KDM6A and KDM6B play contrasting roles in nuclear transfer embryos revealed by MERVL reporter system

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Abstract

Despite the success of animal cloning by somatic cell nuclear transfer (SCNT) in many species, the method is limited by its low efficiency. After zygotic genome activation (ZGA) during mouse development, a large number of endogenous retroviruses (ERVs) are expressed, including the murine endogenous retrovirus-L (MuERVL/MERVL). In this study, we generate a series of MERVL reporter mouse strains to detect the ZGA event in embryos. We show that the majority of SCNT embryos do not undergo ZGA, and H3K27me3 prevents SCNT reprogramming. Overexpression of the H3K27me3-specific demethylase KDM6A, but not of KDM6B, improves the efficiency of SCNT. Conversely, knockdown of KDM6B not only facilitates ZGA, but also impedes ectopic Xist expression in SCNT reprogramming. Furthermore, knockdown of KDM6B increases the rate of SCNT-derived embryonic stem cells from Duchenne muscular dystrophy embryos. These results not only provide insight into the mechanisms underlying failures of SCNT, but also may extend the applications of SCNT.

Keywords H3K27me3, KDM6A; KDM6B; MERVL; nuclear reprogramming

Introduction

The metaphase II (MII) oocyte cytoplasm can reprogram somatic cell nuclei to the totipotent or pluripotent state via a series of sequential epigenetic events, including histone modifications, X chromosome reactivation, and pluripotency gene reactivation [1–4]. Somatic cell nuclear transfer (SCNT) has obvious advantages over other similar biotechnology techniques by enabling the generation of a new individual with an identical genome to that of the donor cell [5,6]. However, SCNT-mediated reprogramming has a very low efficiency [7]. In particular, in mice, nearly half of SCNT embryos arrest at the preimplantation stage and only 1–2% of SCNT embryos develop to term [8]. The molecular mechanisms underlying SCNT reprogramming are still unknown. Nevertheless, the successful reprogramming of human somatic cells by SCNT and the derivation of nuclear transfer embryonic stem cells (ntES) suggest that this is a promising approach [9–12].

A major feature of SCNT reprogramming is the global shift in gene expression from the somatic to the embryonic state. Zygotic genome activation (ZGA) occurs at the 2-cell stage in mice and at the 4- to 8-cell stage in pigs, bovines, and humans [13]. When the zygotic genome is first transcribed, a large number of retrotransposons are expressed, including endogenous retroviruses (ERVs), long interspersed nuclear elements, and non-autonomous short interspersed nuclear elements [14,15]. MERVL repeats belong to type III ERVs and are specifically expressed at the 2-cell stage [16–21]. Hundreds of genes express chimeric transcripts with junctions to MERVL at the 5’ end, indicating that the long terminal repeats (LTRs) of MERVL serve as functional promoters [22,23]. In the present study, we generated transgenic mouse lines containing a red fluorescent protein tandem dimeric tomato (tdTomato) reporter under the control of MERVL-LTR (MERVL::tdTomato). We used this unique reporting system to monitor ZGA in SCNT-reconstructed embryos. Recent studies have indicated that ZGA in SCNT embryos is limited by histone H3 lysine 9 trimethylation (H3K9me3) barriers that preexist in the genome of donor cells [7,24]. Previous studies have also indicated that treatment with pharmacological histone deacetylase and DNA methyltransferase inhibitors improves SCNT efficiency [25,26]. However, SCNT efficiency is still not comparable to normal embryonic development, and it is likely that additional obstacles to SCNT reprogramming exist.

In this study, we demonstrated that ZGA failure is frequent in SCNT-generated embryos, and another prominent silencing marker, H3K27me3, is an obstacle for SCNT reprogramming. The overexpression of KDM6A, a H3K27me3-specific demethylase, facilitates ZGA-related gene expression in SCNT embryos. However, KDM6A-overexpressing SCNT embryos did not exhibit more efficient full-term development. On the contrary, KDM6B knockdown not only improved the blastocyst formation rate, but also increased the cloned embryo birth rate and ntES establishment efficiency. For future clinical applications of KDM6B knockdown-assisted SCNT, we derived blastocysts from...
DMD-deficient (X chromosome-linked muscular dystrophy, mdx) somatic cells and efficiently generated si6B-mdx-nE. Thus, we established a highly efficient reprogramming method to improve SCNT for reproductive and therapeutic cloning.

Results

Most SCNT embryos exhibited ZGA and developmental failure

For the sensitive and convenient detection of ZGA events, we generated transgenic mouse lines containing a MERVL::tdTomato reporter (Fig 1A and Appendix Fig S1A). The cumulus cells from MERVL::tdTomato transgenic mice were used as nuclear donors for SCNT. As controls, intracytoplasmic sperm injection (ICSI) embryos were produced using the littersmates of transgenic mice (Fig 1A and Appendix Fig S1B). As expected, the MERVL::tdTomato reporter was expressed at the late 2-cell stage (Fig 1B and C, Appendix Fig S1C and D, Movie EV1). We found that only 12% of SCNT embryos exhibited reactivation somatic MERVL::tdTomato at the 2-cell stage, while 92% of ICSI embryos exhibited reactivation (Fig 1D and E). MERVL encodes a canonical retroviral Gag protein [19]. We next verified the accuracy of the MERVL::tdTomato reporter by immunofluorescence (IF) and real-time

Figure 1.
quantitative PCR (qPCR), and these results are in accordance with the fluorescence images (Fig 1B and F, Appendix Fig S1E). To further confirm that the MERVL::tdTomato reporter can capture ZGA events, embryos were divided into tdTomato⁻ and tdTomato⁺ groups according to MERVL::tdTomato expression. The qPCR results showed that the expression levels of ZGA-related genes in tdTomato⁺ were significantly higher than those in the tdTomato⁻ group (Fig 1G and Appendix Fig S1F). After in vitro culture, for both ICSI and SCNT embryos, most tdTomato⁻ embryos developed to the blastocyst stage (97 and 89%, respectively). Surprisingly, we found that 18% SCNT-tdTomato⁺ embryos developed to the blastocyst stage, but none of the ICSI-tdTomato⁻ embryos reached the blastocyst stage, and most of them were blocked at the 2-cell stage (Fig 1H and 1, Appendix Table S1). Notably, previous studies have shown that ZGA is essential for mouse embryonic development, as embryos will arrest at the 2-cell stage if ZGA is blocked [27]. Thus, MERVL::tdTomato could be used to monitor ZGA events in real time. Compared with ICSI embryos, a number of SCNT embryos arrested at various developmental stages (not limited to the 2-cell stage). Moreover, SCNT embryos are usually incapable of repressing some somatic genes inherited from donor cells [28,29]. The expression of donor cell-specific genes in SCNT embryos could also lead to the development of a few SCNT-tdTomato⁻ blastocysts.

Effect of ZGA on SCNT embryonic development and ntES derivation

Having established a correlation between MERVL::tdTomato and blastocyst formation, we next evaluated whether SCNT-tdTomato⁻ could develop to term. Because the IF assay requires fixation and/or denaturation, thereby preventing development, we used a live-cell imaging system to assess the full-term developmental ability of SCNT embryos (Fig 2A and B, Movie EV2). Based on tdTomato fluorescence, the SCNT blastocysts were grouped into SCNT-tdTomato⁻ and SCNT-tdTomato⁺. We detected fewer nuclei in SCNT-tdTomato⁻ blastocysts than in tdTomato⁺ blastocysts (Fig 2C and D). To gain further insights into blastocyst lineage segregation, the blastocysts derived from SCNT were subjected to IF staining of Nanog and Cdx2 (Fig 1E). In the SCNT-tdTomato⁺ blastocysts, Nanog and Cdx2 were exclusively localized to the nuclei of the ICM and TE, as previously reported in normal embryos [30]. By contrast, the Nanog and Cdx2 were localized to the cytoplasm of the ICM and TE in the SCNT-derived tdTomato⁻ blastocysts. Thus, the Nanog and Cdx2 in SCNT-tdTomato⁺ embryos are mislocalization in a spatial manner. To further evaluate the developmental ability in vivo, the SCNT-tdTomato⁺ and SCNT-tdTomato⁻ blastocysts with normal morphologies were used for embryo transfer. At embryonic day E6.5, no difference was observed between the SCNT-tdTomato⁺ and SCNT-tdTomato⁻ blastocysts in the implantation rate, as determined by the embryo retrieval rate (Appendix Fig S2A and B). However, 84% (16/19) of fetuses retrieved from tdTomato⁻ blastocysts had the typical morphology, with distinct embryonic and extraembryonic compartments, while none of the tdTomato⁺ fetuses were normal (0/29, Fig 2F). Furthermore, with respect to the ntES derivation efficiency, SCNT-tdTomato⁺ blastocysts had higher rates of attachment and ES establishment than those of SCNT-tdTomato⁻ blastocysts (Fig 2G and H, Appendix Fig S2C and D). We next examined the expression of somatic genes that have been reported to inhibit SCNT reprogramming (Fig 2I and Appendix Fig S2E) [28,31–34]. We found significant suppression of the expression of somatic cell genes at the 2-cell stage in the SCNT-tdTomato⁻ group, suggesting that these embryos have a greater degree of reprogramming than that of SCNT-tdTomato⁺ embryos.

