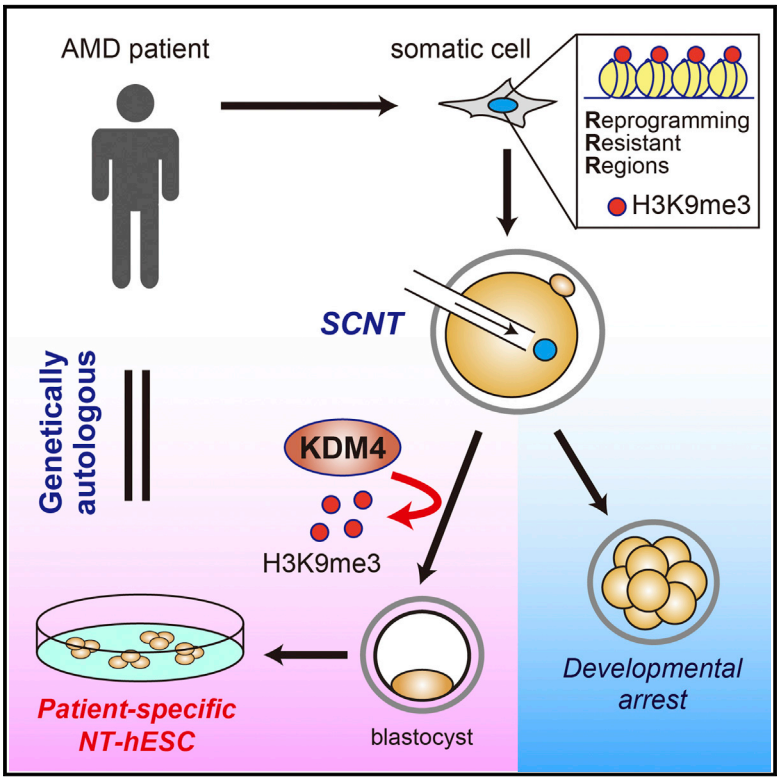
Short Article

Cell Stem Cell

Histone Demethylase Expression Enhances Human Somatic Cell Nuclear Transfer Efficiency and Promotes Derivation of Pluripotent Stem Cells

Graphical Abstract



Highlights

- H3K9me3 is a barrier for human SCNT reprogramming as in mouse
- Injection of human KDM4A mRNA improves SCNT success in mouse oocytes
- KDM4A also improves human SCNT blastocyst formation and NT-ESC derivation
- H3K9me3 removal facilitates zygotic genome activation in human SCNT embryos

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In Brief

Zhang and colleagues show that, as they found previously in mouse, injection of a histone demethylase improves the efficiency of human SCNT reprogramming. Using this approach they were able to reduce variability in success from different egg donors and derive human NT-ESC lines from patients with age-related macular degeneration.

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Short Article

Histone Demethylase Expression Enhances Human Somatic Cell Nuclear Transfer Efficiency and Promotes Derivation of Pluripotent Stem Cells

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SUMMARY

The extremely low efficiency of human embryonic stem cell (hESC) derivation using somatic cell nuclear transfer (SCNT) limits its potential application. Blastocyst formation from human SCNT embryos occurs at a low rate and with only some oocyte donors. We previously showed in mice that reduction of histone H3 lysine 9 trimethylation (H3K9me3) through ectopic expression of the H3K9me3 demethylase Kdm4d greatly improves SCNT embryo development. Here we show that overexpression of a related H3K9me3 demethylase KDM4A improves human SCNT, and that, as in mice, H3K9me3 in the human somatic cell genome is an SCNT reprogramming barrier. Overexpression of KDM4A significantly improves the blastocyst formation rate in human SCNT embryos by facilitating transcriptional reprogramming, allowing efficient derivation of SCNT-derived ESCs using adult Age-related Macular Degeneration (AMD) patient somatic nuclei donors. This conserved mechanistic insight has potential applications for improving SCNT in a variety of contexts, including regenerative medicine.

INTRODUCTION

The differentiated somatic cell genome can be reprogramed back into an embryonic state when the nucleus is exposed to the molecular milieu of the oocyte cytoplasm via somatic cell nuclear transfer (SCNT) (Gurdon, 1962), thereby enabling the generation of pluripotent embryonic stem cells (ESCs) from terminally differentiated somatic cells (Wakayama et al., 2001). Because SCNT-derived ESCs (NT-ESCs) are genetically autologous to the nuclear donor somatic cells, SCNT has great potential in therapeutic and regenerative medicine, including disease modeling and cell/tissue replacement therapy (Hochedlinger and Jaenisch, 2003; Yang et al., 2007). Thus, SCNT can be used to fix mitochondria-gene-related defects, which cannot be done through transcription-factor-based reprogramming (Ma et al., 2015). Despite the great potential of human NT-ESCs, technical difficulties makes its application to human therapeutics extremely difficult (French et al., 2008; Noggle et al., 2011; Simerly et al., 2003).

The first NT-ESCs were generated by the Mitalipov group using differentiated fetal and infant fibroblasts as nuclear donors (Tachibana et al., 2013). Using their optimized conditions, we and others succeeded in deriving human NT-ESCs from adult and aged patient somatic cells (Chung et al., 2014; Yamada et al., 2014). However, derivation of NT-ESCs still remains a very difficult task due to the extremely low rate of SCNT embryos developing to the blastocyst stage. Currently only oocytes with the highest quality from certain females can support the development of SCNT embryos to the blastocyst stage (Chung et al., 2014; Tachibana et al., 2013), limiting the useful oocyte donor pools.

