sci. *72*, 1175–1184.