Aberrant reprogramming of H3K27me3 in the SCNT embryos at the 2-cell stage

In the SCNT mouse embryos, abnormalities in gene expression have been observed at the 2-cell stage, which corresponds to ZGA events. Furthermore, the epigenetic reprogramming of the somatic cell genome has been suggested as a key event in SCNT. We next...
determined the difference in epigenetic modifications between the SCNT and ICSI embryos at the 2-cell stage. Because histones H3K9me3 and H3K27me3 are correlated with gene silencing, histone H3K4me3 leads to the initiation of gene transcription. The H3K4me3, H3K9me3, and H3K27me3 modifications of both ICSI and SCNT embryos were investigated (Fig 3A and B, Appendix Fig S3A–C). The IF assay indicated that H3K4me3 and H3K9me3 did not markedly differ between ICSI and SCNT-tdToma+/− tdTomato− 2-cell embryos. In contrast, we found that H3K27me3 was specifically enriched in SCNT-tdToma− embryos, but moderate stain in SCNT-tdToma+ and ICSI embryos. Contrary to H3K27me3 modification, the H3K27me2 did not differ between ICSI- and SCNT-derived...
embryos (Appendix Fig S3D). To further consolidate the IF results, we compared the H3K27me3 between different type embryos by Western blot (WB). In the first set of experiments, SCNT-tdTomato, SCNT-tdTomato−, and ICS embryos were collected at 2-cell stage, and the numbers of the embryos harvested for WB are 500, respectively. Furthermore, the polar bodies were also removed to avoid histone contamination. As IF results, in the short-exposure condition, H3K27me3 modification was effectively detected in the SCNT-tdTomato− and cumulus cell (Fig 3C and Appendix Fig S3E). Therefore, the H3K27me3 modification in the SCNT-tdTomato− and ICSI 2-cell embryos is present at very low levels, but it can be detected. In addition, irrespective of whether female cumulus cells, male Sertoli cells, or mouse embryonic fibroblasts (MEFs) were used, the difference in H3K27me3 staining between the two types of SCNT embryos was also observed (Appendix Fig S3F). It is well known that fertilization unites two highly specialized haploid genomes with markedly different chromatin modifications within a single cell to form a diploid zygote. In the short period of the 1-cell stage, the two haploid genomes undergo dramatic asymmetric chromatin remodeling to reestablish transcriptional activation of zygotic gene expression [35]. We further investigated whether the difference in H3K27me3 modification also exists at the 1-cell zygote stage. We found that in 1-cell ICSI embryos, H3K27me3 signals were prominent in the paternal pronuclei, but not in the paternal pronuclei (Fig 3D and Appendix Fig S3G), which are consistent with previous study [36]. Unlike the asymmetric modifications in ICSI embryos, we detected strong H3K27me3 signals in all pseudo-pronuclei of SCNT embryos (Fig 3E). Furthermore, we also found that H3K27me3 levels were much higher in SCNT-tdTomato− embryos than in SCNT-tdTomato+ embryos or ICSI embryos at the morula stage (Fig 3F). According to the above results, we speculated that H3K27me3 is a natural key barrier preventing somatic cell nuclear reprogramming. We further examined the presence of H3K27me3 in bovine embryos, in which ZGA takes place during the 8-cell stage. As expected, the bovine intraspecific SCNT embryos also had much higher levels of H3K27me3 in the nuclei compared to those in the in vitro fertilization embryos at the 8-cell stage (Fig 3G and H). These results indicated that the H3K27me3 epigenetic barrier for SCNT-mediated reprogramming is shared across taxa.

**Overexpression of KDM6A, but not KDM6B, improves preimplantation development in SCNT embryos**

Having established that H3K27me3 is a barrier to somatic cell reprogramming, we next evaluated whether the removal of H3K27me3 could facilitate ZGA in SCNT embryos. We compared the expression levels of KDM6A and KDM6B, which are H3K27me3-specific demethylases, between ICSI embryos and SCNT embryos by RT–qPCR (Fig 4A and B). Neither KDM6A nor KDM6B was adequately activated in SCNT embryos. In addition, the expression levels of other KDMs in SCNT embryos were also lower than those in ICSI embryo (Appendix Fig S4A). To correct the H3K27me3 modification, the in vitro transcription vectors KDM6A and KDM6B tagged C-terminally with the hemagglutinin epitope (KDM6A-HA and KDM6B-HA) were constructed (Fig 4C). The exogenous HA ectopic expression vectors allowed us to track the KDM6A and KDM6B proteins in early embryos, without the use of specific antibodies. Strikingly, IF staining showed that ectopic expression of KDM6A or KDM6B markedly reduced the levels of H3K27me3 (Fig 4D and Appendix Fig S4D). Furthermore, other lysine methylation marks, including H3K9me3 and H3K4me3, were not affected (Appendix Fig S4B). We first injected KDM6A mRNA into enucleated MIL oocytes (Fig 4E) and found that the overexpression of KDM6A mRNA significantly increased the SCNT blastocyst formation rate (Fig 4F). Contrarily, the efficiency of SCNT was greatly decreased.
Figure 3.
Figure 4. Overexpression of KDM6A only improves the blastocyst formation rate of SCNT embryos, but not full-term development.

A, B RT–qPCR analysis of KDM6A (A) and KDM6B (B) mRNA levels in SCNT 2-cell embryo. Data shown are mean expression values relative to Gapdh. The value in ICSI control was set as 1. Error bars, SEM, n ≥ 3. **P < 0.01 by Student’s t-test.

c The sketch of KDM6A and KDM6B in vitro transcription vector (right), and the integrity of in vitro transcribed mRNA, was confirmed by electrophoresis with formaldehyde gels (left). M, marker; T7, in vitro transcription promoter; HA, hemagglutinin epitope tag.

D Immunostaining of SCNT embryo for H3K27me3 and HA epitope tag after injection of different mRNA as indicated. Representative images from ≥ 187 embryos analyzed in four independent micromanipulations for each condition are shown. Scale bar, 20 μm.

E Schematic illustration of mRNA injection into oocytes and SCNT.

F Representative images of SCNT embryos at 115 h after injection of different mRNA as indicated. Scale bar, 20 μm.

G The bar chart showing the efficiency of blastocyst formation. Injection of KDM6A mRNA improved the preimplantation development rate of SCNT embryos. Error bars, SEM, n ≥ 3. **P < 0.01, ***P < 0.001 by two-tailed Student’s t-test. n.s., not significant.

H Phenotypic analysis of E19.5 SCNT mouse embryos injection with 20 ng/μl or 50 ng/μl KDM6A mRNA (left). Representative images of the KDM6A-injected embryos at E19.5 (right). The injected SCNT embryos were only obtained degenerated embryos. AI, allantois; SM, somite. Scale bar, 50 μm.

I The retrieved rate of embryo assessed at E8.5. Five independent experiment replications were performed. The numbers at the bottom of the bars indicated the total number of transferred embryos.

J Schematic diagram depicts the position of highly conserved TPR and JmjC domain. The KDM6A is broadly expressed proteins characterized by N-terminal TPRs and C-terminal JmjC domain. In KDM6B, the only clearly identifiable domain is the C-terminal JmjC domain. White asterisk indicates the mutation point.

K Preimplantation development rates in the KDM6A-HA, KDM6B-HA, or KDM6A-cM/-nM/-ncM-HA mRNA-injected and non-injected control SCNT groups. The efficiency was calculated based on the number of cleavage embryo. Error bars, SD, the total numbers of cleavage embryos in each condition (KDM6A-HA, KDM6B-HA, KDM6A-cM/-nM/-ncM-HA, and control) from three independent experiments were 275, 199, 290, 221, 244, and 286, respectively.
reduced by injecting KDM6B mRNA into enucleated MI oocytes prior to SCNT (even at low doses, Fig 4F and Appendix Fig S4C). We also noticed that a KDM6A concentration of 20 or 50 ng/μl substantially improved the SCNT blastocyst development rate, while concentrations of KDM6A mRNA over 200 ng/μl were detrimental to embryonic development (Fig 4G and Appendix Fig S4C). To further investigate whether KDM6A overexpression improved the efficiency of full-term development, we transferred the SCNT embryos derived above into surrogates. For most transfers, pregnancies were established and maintained until day E8.5 and the fetuses were retrieved on that day (Fig 4H). We found that the embryo retrieval rate for the group injected with KDM6a mRNA was substantially greater than that of directly transferred SCNT embryos (Fig 4I). Unexpectedly, only implantedation sites and degenerated embryos were observed on day E19.5, suggesting that KDM6A-treated SCNT fetuses failed and were reabsorbed at E8.5–19.5 (Fig 4H). These results indicate that the overexpression of KDM6A (but not KDM6B) improved implantation development, but could not improve the rate of full-term development in SCNT fetuses.