The poor developmental potential of SCNT embryos is not limited to human; it is also commonly observed in all examined mammalian species (Rodriguez-Osorio et al., 2012). Through comparative transcriptomic and epigenomic analyses of mouse in vitro fertilization (IVF) and SCNT embryos, we have recently revealed that histone H3 lysine 9 trimethylation (H3K9me3) in the donor somatic cell genome functions as a barrier preventing transcriptional reprogramming by SCNT, leading to failure of zygotic genome activation (ZGA) and preimplantation development (Matoba et al., 2014). Importantly, we demonstrated that this epigenetic barrier could be removed by the ectopic overexpression of mouse Kdm4d, an H3K9me3 demethylase. Removal of H3K9me3 facilitates ZGA and consequently improves development of mouse SCNT embryos to reach the blastocyst stage, leading to an increased rate of mouse NT-ESC establishment

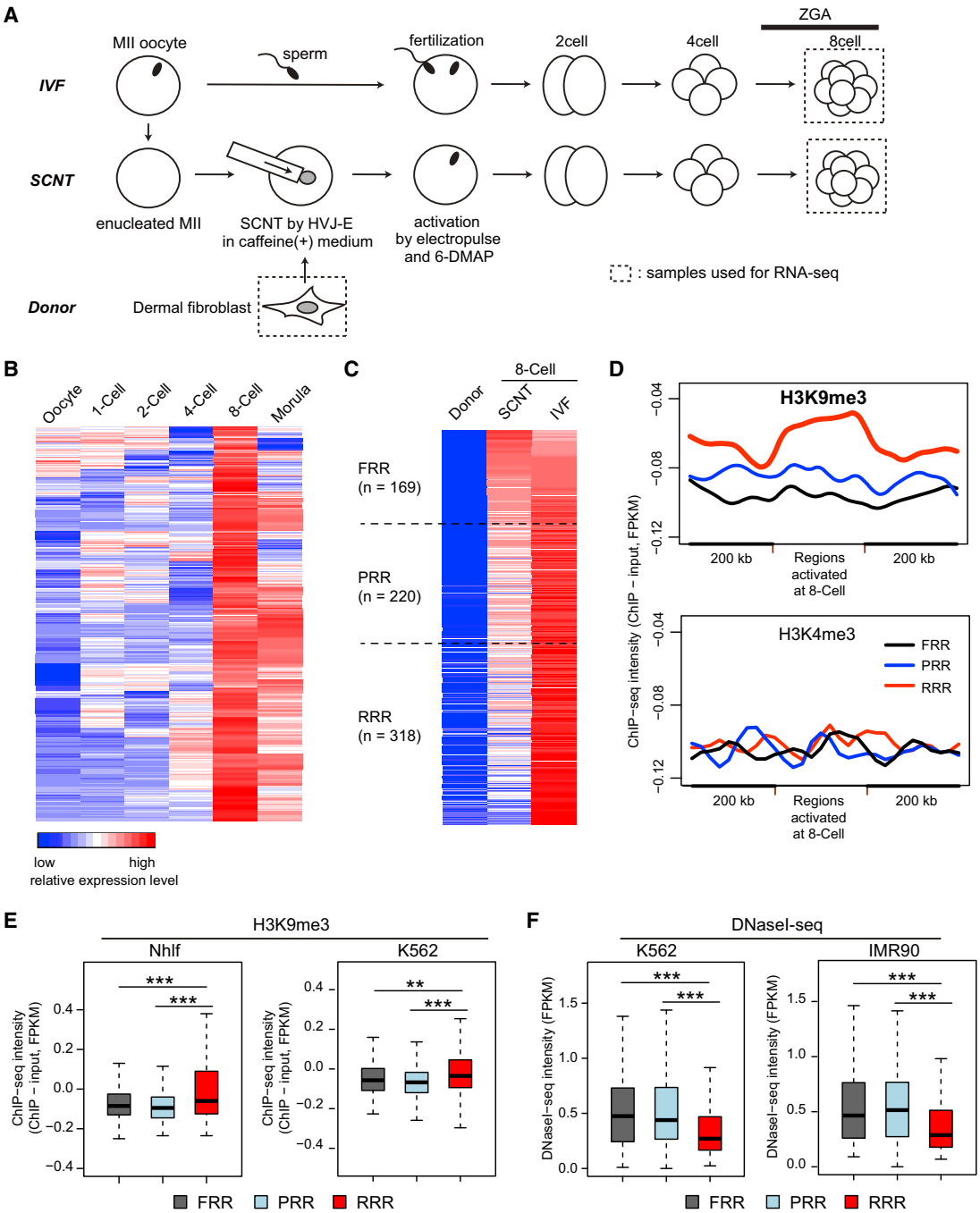

Figure 1. Human RRRs Are Enriched for H3K9me3 in Somatic Cells

(A) Schematic illustration of the experimental procedures. Samples used for RNA-seq are marked by dashed rectangles.

(B) Heatmap illustration of the transcriptome of IVF human preimplantation embryos. Each tile represents an average of peaks within the region obtained by sliding-window analysis. Shown are the 707 regions that are activated from the four-cell to the eight-cell stage in IVF embryos. RNA-seq datasets were obtained from a previous publication (Xue et al., 2013).

(C) Heatmap illustration of the transcriptome comparing donor somatic cells, IVF embryos, and SCNT embryos at the eight-cell stage. Shown are the 707 regions identified in (A). These regions were classified into three groups based on the fold-change (FC) in transcription levels between SCNT and IVF eight-cell embryos. FRRs, PRRs, and RRRs indicate fully reprogrammed regions (FC $\leq$ 2), partially reprogrammed regions (2 < FC $\leq$ 5), and reprogramming-resistant regions (FC > 5), respectively.

(D) The average ChIP-seq intensity of H3K9me3 and H3K4me3 in human fibroblast cells (Nhlf) are shown within FRR, PRR, and RRR compared with 200 kb flanking regions. Histone modification ChIP-seq datasets were obtained from the ENCODE project (Bernstein et al., 2012; ENCODE Project Consortium, 2011).

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(Matoba et al., 2014). These findings prompted us to ask whether the principle we discovered in mouse can be applied to human SCNT.

Here we report that somatic cell H3K9me3 also serves as a barrier in human SCNT reprogramming. We demonstrate that KDM4A overexpression significantly improves human SCNT embryo development, allowing efficient derivation of patient-specific NT-ESCs using oocytes obtained from donors whose oocytes failed to develop into blastocysts without the help of KDM4A overexpression. Thus, our study expands the usability of oocyte donors and establishes histone-demethylase-assisted SCNT as a general method for improving mammalian SCNT for reproductive and therapeutic cloning.

RESULTS

Identification of Reprogramming-Resistant Regions in Eight-Cell Human SCNT Embryos

Human ZGA takes place during the late four-cell to the late eight-cell stages (Niakan et al., 2012) (Figure 1A). To identify the genomic regions activated during ZGA of normal human IVF embryos, we analyzed published human preimplantation embryo RNA-sequencing (RNA-seq) datasets (Xue et al., 2013) and identified 707 genomic regions ranging from 20 to 160 kb in size (Table S1) that were activated at least 5-fold at the eight-cell stage compared to the four-cell stage (Figure 1B).

To determine whether ZGA takes place properly in human SCNT, we collected late eight-cell stage embryos (five/group), derived either from SCNT or IVF, and performed RNA-seq (Figure 1A). In parallel, we also performed RNA-seq of the donor dermal fibroblast cells (DFB-8, see Experimental Procedures). Analysis of the 707 genomic regions defined above (Figure 1B, Table S1) indicates that the majority of the ZGA regions are activated in the SCNT embryos compared to those in donor fibroblasts (Figure 1C). However, the level of activation is not comparable to that in IVF embryos (Figure 1C). Of the 707 genomic regions, 169 were activated at a level comparable to those in IVF embryos (FC $\leq$ 2, IVF versus SCNT), and were thus termed fully reprogrammed regions (FRRs) following our previous definition (Matoba et al., 2014). Similarly, 220 regions were partially activated (2 < FC $\leq$ 5) in SCNT compared to IVF embryos and were termed partially reprogrammed regions (PRRs). However, the remaining 318 regions (Table S2), termed reprogramming-resistant regions (RRRs), failed to be activated in SCNT embryos (FC > 5). Thus, comparative transcriptome analysis allowed us to identify 318 RRRs that were refractory to transcriptional reprogramming in human eight-cell SCNT embryos.

The Heterochromatin Features of RRRs Are Conserved in Human Somatic Cells

We next asked whether the human RRRs possess the heterochromatin features like those of the mouse RRRs. Analysis of the publically available ChIP-seq datasets of eight major histone modifications from human fibroblast cells (Bernstein et al., 2012; ENCODE Project Consortium, 2011) revealed specific enrichment of H3K9me3 in human RRRs (Figures 1D and S1A). The enrichment of H3K9me3 is unique to RRRs, as a similar enrichment was not observed in FRRs or PRRs (Figures 1D and S1A). Similar analysis also revealed the enrichment of H3K9me3 at RRRs in K562 erythroleukemic cells, Hsmm skeletal muscle myoblasts, and Mcf7 breast adenocarcinoma cells (Figures 1E and S1B), indicating that H3K9me3 enrichment in RRRs is a common feature of somatic cells.

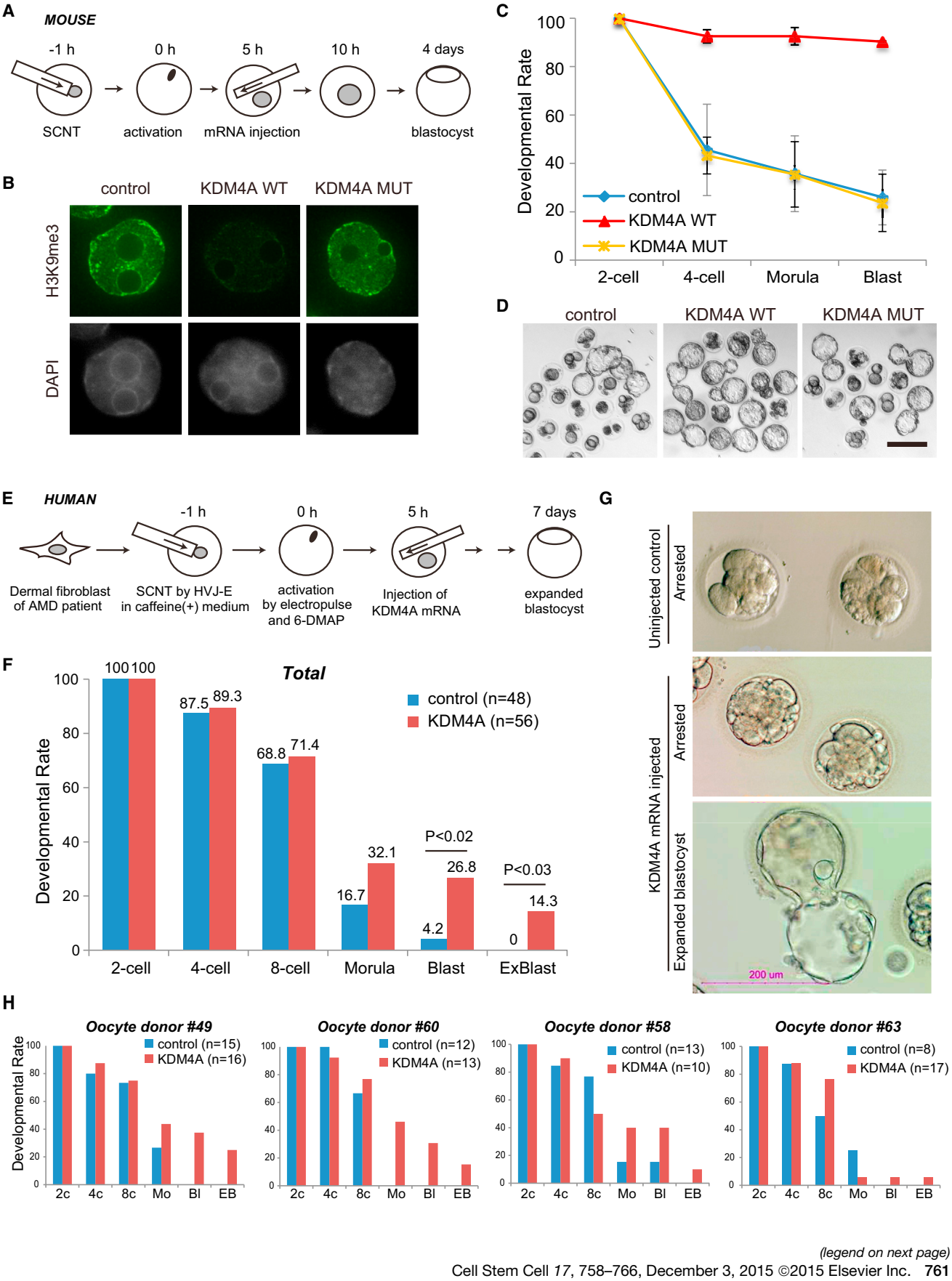
Next, we analyzed the DNaseI hypersensitivity of four different somatic cell types using the datasets generated by the ENCODE project. The analysis revealed that RRRs are significantly less sensitive to DNaseI compared to FRR and PRR in all human somatic cell types analyzed (Figures 1F and S1C). Consistent with their heterochromatin feature, human RRRs are relatively gene-poor compared to FRRs or PRRs (Figure S1D), and they are enriched with specific repeat sequences such as LINE and LTR, but not SINE (Figure S1E). Collectively, these results indicate that the heterochromatin features of RRRs, enrichment of H3K9me3, and decreased accessibility to DNaseI are conserved in both mouse and human somatic cells.