Both KDM6A and KDM6B are jumonji (JmjC) domain-containing proteins and catalyze the removal of trimethylation from histone H3K27 by using a hydroxylation reaction with iron (Fe2+) and α-ketoglutarate (α-KG) as cofactors [37,38]. The jumonji gene was named for a mutation in mice that causes abnormal cruciform neural grooves (in Japanese, jumonji means cruciform). As shown in Fig 4J, KDM6B shows high homology and structural relationship to KDM6A, especially in the JmjC domain, but lacks the tricatricopeptide (TPR) domain, which is assumed to mediate protein–protein interactions [39,40]. In order to further compare the differences between KDM6A and KDM6B in SCNT reprogramming, we synthetized KDM6A-HA expression vectors with different loci mutation and injected different type mRNA into SCNT embryos (Appendix Fig S4E). When KDM6A-cM-HA (JmjC domain mutant) or KDM6A-cm-M-HA (TPR and JmjC double mutant) was ectopically expressed in SCNT embryos, no reduction in H3K27me3 methylation levels was observed (Fig 4D and Appendix Fig S4D), which demonstrates that the demethylation activity is dependent on JmjC domain. Furthermore, the blastocyst formation rate of SCNT embryos was greatly reduced when KDM6A-nM-HA was injected, which was similar to that of KDM6B-injected SCNT embryos (Fig 4F and K, Appendix Table S2). Compared with the control group, the efficiency of SCNT was no different by injecting KDM6A-CM-HA or KDM6A-cm-M-HA into SCNT embryos. These results suggest that the TPR and JmjC domain were required for KDM6A rescue the poor developmental phenotype of SCNT embryos and indirectly indicate that TPR domain may mediate protein–protein interactions for moderate KDM6A activity in the SCNT reprogramming.

KDM6B knockdown increased the expression of KDM6A and blastocyst formation rate

As described above, the ectopic overexpression of KDM6A mRNA at low concentrations improved the SCNT efficiency. We have previously shown that mouse parthenogenetic embryos in which KDM6B is knocked down exhibited a moderate increase in KDM6A expression [41]. We speculated that KDM6B knockdown could facilitate ZGA and improve SCNT efficiency. To verify this hypothesis, we designed and constructed short interfering RNA (siRNA) specifically targeting KDM6A and KDM6B (Fig 5A and Appendix Fig S5A). A siRNA without any specificity to KDM6A/B or other genes was constructed as an siRNA-control. As expected, the qPCR results demonstrated that the decrease in KDM6A or KDM6B expression was accompanied by an increase in KDM6B or KDM6A expression, respectively (Fig 5B). Furthermore, a marked decrease in H3K27me3 levels was observed when injected with either KDM6A or KDM6B siRNA (Fig 5C and Appendix Fig S5B). The WB results also confirmed this phenomenon at another protein levels (Fig 5D and Appendix Fig S5C). These findings suggest that KDM6A and KDM6B are functionally redundant and compensate for each other in SCNT embryos. At the beginning of the knockdown assay, we noticed that the pluripotency genes Oct4, Sox2, and Nanog acquire the H3K27me3 mark as they get repressed during ESC differentiation [42,43]. In addition, KDM6B also regulates the Hox gene expression,

Figure 5. KDM6B knockdown greatly improved the preimplantation development rate of SCNT embryos. A. RT-qPCR analysis of KDM6A/B in SCNT embryos injected with siRNA-6A or siRNA-6B. The SCNT embryos were subject to injection of 10 μM or 20 μM siRNA as indicated. Data are mean expression relative to Gapdh with siRNA-control normalized to 1. Error bars, SEM, n = 3. **P < 0.01, ***P < 0.001 according to two-tailed Student’s t-test. n.s., not significant. B. The bar chart shows the KDM6A/B transcript levels in SCNT embryo with 20 μM siRNA-6A/B. All data are mean expression relative to Gapdh with control siRNA-injected SCNT embryos normalized to 1. Error bars, SEM, n = 3. **P < 0.01 by Student’s t-test. C. Immunofluorescence staining results showing H3K27me3 of 2-stage SCNT embryos. Embryo was injected with siRNA as indicated. The H3K27me3 levels between control and double injected with siRNA-6A-6B cannot observe any difference, but a marked decrease was observed when injected with either siRNA-6A or siRNA-6B. Scale bar, 20 μm. D. Western blot showing the expression of KDM6A and KDM6B in SCNT embryos after injection of different siRNA as indicated. Protein lysates from 1,500 embryos were loaded in each lane. The results of one representative of two independent experiments are presented. The Ponceau S staining demonstrates equivalent loading of each lane is shown in Appendix Fig S5C. E. Schematic illustration of siRNA injection into oocytes and SCNT. F. Representative images of SCNT embryos at 115 h after injection of different siRNA as indicated. These images are representative examples of the quantification shown in Fig S5C. n ≥ 3. Scale bar, 50 μm. G. Injection of siRNA-6B improved the preimplantation development rate of SCNT embryos. Both cumulus cells, Sertoli cells, and C57 MEF cells were used as donor cells. The bovine intraspecies SCNT embryos derived from bovine ear fibroblast cells. Shown is the percentage of embryos that reached the indicated stages: d and ♀ indicated the male and female, respectively. Error bars, SD, n ≥ 3. H. RT-qPCR analysis for select ZGA genes in ICSI and SCNT embryos. The SCNT embryo was injected with siRNA as indicated. Results were normalized based on the geometric mean of the expression levels of two reference genes (Ywhaz and Gapdh). Error bars, SEM, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001 according to two-tailed Student’s t-test. n.s., not significant.
which is essential for regulating cell differentiation and the formation of body structures during early embryonic development. In order to avoid injuries caused by knockdown KDM6B, we next tested a serial dilution of siRNA-6B to determine the knockdown efficiency (Fig 5A). Briefly, the optimal injection concentration of siRNA-6B in our experiment was 10 μM. We then injected siRNA-6B into recipient MII oocytes before SCNT (Fig 5E). Notably, the injection of KDM6B siRNA before SCNT increased the blastocyst rate to 70.8%, which did not differ significantly from the rate observed for KDM6A mRNA injection alone (70.3%, Fig 5F and G, Appendix Table S3). Furthermore, using Sertoli or MEF cells, the injection of siRNA-6B before SCNT also increased the blastocyst formation rate. When we performed the same trials for bovine intraspecific SCNT, siRNA-6B injection also improved the developmental efficiency (Fig 5G, Appendix Fig S5D, and Appendix Table S3). When KDM6A was knocked down, the rate of SCNT blastocyst formation was significantly decreased (Fig 5F and G, Appendix Table S3). To further examine whether the positive effect of siRNA-6B on SCNT embryonic development is dependent on the observed increase KDM6A expression, we next double

Figure 5.
Figure 6. KDM6B knockdown improved the SCNT embryo birth rate and DMD-ntES derivation.
A Experimental scheme for generating dual-reporter mice and SCNT.
B Representative fluorescence images of siRNA-control or siRNA-6B-injected SCNT embryos. Scale bar, 50 μm.
C Quantification of embryos that expression tdTomato after injection with siRNA-control or siRNA-6B. Numbers of observed embryos are indicated. n ≥ 3, Error bars, SEM, ***P < 0.001 according to two-tailed Student’s t-test.
D Representative images of green fluorescence in Oct4::EGFP-reconstructed blastocysts derived from injected siRNA-control or siRNA-6B SCNT embryos. Representative images from ≥ 25 embryos analyzed in three independent micromanipulations are shown. Green fluorescence indicates that the Oct4::EGFP transgene has been expressed. Scale bar, 50 μm.
E Oct4::EGFP was upregulated significantly in ICSI embryos compared with SCNT embryos at blastocyst stages, as determined by RT–qPCR. Data shown are mean expression values relative to Gapdh. The value in ICSI embryos was set as 1. Error bars, SEM, n ≥ 3. *P < 0.05, ***,P < 0.001 by two-tailed Student’s t-test.
F Representative image of cloned mice derived by siRNA-6B-injected SCNT embryos.
G Bar graph showing the efficiency of attachment to the feeder cells (left) and si6B-mdx-ntES derivation (right). The mdx sick mice tail-tip fibroblasts as nuclear donors. N, total number of embryos analyzed for each condition. Results are from four replicate experiments.
H Immunostaining images of si6B-mdx-ntES expressed pluripotency markers. Scale bar, 50 μm.
I The si6B-mdx-ntES possessed multiple differentiation potential, as shown in embryoid body. Scale bar, 100 μm.
J An image of a chimeric mouse derived from si6B-mdx-ntES.
injected siRNA-6A-6B into SCNT embryo. We observed significantly lower developmental potential for siRNA-6A-6B-injected SCNT embryos, with the majority arresting at the 2-cell stage and only a few reaching the blastocyst stage (Fig SF and G, Appendix Table S3). We also compared the ZGA-related gene expression between different type siRNA-injected SCNT embryos via RT–qPCR. Similarly, the qPCR results showed that the expression of ZGA-related genes is decreased in SCNT embryo with siRNA-6A or siRNA-6A-6B-injected compared with the control (Fig 5H). These results suggest that the overexpression of KDM6A or knockdown of KDM6B can improve the efficiency of SCNT reprogramming.