Human KDM4A mRNA Injection Improves Development of Mouse SCNT Embryos

Having established that human RRRs are enriched for H3K9me3, we next asked whether removal of H3K9me3 could help overcome the reprogramming barrier in human SCNT embryos. We previously demonstrated using a mouse SCNT model that the H3K9me3 barrier could be removed by injection of mRNAs encoding the mouse H3K9me3 demethylase Kdm4d (Matoba et al., 2014). Before moving into a human SCNT model, given that multiple members of the KDM4 family with H3K9me3 demethylase activity exist in mouse and human (Klose et al., 2006; Krishnan and Trievel, 2013; Whetstine et al., 2006), we were interested in determining whether the effect of Kdm4d in facilitating SCNT reprogramming could be extended to other members of the KDM4 family. In addition, we were also interested in testing whether KDM4 family members could function across species.

To this end, we performed SCNT using cumulus cells of adult female mice as nuclear donors and injected human KDM4A mRNA at 5 hr post-activation (hpa) following the same procedure used in our previous study (Figure 2A) (Matoba et al., 2014). Immunostaining revealed that injection of WT, but not catalytic mutant, human KDM4A mRNA greatly reduced H3K9me3 levels in the nucleus of mouse SCNT embryos (Figure 2B). Importantly, injection of KDM4A mRNA greatly increased the developmental potential of SCNT embryos with 90.3% of them developing to the blastocyst stage, which is in contrast to the 26% blastocyst formation rate in controls (Figures 2C and 2D, Table S3). The extremely high efficiency of blastocyst formation is similar to the 88.6% observed in Kdm4d-injected mouse SCNT embryos (Matoba et al., 2014). These results suggest that the

(E and F) Boxplots comparing the average intensity of H3K9me3-ChIP-seq (E) and DNaseI-seq (F) within FRR, PRR, and RRR in different somatic cell types. ChIP-seq and DNaseI-seq datasets were obtained from the ENCODE projects (ENCODE Project Consortium, 2011). Middle line in the colored space indicates the median, the edges indicate the 25th/75th percentiles, and the whiskers indicate the 2.5th/97.5th percentiles. ***p < 0.001, **p < 0.01.
See also Figure S1, Table S1, and Table S2.



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reprogramming barrier, H3K9me3 in the somatic cell genome, can be removed by any member of the KDM4 family demethylases as long as it contains H3K9me3 demethylase activity.

KDM4A mRNA Injection Significantly Increases the Blastocyst Formation Rate of Human SCNT Embryos

We next asked whether KDM4A mRNA injection could also help overcome the reprogramming barrier in human SCNT using the optimized SCNT conditions including the use of the histone deacetylase inhibitor Trichostatin A (TSA) (Tachibana et al., 2013). With the future clinical application of KDM4A-assisted SCNT in mind, we used dermal fibroblasts of Age-related Macular Degeneration (AMD) patients as nuclear donors.

To reaffirm the beneficial effect of KDM4A on human SCNT, we chose oocyte donors whose oocytes failed to develop to the expanded blastocyst in our past attempts using the regular procedures (Chung et al., 2014). Following enucleation, a total of 114 MII oocytes collected from four oocyte donors were fused to donor fibroblast cells by HVJ-E (Hemagglutinating virus of Japan envelope). Upon activation, 63 of the reconstructed SCNT oocytes were injected with human KDM4A mRNA, and the rest (51) served as non-injected controls (Figure 2E, Table S4). We monitored the developmental process of these SCNT embryos and found that the two groups featured similar cleavage efficiencies to form two-cell embryos (control: 48/51 = 94.1%, KDM4A: 56/63 = 88.9%) (Table S4). As expected, KDM4A mRNA injection did not show any beneficial effect on the developmental rate of SCNT embryos before ZGA finishes at the end of the eight-cell stage (68.8% versus 71.4%) (Figure 2F and Table S4). However, the beneficial effect became clear at the morula stage (16.7% versus 32.1%) (Figure 2F and Table S4). Strikingly, at day 6, 26.8% (15/56) of the KDM4A-injected embryos had successfully reached the blastocyst stage, as compared to only 4.2% (2/48) of control embryos. On day 7, 14.3% of KDM4A-injected embryos developed to the expanded blastocyst stage, while none of the control embryos developed into this stage (Figures 2F and 2G). Importantly, the beneficial effect of KDM4A was observed in all four donors examined (Figure 2H). Thus we conclude that KDM4A mRNA injection can improve the developmental potential of human SCNT embryos, especially beyond ZGA.