KDM6B knockdown increased the SCNT embryo birth rate as well as the efficiency of DMD-specific ntES derivation

Reactivation of pluripotency genes is a major event for the successful reprogramming of somatic cells to the blastocyst stage. In particular, the transcription factor Oct3/4 (Pou5f1) is expressed in the ICM of the blastocyst stage, which is an effective indicator of embryonic quality. To determine the extent to which the injection of siRNA-6B could overcome ZGA defects in the SCNT embryos, we intercrossed MERVL::tdTomato with Oct4::EGFP transgenic mice (expressing the enhanced green fluorescence protein controlled by the Oct3/4 promoter) to produce MERVL::tdTomato/Oct4::EGFP dual-reporter mice (Fig 6A and Appendix Fig S6A). Similar to other somatic cells, the cumulus cells and sperm did not express tdTomato and EGFP (Appendix Fig S6B). As expected, 40.7% of the siRNA-6B-injected SCNT embryos exhibited tdTomato expression at the 2-cell stage, whereas only 3.5% of the siRNA-control group exhibited tdTomato fluorescence (Fig 6B and C). We also found weak, but substantial expression of Oct4::EGFP in the siRNA-6B-injected blastocysts (25/33, 75.6%), but not in the siRNA-control-injected embryos (0/25, Fig 6D). We further compared the Oct4::EGFP mRNA levels between ICSI- and siRNA-injected SCNT blastocysts. The RT–qPCR results also showed that Oct4::EGFP mRNA expression was higher in SCNT blastocysts injected with siRNA-6B than in controls (Fig 6E). Moreover, the siRNA-6B-injected embryos contained a greater total cell number than that of the control blastocysts (118 in siRNA-6B injected versus 67 in control, Appendix Fig S6C). We next investigate whether this positive effect could be contributing to cloned mice birth. For this purpose, siRNA-6B-injected SCNT embryos were transferred at the 2-cell stage into pseudopregnant females. Caesarian section at E19.5 revealed that the 6.0% (16/265, six twins) of transferred siRNA-6B-injected SCNT embryos developed to term, while none of the 120 transferred control embryos developed to term (Fig 6F). After E15.5. The retrieved the siRNA-6B-injected SCNT conceptus at E15.5. The SCNT efficiency, we next evaluated corresponding changes at the molecular level. We first used a qPCR assay to detect the 2-cell embryo-specific transcripts (Appendix Fig S7A). We found that Zscan4, Gm6763, Eif1a, and MERVL levels were higher in NT blastocysts with siRNA-6B injection than in the control. Interestingly, MERVL was strongly upregulated, but other repeat elements, such as LINE-1 and IAP (intracisternal A particles), were unaffected. These results suggested that the knockdown of KDM6B improved the developmental potential of SCNT embryos by increasing ZGA-related transcripts. To further verify this result, we used single-cell RNA sequencing (scRNA-seq) to evaluate the transcriptome in siRNA-6B-injected SCNT embryos. We also noticed that injection of siRNA-6B does not make every SCNT embryos active MERVL::tdTomato expression and reach the blastocyst. We combined live-cell imaging, blastomeric biopsy, and scRNA-seq to accurately characterize the molecular characteristics (Fig 7A and Movie EV3). We first confirmed that the removal of a single blastomere at the 2-cell stage did not influence the developmental capacity (Appendix Fig S7B and C, Movie EV4). Using this system, we removed one blastomere from siRNA-6B-injected MERVL::tdTomato-SCNT 2-cell stage embryos for scRNA-seq (referred to as si6B-NT); the remaining blastomeres were monitored by live-cell imaging system for blastocyst formation and tdTomato expression. We also generated scRNA-seq profiles for normal SCNT 2-cell embryos (NT-2), and the publicly available fertilized 2-cell embryo RNA-seq dataset was harvested as WT-2 [44]. Finally, we obtained > 65 million 90-bp reads per sample, with at least 72.8% of the reads aligning to the mouse genome. Two biological replicates for each sample demonstrated high reproducibility (Appendix Fig S7D). Compared to NT-2 embryos, 1,175 genes were highly expressed in siRNA-6B-injected embryos (FC > 5, FPKM > 5, Fig 7C). We next focused on the expression of 7,773 representative ZGA-related genes [44], because we supposed that knockdown of KDM6B to promote ZGA in SCNT embryos. A pairwise comparison of the transcriptomes of NT-2, si6B-NT, and WT-2 embryos identified 1,813 differentially expressed ZGA-related genes (FC > 5, FPKM > 5), and these DEGs (differentially expressed gene) could be classified into two groups (designated Group1 and Group2) by an unsupervised hierarchical cluster analysis (Fig 7B, Datasets EV1 and EV2). Group2 genes were...
significantly more highly expressed in SCNT embryos injected with siRNA-6B than in the NT-2 embryo. To further investigate whether these DEGs cause developmental issues in SCNT embryos, we used GO (Gene Ontology) and KEGG (Kyoto encyclopedia of genes and genomes) to analyze enrichment for biological processes and pathways. Group2 genes were enriched for cell cycle, methyltransferase activity, ribosome, and mitochondrion categories. These results suggest that the dysregulation of these developmentally important genes might be a cause of SCNT failure.

It is well known that Zscan4 plays an important role in lengthening telomeres by recombination-based mechanisms and in maintaining genomic stability during embryonic development; the depletion of Zscan4 causes a severe delay in preimplantation development [45]. Therefore, we further examined the DEGs between si6B-NT and NT-2 (Fig 7D and Appendix Fig S7E). We found that the knockdown of KDM6B expression increased the expression of Zscan4 and Eifla-like genes, suggesting that the knockdown of KDM6B increases the efficiency of SCNT reprogramming. Furthermore, we also identified 319 genes that were not activated in 2-cell SCNT embryos and were derepressed by KDM6B knockdown (FC > 5, FPKM > 5, Fig 7E and Dataset EV3). KEGG and GO analyses indicated these genes are enriched for methyltransferase activity, metabolic pathways, and RNA processes (Fig 7E). Taken together, these results indicate that KDM6B knockdown can facilitate the activation of the embryonic genome in SCNT reprogramming.

**Knockdown KDM6B not only facilitates ZGA in SCNT, but also impedes ectopic xist expression**

The results above showed that 2-cell stage aberrant epigenetic reprogramming can be rescued through overexpression of KDM6A or knockdown of KDM6B. Although aberrant SCNT-ZGA is believed to be the main reason for low cloning efficiency, another error identified in SCNT embryo is ectopic expression of the Xist (X-inactive specific transcript), which initiates X chromosome inactivation. Recently, H3K27me3 was identified as an imprinting mark for Xist [46], which prompted us to ask whether it is also responsible for fine-tuning KDM6A/B improved development of SCNT embryo. As exact adjustment by siRNA is technically difficult, we next primarily focused on male SCNT embryos with only a single X chromosome and never expressed at 4-cell stage. According to a previous report [47], sex screening of early mouse embryos was determined by PCR using a single blastomere biopsy at the 4-cell stage (Fig 7F). To determine whether loss of H3K27me3 modification can induce Xist derepression in embryos, we first injected KDM6A and KDM6B mRNA into ICSI-derived embryos. As Inoue A et al report [46], RNA fluorescent in situ hybridization (FISH) analysis confirmed that KDM6A/B mRNA injection induces ectopic expression of Xist and only KDM6A in a concentration-dependent manner (Appendix Fig S7F). To evaluate the effect of fine-tuning KDM6A/B on Xist expression in SCNT embryos, we harvested Sertoli cell-derived SCNT embryos for Xist RNA detection via FISH assay. As shown in Fig 7G, the majority of SCNT-derived blastomere showed Xist RNA signal, and ICSI-derived embryos showed no Xist signal. As expected, KDM6B knockdown by siRNA-6B led to Xist downregulation and loss of Xist signal within the nucleus of SCNT embryos. In contrast, most of the siRNA-6A-injected SCNT embryos still showed one strong Xist signal in blastomeres. Previous studies demonstrated that ectopic expression of Xist will lead to large-scale downregulation of X chromosome-linked genes in the SCNT embryos [3,48]. The effect of siRNA-6B on ectopic Xist expression was further examined by the expression levels of Xist and X-linked genes (Tsix, Rnf12, Pkg1, Fmr1nb, Atrx, Uba1, Mecp2, and Plac1) via single embryo RT–qPCR (Fig 7H). Consistent with the FISH results, significant downregulation of Xist was observed in SCNT embryos that had been injected with siRNA-6B. In contrast, Xist was significantly upregulated in KDM6A mRNA-injected SCNT embryos, and the X-linked genes were also upregulated in siRNA-6B-injected embryos.