Establishment and Characterization of Human ESCs Derived from KDM4A-Injected SCNT Blastocysts

We next attempted to derive NT-ESCs from KDM4A-injected SCNT blastocysts. We obtained a total of eight expanded blastocysts from KDM4A-injected SCNT embryos (Figure 3A and Table S4). After we removed the zona pellucida, the expanded blastocysts were cultured on irradiated mouse embryonic fibroblasts (MEFs) in a conventional ESC derivation medium. Seven out of the eight blastocysts attached to the MEF feeder cells and initiated outgrowth. After five passages, we successfully derived four stable NT-ESC lines, which were designated as NTK (NT assisted by KDM4A)-ESC #6–9, respectively (Figure 3A, also named CHA-NT #6–9).

Immunostaining revealed that OCT4, NANOG, SOX2, SSEA-4, and TRA1-60 were all expressed with similar patterns to those of a control human ESC line derived by IVF (Figures 3B, S2A, and S2B). RNA-seq (Figure S2C) revealed that the NTK-ESCs express pluripotency marker genes at levels similar to those of control ESCs (Figure 3C). Pairwise comparison of global transcriptomes revealed a high correlation between NTK-ESCs and control ESCs (Figures 3D and S2D). Hierarchical clustering analyses of transcriptomes revealed that NTK-ESCs are clustered together with control ESCs (Figure 3E). These results suggest that NTK-ESCs are indistinguishable from control ESCs at the molecular level.

We next examined the differentiation capacity of the NTK-ESCs by in vitro differentiation and in vivo teratoma assays. Immunostaining of embryoid bodies (EBs) after 2 weeks of in vitro culture revealed that the NTK-ESCs could efficiently give rise to all three germ layer cells (Figures 3F and S2E). Moreover, the NTK-ESCs formed teratomas containing all three germ layer cells within 12 weeks of transplantation (Figures 3G and S2F). These results indicate that the NTK-ESCs are pluripotent.

Karyotyping demonstrated that these NTK-ESCs maintain a normal number of chromosomes and have the same expected pair of sex chromosomes as those of the nuclear donor somatic cells (46, XX for NTK6/7; 46, XY for NTK8; Figures 3H and S3A). Short Tandem Repeat (STR) analysis demonstrated that all of the 16 repeat markers located across the genome showed a perfect match between donor somatic cells and their derivative NTK-ESCs (Figures 3I and S3B). mtDNA sequence analysis revealed that both SNPs of NTK-ESCs matched exactly those of oocyte donors, but not those of nuclear donors (Figures 3J and S3C). Collectively, these results establish the reliability of our SCNT method, and they demonstrate that KDM4A mRNA injection improves SCNT-mediated ESC derivation without compromising pluripotency or genomic stability of the established NTK-ESCs.

KDM4A Facilitates ZGA of RRRs in Eight-Cell SCNT Embryos

The fact that KDM4A mRNA injection significantly improves SCNT embryo development after ZGA suggested that H3K9me3

Figure 2. Injection of Human KDM4A mRNA Improves Development of Mouse and Human SCNT Embryos

(A) Schematic illustration of the mouse SCNT procedures.

(B) Representative nuclear images of one-cell stage SCNT embryos stained with anti-H3K9me3 and DAPI at 5 hr after mRNA injection.

(C) KDM4A mRNA injection greatly improves preimplantation development of mouse SCNT embryos. Shown is the percentage of embryos that reached the indicated stages. Error bars indicate SD.

(D) Representative images of SCNT embryos after 120 hr of culturing in vitro. Scale bar, 100 μm.

(E) Schematic illustration of the human SCNT procedures.

(F) Bar graph showing the average developmental efficiency of human SCNT embryos obtained using oocytes from four different donors during 7 days of in vitro culture. The efficiency was calculated using the number of embryos that reached the two-cell stage. Blast, blastocyst; ExBlast, expanded blastocyst. Developmental rates were statistically analyzed by Fisher's exact test.

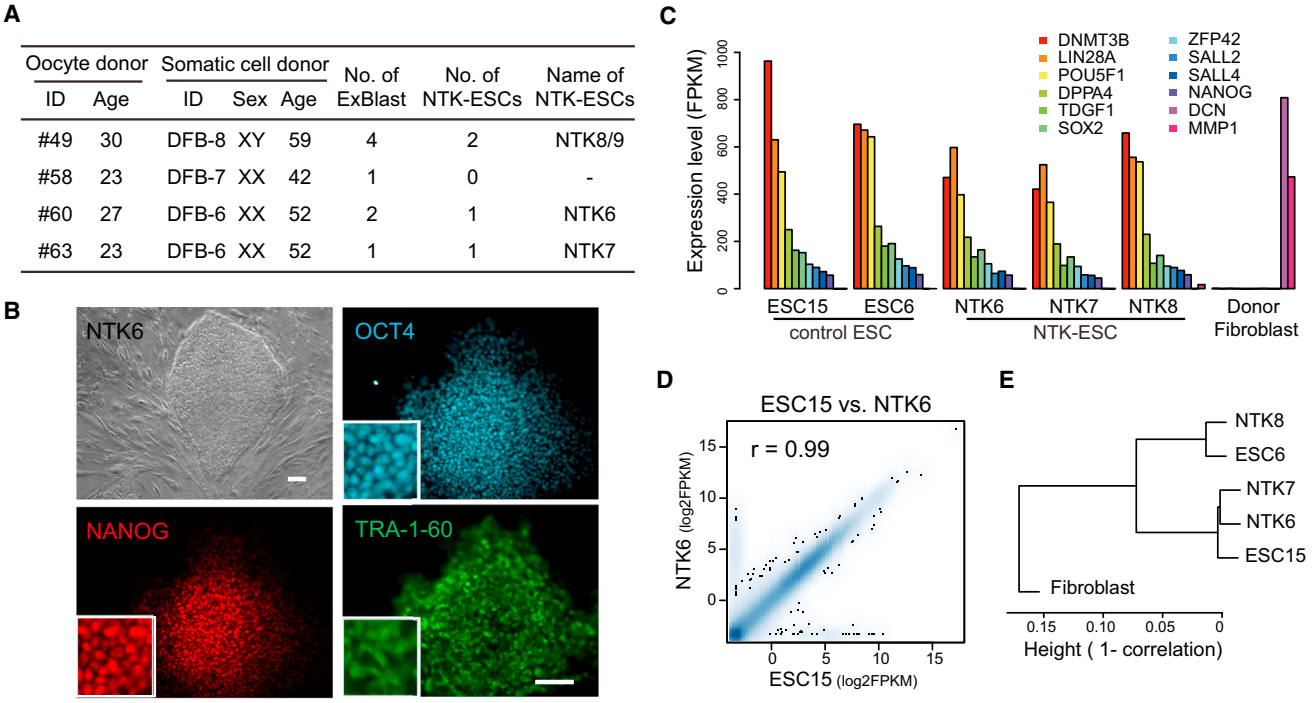
(G) Representative images of SCNT embryos after 7 days of culturing in vitro.