Related studies have demonstrated that the ectopic expression of Xist in SCNT-derived embryos could be corrected by siRNA-Xist, leading to more than a 10-fold increase in the birth rate of male clones [3,48,49]. To examine whether the combination of siRNA-Xist and siRNA-6B could further improve SCNT embryonic full-term development, we then performed embryo transfer experiments to assess the full-term developmental ability of siRNA-Xist-6B-coinjected SCNT embryos. Similar to previous report [3], injection with siRNA-Xist alone improved the birth rate from 1.3% (1/77) to 11.7% (12/103) (Fig 7I and Appendix Table S4). Importantly, siRNA-Xist-6B coinjection further increased the SCNT birth rate to 21.1% (16/76). This result indicates that siRNA-6B and siRNA-Xist exert a synergistic effect on the SCNT reprogramming. Thus,
knockdown of KDM6B not only facilitates the cloned embryos ZGA, but also impedes ectopic Xist expression in SCNT reprogramming.

**Discussion**

Preimplantation embryogenesis encompasses several critical events, especially the activation of ZGA-related genes. In 2014, Matoba and colleagues identified reprogramming resistant regions (RRRs), which are enriched for the histone modification H3K9me3 [7]. Liu and colleagues proved that excessive H3K9me3 modifications would lead to ZGA failure [24]. Therefore, ZGA is indispensable for somatic cell reprogramming [26]. It is noteworthy that a lack of relevant animal models has hampered precise spatiotemporal detection and critical evaluations of the efficacy of ZGA in SCNT reprogramming. Immunocytochemistry requires sample fixation and is insufficient for real-time monitoring of ZGA events. To the best of our knowledge, the present study generated the first MERVL::tdTomato transgenic

![Image](image_url)
mice. To detect the efficiency of SCNT reprogramming, we crossed the MERVL::tdTomato mouse strain with the Oct4::EGFP transgenic mouse strain (also known as OG2) [50]. The compound homozygous MERVL::tdTomato/Oct4::EGFP double transgenic mice provide the opportunity for serial real-time monitoring of ZGA and reprogramming efficiency.

The MERVL::tdTomato/Oct4::EGFP SCNT embryos can be divided into three groups: MERVL−/Oct4−, MERVL+/Oct4−, and MERVL+/Oct4+. Only a small proportion of reconstructed embryos were labeled by both reporters (MERVL+/Oct4−), and we never found MERVL−/Oct4− SCNT embryo. We only detect moderate H3K27me3 modifications in the MERVL− SCNT- and ICSI embryos at the 2-cell stage, but we clearly detected strong H3K27me3 staining in the MERVL− SCNT embryos. Although the H3K27me3 defect in SCNT embryos has been observed, previous studies have reported the loss of H3K27me3 in ICM cells of most SCNT embryos [51]. This difference might be explained by a difference in the time of embryo collection between studies. Our scRNA-seq transcriptome also demonstrated that ZGA explained by a difference in the time of embryo collection between SCNT embryos. Although the H3K27me3 defect in SCNT embryos has an opportunity for serial real-time monitoring of ZGA and reprogramming efficiency. Thus, knockdown of KDM6B not only facilitates the cloned embryos ZGA, but also impedes ectopic Xist expression [46,49]. To further determine the role of KDM6A in the XCI of SCNT, we injected KDM6A mRNA (1,000 ng/μl) into SCNT embryos and found that the developmental efficiency of SCNT embryos was reduced, while many X-linked genes were consistently repressed. In contrast, knockdown of KDM6B could increase the SCNT embryo birth rate as well as the efficiency of DMD-specific ntES derivation. Thus, knockdown of KDM6B not only facilitates the cloned embryos ZGA, but also impedes ectopic Xist expression in SCNT reprogramming.

Previous studies have shown that the knockdown of KDM6B or overexpression of KDM6A in MEFs results in significantly more iPSC colonies compared with wild-type cells [55,56]. Interestingly, the knockdown of KDM6B in SCNT embryos leads to a moderate increase in the expression of KDM6A, consistent with our previous findings in mouse parthenogenetic embryos [41]. While our paper was under preparation, another study reported the identification of H3K27me3-dependent imprinting genes (which include Gab1, Sfnb12, and Slc38a4), and previous studies have shown that these genes exhibit a loss of imprinting in SCNT embryos [57,58]. This provides an explanation for why the knockdown of the H3K27me3 demethylase KDM6B promotes SCNT efficiency. In addition to the silencing of the histone modification, a recent study found that H3K4me3, an activating modification, is also obstacle to reprogramming [29]. The findings of the present study in combination with previous results in the field indicate that there are many obstacles in SCNT reprogramming. Further studies should focus on identifying the core obstacle.

Materials and Methods

Ethics statement

All studies adhered to procedures consistent with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Inner Mongolia University.

Animals

C57BL/6N, DBA/2, and BDF1 (C57BL/6N × DBA/2) F1 strains of mice were purchased from Vital River Laboratories (China). Pseudopregnant CD1 or Kun-Ming (KM) mice were used as embryo recipients. In order to detect reprogramming by means of Oct4 promoter-driven EGFP, BDF1 mice were replaced with OG2 mice that carry an Oct4-EGFP transgene (JAX stock number 004654). All the MERLV::tdTomato transgenic mice are syngeneic and bred by the same positive founder (F0). All the embryos used in the experiment were produced by MERLV::tdTomato sperm and MII oocytes from the littermates of transgenic mice. The copy numbers of MERLV::tdTomato were detected by previously reported methods [59,60]. In brief, we detected approximately 200 copies of MERLV::tdTomato in reporter transgenic mice as determined by quantitative PCR. Furthermore, the MERLV::tdTomato transgene copy number was stable throughout the F20 generations.

Superovulation and in vivo fertilization

Superovulation was done as previously described [41]. Briefly, BDF1 female (6–8 weeks old) mice were superovulated by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG, Sansheng, China, 10 IU) and human chorionic gonadotropin (hCG, Sansheng, China, 10 IU) 48 h apart. Mice were sacrificed by cervical dislocation, and cumulus–oocyte complexes (COCs) were collected from oviducts 14 h post-hCG. For zygotes, superovulation of 7–8-week-old BDF1 females mated with males of the same strain. Successful mating was confirmed by the presence of vaginal plugs. The cumulus cells were dispersed by 0.3 mg/ml hyaluronidase in M2 medium (Millipore, USA).
**In vitro mRNA synthesis, siRNA construction, and microinjection in oocytes**

The coding region of KDM6A and KDM6B was amplified from mouse tail-tip genome. Forward and reverse primers contained T7 promoter and HA sequences, respectively. To prepare mRNAs for microinjection, pT7-Cas9 (OriGene, GE100014), KKDMD6A and KKDMD6B expression vectors were linearized and subjected to phenol–chloroform extraction and ethanol precipitation. The mRNA synthesized with the nMESSAGE-mMACHINE T7 Ultra Kit (Thermo, USA) according to the manufacturer’s instructions. Two different siRNA species targeting KDM6B were designed and synthesized using the Silencer siRNA Construction Kit (Ambion, USA) following the manufacturer’s instructions. A commercially available siRNA without any specificity to known genes was used as control. As previously described [41], with minor modifications, 8 pl of siRNA or mRNA was microinjected into the cytoplasm of denuded oocytes. Oocytes were injected using Piezo-operated blunt-end micropipette (3–5 µm internal diameter). After injection, oocytes were kept at RT for 30 min and then moved into the incubator.

**Transgenic mice generation**

The MERVL::tdTomato vector was a gift from Samuel Pfaff (Addgene 40281). The vector was linearized with the enzyme. The pronuclear microinjection for the production of transgenic mice followed previously published studies [61]. Briefly, the linearized vector was injected into the well-recognized pronuclei. Injected zygotes were transferred into pseudopregnant female mice (~30 zygotes per mouse) after 4-h recovery culture in KSOM-AA medium. For founder identification, tail tips were subjected to standard DNA extraction procedures. The amplified DNA fragments were subjected to TA cloning and sequencing. The founder mice were crossed to the littermates of founder mice for four generations to produce homozygous MERVL::tdTomato mice. We also intercrossed MERVL::tdTomato mice with homozygous Oct4::EGFP transgenic mice (OG2) for six generations to produce MERVL::tdTomato/Oct4::EGFP dual-reporter mice.