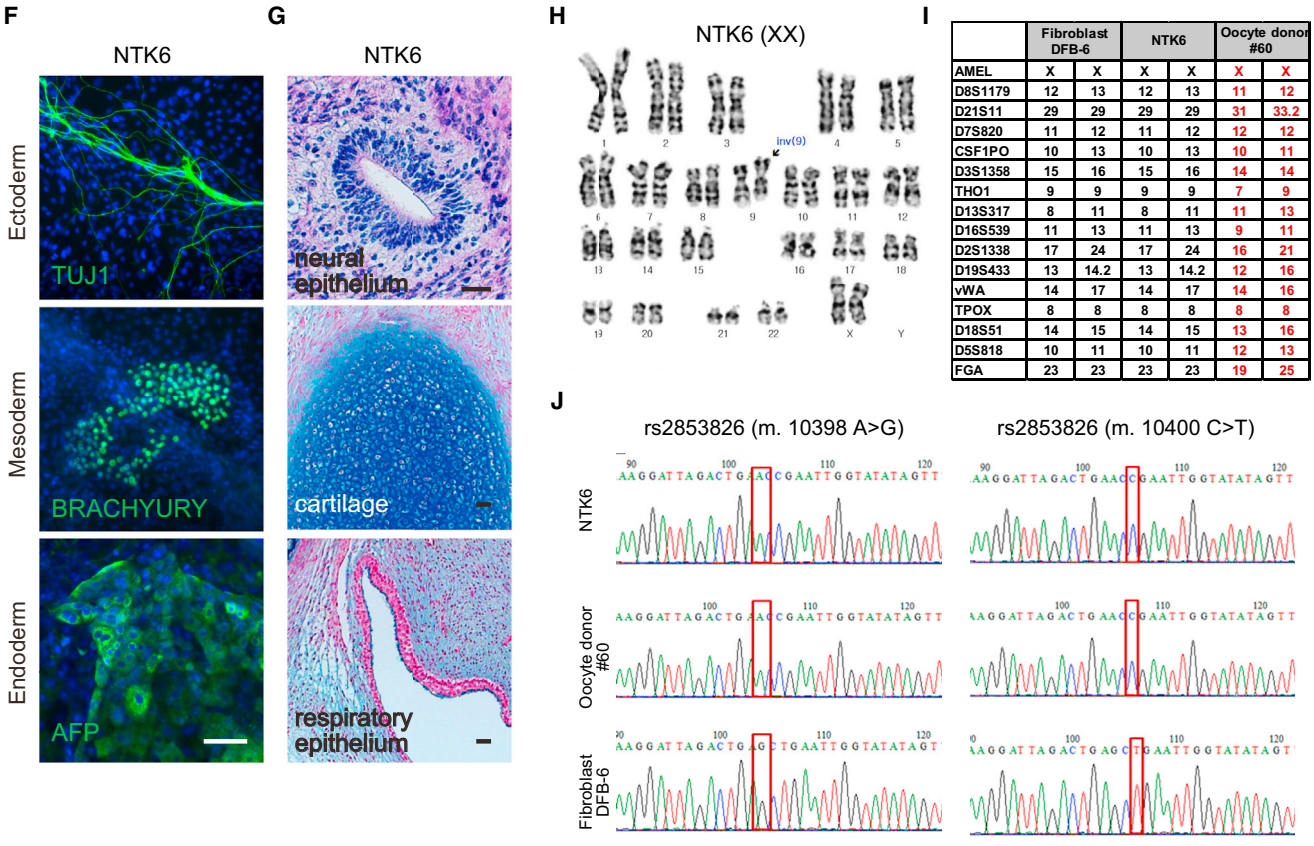
(H) Bar graphs showing the developmental rate of human SCNT embryos derived from each oocyte-donor female.

See also Tables S3 and S4.