**SCNT, ICSI, and IVF**

The mouse-SCNT was done as previously described [62]. Briefly, groups of ~50 MI oocytes were transferred to a chamber containing oil-covered M2 supplemented with 5 µg/ml cytochalasin B (CB). The spindle-chromosome complex (SCC) was removed by a blunt Piezo-driven pipette (~10 µm internal diameter) on a 37°C heating stage of an inverted microscope (Nikon, Japan). The nuclei of donor cumulus cells, Sertoli cells, or C57-MEF cells were drawn in and out of the injection pipette until its plasma membrane was broken and then injected into enucleated oocytes. For the mdx-MEF cells, live cells with a diameter of 10–15 µm were selected. The reconstituted embryos were cultured in aMEM medium (Thermo, USA) containing 10% fetal calf serum (FCS, Hyclone, USA) for 1–3 h before activation treatment. The reconstructed embryos were activated in Ca2+ free KSOM medium containing 10 mM strontium and 5 µg/ml CB for 6 h. Activated embryos were thoroughly washed and cultured in G1/G2 medium (Vitrolife, Sweden). The bovine SCNT, bovine oocytes obtained by aspirating follicles on slaughter-house-derived ovaries. We cultured immature COCs in M199 medium supplemented with 10% FCS, 0.2 mM pyruvate, 200 µg/ml gentamicin, 0.5 mg/ml luteinizing hormone, and 1 mg/ml estradiol for 16–18 h at 38.5°C with 5% CO2 in the air. 18 h after the start of maturation, cumulus cells were removed from the oocytes, and oocytes with extruded first polar bodies were selected as MI oocyte. Oocytes enucleated using a beveled glass pipette by aspirating the first polar body and the MII plate in a small amount of surrounding cytoplasm in M199-HEPES medium containing 5 µg/ml CB. In some experiments, we labeled oocytes with DNA fluorochrome (Hoechst 33342) before enucleation. To ensure removal of the oocyte chromatin, we exposed the aspirated cytoplasm to UV light to examine the enucleation. The donor cells were injected into the perivitelline space of each enucleated oocyte by using the same slit in the zona pellucida as made during enucleation. Then, we fused nuclear transfer couples in fusion medium by applying a single electric pulse (1.2 kV/cm for 30 µs). One hour after fusion, the fused embryos using 5 µM ionomycin for 5 min, followed by 5 h of treatment with 10 µg/ml cycloheximide (CHX). The mouse-ICSI was done as previously described [63]. Only the sperm head was injected into the oocyte. After 30 min of recovery, the ICSI-generated embryos were washed several times and cultured in KSOM-AA medium at 37°C in a 5% CO2 in air. The bovine-IVF, COCs matured for 24 h were co-incubated with sperm (108 spermatozoa/ml; thawing semen in 37°C water) in IVF medium at 38.5°C with 5% CO2 in the air for 20 h. The IVF medium consisted of NaCl (114 mM), KCl (3.15 mM), NaH2PO4 (0.39 mM), Na-Lactate (13.3 mM), CaCl2 (2 mM), MgCl2 (0.5 mM), Na-Pyruvate (0.2 mM), penicillin (50 IU/ml), streptomycin (50 µg/ml), NaHCO3 (25 mM), heparin (10 µg/ml), penicillamine (20 µM), hypotaurine (10 µM), epinephrine (1 µM), and bovine serum albumin (6 mg/ml). Presumptive zygotes were vortexed for 2 min to separate cumulus cells. Groups of ~40 presumptive zygotes were cultured in 500 µl drops of SOF medium under mineral oil at 38.5°C, 5% CO2 in humidified air. 72 h after insemination, 5% FCS was added to the culture media.

**ntES derivation, chimeric mice, and embryo transfer**

Blastocysts were denuded by acidic Tyrode’s solution and plated on mitomycin-treated MEF feeder layers in a 96-well plate. The ntES cell derivation medium contains KnockOut-DMEM (Thermo, USA) supplemented with 15% (v/v) KnockOut Serum Replacement (KSR; Thermo, USA), 1 mM GlutaMAX (Thermo, USA), 0.1 mM mercaptoethanol, 1% nonessential amino acid (Thermo, USA), penicillin/streptomycin (100×; Thermo, USA), nucleosides (100×; Thermo, USA), and 1,000 U/ml LIF (Thermo, USA). The ntES colonies formed with culturing for 10 days were picked and transferred for cell passage. For chimeric experiments, ntES were used one day before passaging, which showed an optimal undifferentiated morphology. The ntES were microinjected into CD1/KM blastocysts using a Piezo microinjection pipette. After culturing for 3 h, the embryos were transplanted into the uterus of pseudopregnant mice (~20 embryos per mouse). The 2-cell stage SCNT-, siRNA-6B-, or KDM6A/B-injected embryos were transferred to the oviducts of E0.5 pseudopregnant (~20 embryos per mouse). The embryos were recovered by caesarian section on the E8.5, E14.5, or E19.5. The cloned pups nursed by lactating CD1/KM females. SSLP analysis was performed for D6Mit15, D2Mit102, D11Mit236, D4Mit204, and EGFP. The primer information is presented in Appendix Table S8.  

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Immunofluorescence staining

Embryos and nESs were rinsed three times in phosphate-buffered saline (PBS) with 0.3% BSA, fixed with 4% paraformaldehyde (PFA) overnight at 4°C, and then permeabilized with 0.2% (vol./vol.) Triton X-100 for 15 min at room temperature, followed by washing thoroughly in PBS containing 0.3% BSA. Fixed samples were blocked in 0.05% Tween 20 in PBS containing 3% BSA (PBST) at 37°C for 1 h and then incubated with the primary antibodies overnight at 4°C. Embryos were incubated with primary antibodies against H3K27me3 (Millipore, ABE44, USA), H3K27me2 (Abcam, ab24684, USA), H3K4me3 (Abcam, ab213224, USA), H3K9me3 (Abcam, ab176916, USA), HA (Santa Cruz, sc-7392, USA), MuERVL-Gag (EpiGentek, A-2801-100, USA), Oct4 (Santa Cruz, sc-8629, USA), Sox2 (Santa Cruz, sc-17319, USA), Cdx2 (Abcam, ab76541, USA), Nanog (Abcam, ab107156, USA), Ssea1 (Santa Cruz, sc-21702, USA), E-cadherin (Abcam, ab40772, USA), Nestin (Santa Cruz, sc-21247, USA), Brachyury (Santa Cruz, sc-17745, USA), and Gata4 (Santa Cruz, sc-21247, USA), and Gata4 (Santa Cruz, sc-21247, USA), and washed three times with PBST. Bound antibodies were detected with SuperSignal West Femto Substrate (Thermo, USA). The antibodies used are listed in IF section. Quantification analysis of band intensity was calculated by ImageJ software (NIH, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/). In addition, at least three different cytoplasmic areas were delineated for normalization to background. The average pixel intensity of the nuclear areas was calculated by ImageJ and then normalized by dividing by the average pixel intensity of the background areas.

Western blot

Western blotting was performed using Multistrip Western blotting protocols described previously [67]. Briefly, whole cell lysates from 500–1,500 oocytes or reconstructed embryos were lysed in beta-mercaptoethanol-containing loading buffer and heated at 95°C for 8 min. The embryonic lysates were separated by SDS-PAGE (90 V for 30 min, 30–50 V overnight, when the loading buffer is no longer on the gel, stop the electrophoresis). Following electrophoresis, the protein transferred from the gel to nitrocellulose membranes (Bio-sharp, China). We next cut the membrane into two strips and blot separately. The membrane was followed by blocking in TBST containing 5% defatted milk (BD, USA) for 120 min at 4°C. After being probed with primary antibodies (overnight at 4°C), the membranes were washed in TBST, incubated with an HRP-linked secondary antibody for 120 min at 37°C, and washed three times with TBST. Bound antibodies were detected with SuperSignal West Femto Substrate Trial Kit (Thermo, USA). The antibodies used are listed in IF section. Quantification analysis of band intensity was calculated by ImageJ software (NIH, Bethesda, USA, http://rsbweb.nih.gov/ij/).

RNA-FISH

RNA-FISH on preimplantation embryos was performed as previously described [46,68]. Briefly, the embryos were fixed in 2% PFA in PBS containing 0.5% Triton X-100 for 20 min at room temperature. After three washes with 0.1% PVP/PBS, embryos were treated with 0.1 N HCl containing 0.02% Triton X-100 for 15 min at 4°C. After three washes with 0.1% PVP/2× SSC, embryos were incubated in a series of 10, 20, and 50% formamide/2× SSC. The samples were covered with mineral oil, heated for 30 min at 80°C, and then incubated for ~30 min at 37°C. Next, the fixed embryos were performed using ViewRNA ISH Cell Assay Kit (Thermo, USA) based on the manufacturer’s instructions. Custom-designed ViewRNA Cell Plus Probe against Xist (Thermo, YX-06, USA). The embryos were then counterstained with DAPI, and fluorescence was detected under a laser-scanning confocal microscope (Nikon, Japan).