A

Oocyte donor		Somatic cell donor			No. of ExBlast	No. of NTK-ESCs	Name of NTK-ESCs
ID	Age	ID	Sex	Age			
#49	30	DFB-8	XY	59	4	2	NTK8/9
#58	23	DFB-7	XX	42	1	0	-
#60	27	DFB-6	XX	52	2	1	NTK6
#63	23	DFB-6	XX	52	1	1	NTK7





I

	Fibroblast DFB-6		NTK6		Oocyte donor #60	
AMEL	X	X	X	X	X	X
D8S1179	12	13	12	13	11	12
D21S11	29	29	29	29	31	33.2
D7S820	11	12	11	12	12	12
CSF1PO	10	13	10	13	10	11
D3S1358	15	16	15	16	14	14
THO1	9	9	9	9	7	9
D13S317	8	11	8	11	11	13
D16S539	11	13	11	13	9	11
D2S1338	17	24	17	24	16	21
D19S433	13	14.2	13	14.2	12	16
vWA	14	17	14	17	14	16
TPOX	8	8	8	8	8	8
D18S51	14	15	14	15	13	16
D5S818	10	11	10	11	12	13
FGA	23	23	23	23	19	25

Figure 3. Establishment and Characterization of NTK-ESCs from AMD Patients

(A) Summary of established NT-ESC lines using AMD patient fibroblasts as nuclear donors through KDM4A-assisted SCNT.

(B) Representative phase contrast and immunostaining images of NTK-ESCs. Scale bar, 100 μm.

(C) Bar graphs showing expression levels of pluripotency-specific and fibroblast-specific genes based on RNA-seq data.

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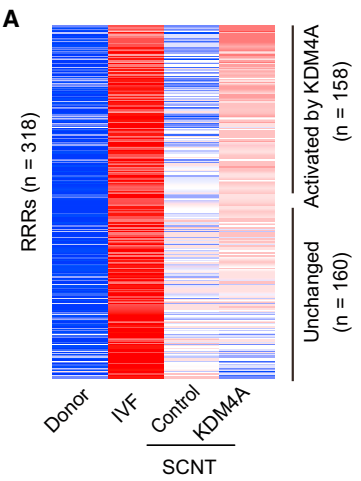

B

GO Terms	P-value
regulation of transcription	4.30E-04
ribonucleoprotein complex biogenesis	7.80E-04
ribosome biogenesis	3.60E-03
RNA processing	1.60E-02

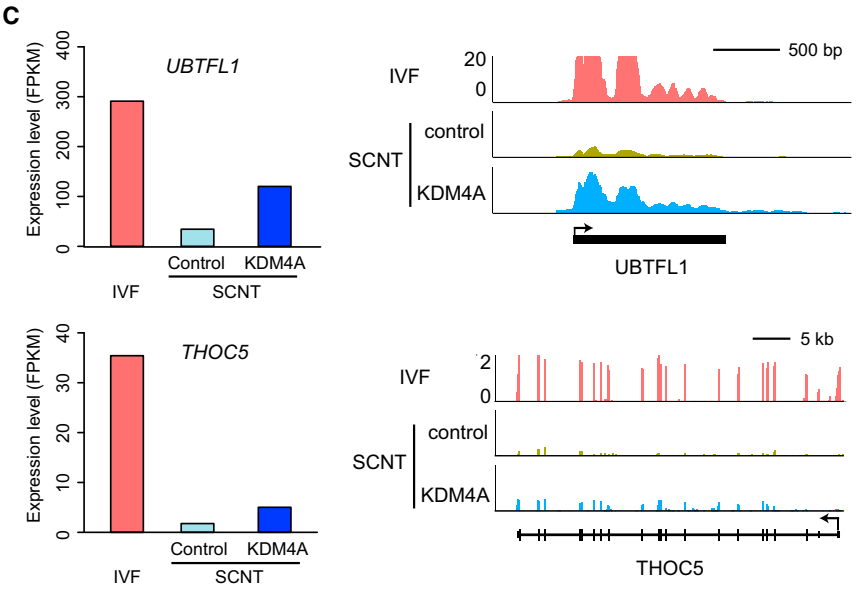

Figure 4. Partial Restoration of Transcription upon KDM4A mRNA Injection in SCNT Eight-Cell Embryos

(A) Heatmap comparing transcription levels of the 318 RRRs at the late eight-cell stage. The expression levels of 158 out of the 318 RRRs are markedly (FC > 2) increased in response to KDM4A mRNA injection.

(B) Gene ontology analysis of the 206 KDM4A-responsive genes (FC > 2).

(C) Bar graphs and genome browser view of transcription levels of two representative KDM4A-responsive genes, UBTFL1 and THOC5, in IVF or SCNT (with or without KDM4A mRNA injection) eight-cell embryos.

See also Table S5.

in donor somatic cell genome indeed functions as a barrier for ZGA in human SCNT embryos, as is the case in mouse. To determine to what extent the injection of KDM4A mRNAs could overcome ZGA defects in the SCNT embryos, we performed RNA-seq of eight-cell SCNT embryos with or without KDM4A injection. Comparative transcriptome analyses indicated that as much as 50% (158) of the 318 RRRs were markedly upregulated by KDM4A mRNA injection (Figure 4A, FC > 2), indicating that erasure of H3K9me3 can at least partly facilitate ZGA in SCNT embryos.

To identify candidate genes that might help explain the improved development of KDM4A-injected SCNT embryos, we focused our analysis on genes (instead of repeats or non-coding regions). We identified 206 genes (Table S5) whose expression was significantly upregulated by KDM4A injection (FPKM > 5, FC > 2). Gene ontology analysis revealed that these genes were enriched for transcriptional regulation, ribosomal biogenesis, and RNA processing (Figure 4B), suggesting that dysregulation of these developmentally important machineries might be a cause of developmental arrest of SCNT embryos. Although the function of the majority of the 206 genes in preimplantation development is currently unknown, two of them, UBTFL1 and THOC5 (Figure 4C), are known to be required for normal preimplantation development in mice (Wang et al., 2013; Yamada et al., 2010). Therefore, defective activation of these genes is at least partly responsible for the poor development of human SCNT embryos.

DISCUSSION

After decades of efforts, human NT-ESCs were finally derived recently (Chung et al., 2014; Tachibana et al., 2013; Yamada et al., 2014). These advances were mainly due to optimization of SCNT derivation conditions. However, the intrinsic defects

(D) Scatterplot comparing gene expression levels between a control ESC line (ESC15) and a representative NTK-ESC, NTK6. Differentially expressed genes (FC > 3.0) are shown as black dots.