RNA extraction and RT-qPCR

As previously described [41], total RNA was extracted using the PicoPure RNA Isolation Kit (Thermo, USA) according to the manufacturer’s instructions. Total RNA was extracted from each pool of embryos (n = 3 pools of 20 oocytes or embryos per time point), and residual genomic DNA was removed by DNase digestion, using an RNase-Free DNase Kit (Qiagen, Germany). Reverse transcription was performed using SuperScript III (Thermo, USA) following the manufacturer’s instructions. Quantitative RT–PCR was performed using a SYBR-Taq Master Mix (Applied Biosystems, USA), and signals were detected with ABI7500 real-time PCR System (Applied Biosystems, USA). Analysis of relative gene expression was measured using the 2^ΔΔCt method. For the single embryo, RT–qPCR was done as previously described [69]. Briefly, embryonic total RNA was extracted using an RNasey Micro Kit (Qiagen, Germany) and treated with DNase following the manufacturer’s instructions. mRNAs were reversed by SuperScript III Reverse Transciptase Kit (Thermo, USA). For quantitative gene expression analysis with high specificity, TaqMan probes (Thermo, USA) were used in single embryo RT–qPCR assays, and the expression levels of all embryos were normalized to the average expression levels of ICSI group. All the TaqMan probes and primer sets used in this study are shown in Appendix Table S5.

Embryo biopsy, library construction, and single-cell RNA-seq

The 2-/4-cell embryos were transferred into Ca2+ and Mg2+ free KSOM-AA medium for 1 h to disrupt cell adhesion and were then transferred to Ca2+ and Mg2+ free M2 medium on the micromanipulation dish. The zona pellucida was penetrated by a blunt Piezo-driven micropipette (~30 μm inner diameter), and one blastomere was gently aspirated from each manipulated embryo, and the rest of the blastomeres were cultured in G1+G2 (1:1) medium with 5% CO2 at 37°C. The isolated single blastomere was washed twice in PBS-BSA (0.1%) and hold individual blastomeres before placing in lysis buffer and stored in liquid nitrogen. The single-cell RNA-seq method followed previously published studies [24], only capture mRNAs with a poly (A) tail. Library construction was performed following the Illumina manufacturer’s instructions, and sequencing was performed at the BGI (China). Paired-end sequencing was further performed on the Illumina Hiseq 2000 platform. The sequencing reads that low quality
and adapters were pre-filtered before mapping. Filtered reads were mapped to the mm9 genome using TopHat (v1.3.3) with default parameters. Transcriptional profiling was done as described [24]. Briefly, data normalization was performed by transforming uniquely mapped transcript reads. Genes with low expression in all stages were filtered out and quantified to FPKM (fragments per kilobase of exon model per million mapped reads) using Cufflinks (v1.2.0) to eliminate the effects of sequencing depth and transcript length. Some analyses were performed using R software.

Live-cell imaging procedures

Live-cell imaging was done as previously described [41,70]. Briefly, the embryos were transferred to drops of KSOM-AA medium, placed in the incubator (Tokai Hit, Japan) on the microscope stage (Nikon, Japan), and incubated at 37°C under 5% CO2 in air. Images were acquired by an electron multiplying charge-coupled device (EM-CCD) camera (iXON 897, Andor, UK). Images were taken over 96 h at 10- or 15-min intervals. Live-cell imaging system was housed in a dark room at 27°C.

Data availability

Sequencing data have been deposited in the NCBI sequence read archive (SRA) under accession codes: si68-NT-1 (SRR8191122), si68-NT-2 (SRR8191121), NT-2-1 (SRR8191120), and NT-2-2 (SRR8191119)\textsuperscript{a}. All other data are available from the authors upon reasonable request.

Expanded View for this article is available online.

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Author contributions

LY and GL conceived and designed the study. LY, LS, and XL performed the experiments; LY, LS, XL, LB, and GL analyzed the data. LY and GL supervised the project. LY and GL wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Gurdon JB (1962) The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morphol 10: 622–640
2. Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P (2001) Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 292: 740–743
3. Matoba S, Inoue K, Kohda T, Sugimoto M, Mizutani E, Ogurokui N, Nakamura T, Abe K, Nakano T, Ishino F et al (2011) RNAi-mediated knockdown of Xist can rescue the impaired postimplantation development of cloned mouse embryos. Proc Natl Acad Sci USA 108: 20621–20626
4. Wilmot I, Schneier AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810–813
5. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663–676
6. Hochdelinger K, Jaenisch R (2003) Nuclear transplantation, embryonic stem cells, and the potential for cell therapy. N Engl J Med 349: 275–286
7. Matoba S, Liu Y, Lu F, Iwabuchi KA, Shen L, Inoue A, Zhang Y (2014) Embryonic development following somatic cell nuclear transfer impeded by persisting histone methylation. Cell 159: 884–895
8. Ogura A, Inoue K, Wakayama T (2013) Recent advancements in cloning by somatic cell nuclear transfer. Philos Trans R Soc Lond B Biol Sci 368: 20110329
9. Chung YC, Matoba S, Liu Y, Eum JH, Lu F, Jiang W, Lee JE, Sepilian V, Cha KY, Lee DR et al (2015) Histone demethylase expression enhances human somatic cell nuclear transfer efficiency and promotes derivation of pluripotent stem cells. Cell Stem Cell 17: 758–766
10. Chung YC, Eum JH, Lee JE, Shih SM, Sepilian V, Hong SW, Lee Y, Treff NR, Choi YH, Kimbrel EA et al (2014) Human somatic cell nuclear transfer using adult cells. Cell Stem Cell 14: 777–780
11. Yamada M, Johannesson B, Sagi I, Burnett LC, Kort DH, Prosser RW, Paul D, Nestor MW, 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
12. Tachibana M, Amato P, Sparman M, Gutierrez NM, Tipper-Hedges R, Ma H, Kang E, Futari A, Lee HS, Sritanaudomchai H et al (2013) Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 153: 1228–1238
13. Li L, Zheng P, Dean J (2010) Maternal control of early mouse development. Development 137: 859–870
14. Schoorlemmer J, Perez-Palacios R, Climent M, Guillard D, Muniesa P (2014) Regulation of mouse retroelement MuERV-L/MERVL expression by REXL and epigenetic control of stem cell potency. Front Oncol 4: 14
15. Ishiuchi T, Enriquez-Gascas R, Mizutani E, Boskovic A, Ziegler-Birling C, Rodriguez-Terrones D, Wakayama T, Vaqueras JM, Torres-Padilla ME (2015) Early embryonic-like cells are induced by downregulating replication-dependent chromatin assembly. Nat Struct Mol Biol 22: 662–671
16. Eckersley-Maslin MA, Svensson V, Krueger C, Stubbins TM, Giehr P, Krueger F, Miragaia RJ, Kyriakopoulos C, Berrens RV, Milegaar I et al (2016) MERVL/Zscan4 network activation results in transient genome-wide DNA demethylation of mESCs. Cell Rep 17: 179–192
17. Huang Y, Kim JK, Do DV, Lee C, Penfold CA, Zylczik JJ, Marioni JC, Hackett JA, Surani MA (2017) Stella modulates transcriptional and endogenous retrovirus programs during maternal-to-zygotic transition. Elife 6: e22345
18. Iturbide A, Torres-Padilla ME (2017) Starting embryonic transcription for the first time. Nat Genet 49: 820–821
19. Ribet D, Louvet-Vallee S, Harper F, de Parseval N, Dewannieux M, Heidmann O, Pierron G, Maro B, Heidmann T (2008) Murine endogenous

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retrovirus MuERV-L is the progenitor of the "orphan" epsilon viruslike particles of the early mouse embryo. J Virol 82: 1622 – 1625.

20. Svoboda P, Stein P, Anger M, Bernstein E, Hannon GJ, Schultz RM (2004) RNAs and expression of retrotransposons MuERV-L and IAP in preimplantation mouse embryos. Dev Biol 269: 276 – 285.

21. Whiddon JL, Langford AT, Wong CJ, Zhong JW, Tappend Scott SJ (2017) Conservation and innovation in the DXU4-family gene network. Nat Genet 49: 935 – 940.

22. Peaston AE, Evsikov AV, Graber JH, de Vries WN, Holbrook AE, Solter D, Knowles B (2004) Retrotransposons regulate host genes in mouse oocytes and preimplantation embryos. Dev Cell 7: 597 – 606.

23. Hayashi M, Maehara K, Harada A, Semba Y, Kudo K, Takahashi H, Oki S, Meno C, Ichiyanagi K, Akashi K et al (2016) Chd5 regulates MuERV-L/MERVL expression in mouse embryonic stem cells via H3K27me3 modification and histone H3(L)/H3.2. J Cell Biol 117: 780 – 792.

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

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

26. Inoue K, Oikawa M, Kamimura S, Ogonuki N, Matoba S, Shiura H, Abe K, Ogura A (2015) Trichostatin A specifically improves the aberrant histone H3 lysine 27 methylation in human embryonic stem cells. Sci Rep 5: 10127.

27. Warner CM, Verstreegh LP (1974) In vivo and in vitro effect of alphamatin on preimplantation mouse embryo RNA polymerase. Nature 248: 678 – 680.

28. Teperek M, Miyamoto K (2013) Nuclear reprogramming of sperm and somatic nuclei in eggs and oocytes. Reprod Med Biol 12: 133 – 149.

29. Hormanseder E, Simeone A, Allen GE, Bradshaw CR, Figlmuller M, Warner CM, Versteegh LR (2012) Epigenetic memory of an active gene state maintenance. J Genet Genomics 39: 225 – 236.

30. Zernicka-Goetz M, Morris SA, Bruce AW (2009) Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. Nat Rev Genet 10: 467 – 477.

31. Yang CS, Lopez CG, Rana TM (2011) Discovery of nonsteroidal anti-inflammatory drug and anticancer drug enhancing reprogramming and induced pluripotent stem cell generation. Stem Cells 29: 1528 – 1536.

32. Wang L, Teng F, Yuan X, Liu C, Wang J, Li Y, Cui T, Li T, Liu Z, Zhou Q (2017) Overexpression of Stella improves the efficiency of nuclear transfer reprogramming. J Genet Genomics 44: 363 – 366.

33. Gao S, Chung YG, Williams JW, Riley J, Moley K, Latham KE (2003) Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. Biol Reprod 69: 48 – 56.

34. Ng RK, Gurdon JB (2008) Epigenetic memory of an active gene state depends on histone H3.3 incorporation into chromatin in the absence of transcription. Nat Cell Biol 10: 102 – 109.

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

36. Santos F, Peters AH, Otte AP, Reik W, Dean W (2005) Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. Dev Biol 280: 225 – 236.

37. Dimitrova E, Turberfield AH, Klose RJ (2015) Histone demethylases in chromatin biology and beyond. EMBO Rep 16: 1620 – 1639.

38. Mosammareparast N, Shi Y (2010) Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem 79: 155 – 179.

39. Blatch GL, Lassle M (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays 21: 932 – 939.

40. Allan RK, Ratajczak T (2011) Versatile TPR domains accommodate different modes of target protein recognition and function. Cell Stress Chaperones 16: 353 – 367.

41. Yang L, Song LS, Liu XF, Xia Q, Bai LG, Gao L, Gao QG, Wang Y, Wei ZY, Bai CL et al (2016) The maternal effect genes UTX and JMJD3 play contrasting roles in Mus musculus preimplantation embryo development. Sci Rep 6: 26711.

42. Pan G, Tian S, Nie J, Yang C, Ruotti V, Wei H, Jonsdottir GA, Stewart R, Thomson JA (2007) Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. Cell Stem Cell 1: 299 – 312.

43. Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappapier J, Issaieva I, Canaani E, Salcini AE, Helin K (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449: 731 – 734.

44. Macfarlan TS, Gifford WD, Driscoll S, Lettieri K, Rowe HM, Bonomini D, Firth A, Singer O, Trono D, Pfaff SL (2012) Embryonic stem cell potency fluctuates with endogenous retrovirus activity. Nature 487: 57 – 63.

45. Zalzman M, Falco C, Sharova LV, Nishiyama A, Thomas M, Lee SL, Stagg CA, Hoang HC, Yang HT, Indig FE et al (2010) Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature 464: 858 – 863.

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

47. Machaty Z, Paldi A, Csaki T, Varga Z, Kiss I, Barandi Z, Vajta G (1993) Biopsy and sex determination by PCR of IVF bovine embryos. J Reprod Fertil 98: 467 – 470.

48. Inoue K, Kohda T, Sugimoto M, Sado T, Ogguni 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.

49. Matoba S, Wang H, Jiang L, Lu F, Iwabuchi KA, Wu X, Inoue K, Yang L, Press W, Lee JT et al (2018) Loss of H3K27me3 imprinting in somatic cell nuclear transfer embryos disrupts post-implantation development. Cell Stem Cell 23: 343 – 354 e5.

50. Szabo PE, Hubner K, Scholer H, Mann JR (2002) Allele-specific expression of imprinted genes in mouse migratory primordial germ cells. Mech Dev 115: 157 – 160.

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

52. Xie B, Zhang H, Wei R, Li Q, Weng X, Kong Q, Liu Z (2016) Histone H3 lysine 27 trimethylation acts as an epigenetic barrier in porcine nuclear reprogramming. Reproduction 151: 9 – 16.

53. Bai FY, Song SH, Zhang YW, Huang X, Huang XW, Sun RZ, Lei L (2018) Kdm6a overexpression improves the development of cloned mouse embryos. Zygote 26: 24 – 32.

54. Tao S, Miyoshi N, Okamoto I, Jenuwein T, Heard E, Azim Surani M (2015) Initiation of epigenetic reprogramming of the X chromosome in somatic nuclei transplanted to a mouse oocyte. EMBO Rep 6: 748 – 754.

55. Zhao W, Li Q, Ayers S, Gu Y, Shi Z, Zhu Q, Chen Y, Wang HY, Wang RF (2013) JMJD3 inhibits reprogramming by upregulating expression of INK4a/Arf and targeting PHF20 for ubiquitination. Cell 152: 1037 – 1050.
Mansour AA, Gafni O, Weinberger L, Zviran A, Ayyash M, Rais Y, Kruopalnik V, Zerbib M, Amann-Zalcenstein D, Maza I et al (2012) The H3K27 demethylase Utx regulates somatic and germ cell epigenetic reprogramming. Nature 488: 409 – 413

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

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

Mitrecic D, Huzak M, Curlin M, Gajovic S (2005) An improved method for determination of gene copy numbers in transgenic mice by serial dilution curves obtained by real-time quantitative PCR assay. J Biochem Biophys Methods 64: 83 – 98

Marden JJ, Harraz MM, Williams AJ, Nelson K, Luo M, Paulson H, Engelhardt JF (2007) Redox modifier genes in amyotrophic lateral sclerosis in mice. J Clin Invest 117: 2913 – 2919

Ittner LM, Gotz J (2007) Pronuclear injection for the production of transgenic mice. Nat Protoc 2: 1206 – 1215

Kishigami S, Wakayama S, Thuan NV, Ohta H, Mizutani E, Hikichi T, Bui HT, Balbach S, Ogura A, Boiani M et al (2006) Production of cloned mice by somatic cell nuclear transfer. Nat Protoc 1: 125 – 138

Yoshida N, Perry AC (2007) Piezo-actuated mouse intracytoplasmic sperm injection (ICSI). Nat Protoc 2: 296 – 304

Ross PJ, Ragina NP, Rodriguez RM, lager AE, Siripattarapravat K, Lopez-Corrales N, Cibelli JB (2008) Polycomb gene expression and histone H3 lysine 27 trimethylation changes during bovine preimplantation development. Reproduction 136: 777 – 785

Gao Y, Hyttel P, Hall VJ (2010) Regulation of H3K27me3 and H3K4me3 during early porcine embryonic development. Mol Reprod Dev 77: 540 – 549

Aoshima K, Inoue E, Sawa H, Okada Y (2015) Paternal H3K4 methylation is required for minor zygotic gene activation and early mouse embryonic development. EMBO Rep 16: 803 – 812

Kiyatkin A, Aksamitiene E (2009) Multistrip western blotting to increase quantitative data output. Methods Mol Biol 536: 149 – 161

Tan K, An L, Miao K, Ren L, Hou Z, Tao L, Zhang Z, Wang X, Xia W, Liu J et al (2016) Impaired imprinted X chromosome inactivation is responsible for the skewed sex ratio following in vitro fertilization. Proc Natl Acad Sci USA 113: 3197 – 3202

Fukuda A, Tomikawa J, Miura T, Hata K, Nakabayashi K, Eggen K, Akatsu H, Umezawa A (2014) The role of maternal-specific H3K9me3 modification in establishing imprinted X-chromosome inactivation and embryogenesis in mice. Nat Commun 5: 5464

Mizutani E, Yamagata K, Ono T, Akagi S, Geshi M, Wakayama T (2012) Abnormal chromosome segregation at early cleavage is a major cause of the full-term developmental failure of mouse clones. Deu Biol 364: 56 – 65

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