(E) Hierarchical clustering of NTK-ESCs, control ESCs, and donor dermal fibroblast cells based on RNA-seq datasets.

(F) Representative images of immunostained embryoid bodies (EBs) spontaneously differentiated in vitro for 2 weeks. Scale bar, 100 μm.

(G) Representative histological images of teratoma derived from NTK6 at 12 weeks after transplantation. Scale bar, 100 μm.

(H) Representative images of cytogenetic G-banding analysis of NTK6.

(I) Nuclear DNA genotyping using 16 STR markers.

(J) mtDNA genotyping of a representative single nucleotide polymorphism (SNP) site.

See also Figures S2 and S3.

in epigenetic reprogramming that cause the developmental arrest of human SCNT embryos have not been identified. In this study, we demonstrate that similar to mouse, H3K9me3 in somatic cell genome presents a barrier for human SCNT reprogramming. Removal of this barrier by overexpressing the H3K9me3 demethylase KDM4A facilitates transcriptional reprogramming at ZGA, thereby allowing human SCNT embryos to develop more efficiently to generate blastocysts, from which we successfully established multiple AMD-patient-specific NT-ESC lines without compromising genomic stability or pluripotency. Our study thus not only demonstrates that H3K9me3 serves as a general reprogramming barrier, but it also establishes a practical approach for improving cloning efficiency.

It has been well known that the ability of human oocytes to support SCNT embryo development varies greatly among oocyte donors. Indeed, human NT-ESCs can be derived only when high-quality oocytes donated by a small group of females were used as recipients (Chung et al., 2014; Tachibana et al., 2013; Yamada et al., 2014), although the reason for the dependence on oocyte quality remains elusive. Consistently, oocytes from only one (ID #58) out of the four donors supported SCNT blastocyst formation without KDM4A mRNA injection even under the presence of TSA, which has been reported to enhance blastocyst formation (Tachibana et al., 2013) (Figure 2H and Table S4). In contrast, oocytes of all four donors tested supported blastocyst formation when KDM4A mRNAs were injected, indicating that KDM4A can overcome the donor variation problem. Whether KDM4A can improve IVF embryo development remains to be determined.

Although the developmental potential of human SCNT embryos reaching the blastocyst stage was significantly and consistently improved by KDM4A mRNA injection, the magnitude of improvement was not as drastic as that of mice (90% in mice versus 27% in human). One possible explanation to this species difference is that the quality of human oocytes varies greatly even within the same batch of oocytes derived from a single ovulation, and only a fraction of them have the capacity to support development to the blastocyst stage even by IVF, which has a varying success rate of 15%–60% (Shapiro et al., 2002; Stone et al., 2014). This is in clear contrast to mouse IVF where more than 90% of embryos can develop to the blastocyst stage. It is therefore possible that some of the human oocytes that we used in our experiments could not support blastocyst formation even by IVF.

In addition to demonstrating the efficacy of KDM4A in improving human SCNT efficiency and NT-ESC derivation, another important finding of this study is that KDM4A can facilitate both mouse and human SCNT reprogramming. Considering that human KDM4A can function in mouse SCNT embryos to achieve an effect similar to that of Kdm4d, it is likely that all members of the KDM4 family can be used to facilitate SCNT as long as they possess H3K9me3 demethylase activity. Given that H3K9me3 in somatic genome functions as a reprogramming barrier in both mouse and human, it is likely that this reprogramming barrier might be conserved in other mammalian species as well. If this is the case, our KDM4-assisted SCNT method should be generally applicable to the cloning of other mammalian species.

In summary, we have established an improved KDM4-assisted SCNT method. Using this method, we derived blastocysts from adult AMD patient cells and subsequently established multiple NTK-ESCs with genomes identical to those of donor patients. This provides unique and important cell sources for understanding AMD as well as for therapeutic drug screening for AMD treatments. Given that the same strategy can be applied to the studies of other human diseases, we anticipate that our study will have a general impact on human therapeutics. Additionally, since SCNT allows replacement of somatic cell mitochondria with that of the recipient oocyte, as we have demonstrated in this study (Figures 3H–3J and S3), it provides an opportunity to treat mtDNA-related diseases. Indeed, a recent study demonstrated that a metabolic syndrome phenotype caused by mtDNA mutation can be corrected by replacing mtDNA through SCNT (Ma et al., 2015). Thus, our KDM4-assisted SCNT method should be useful for mtDNA-replacement therapies.

EXPERIMENTAL PROCEDURES

Human SCNT Procedure and KDM4A mRNA Injection

All MII stage oocytes with distinctive first polar bodies were enucleated under an inverted microscope equipped with a Poloscope (Oosight, Cambridge Research & Instrumentation). The enucleation and nuclear donor cell fusion were carried out in the presence of caffeine (1.25 mM). For enucleation, oocytes were pre-incubated in Global HTF medium with HEPES (Life Global) containing 0.5 μg/ml cytochalasin B and caffeine (1.25 mM) for 5 min. Then, the spindle complex was removed using a PIEZO actuator (Primetech). Dermal fibroblast cells resuspended in a drop containing HVJ-E extract (Cosmo Bio) were inserted into the perivitelline space of the enucleated oocytes. The reconstructed oocytes were kept in the manipulation medium containing caffeine (1.25 mM) until cell fusion was confirmed, and then the reconstructed oocytes were transferred into Global medium (10% SPS) and incubated for 1–1.5 hr before activation. Activation was carried out by the application of electropulses (2× 50 μs DC pulses, 2.7 kV/cm) in 0.25 M d-sorbitol buffer and 6-DMAP (2 mM, 4 hr) as previously described (Tachibana et al., 2013). The activated embryos were transferred to Global 10% SPS medium supplemented with TSA (10 nM, Sigma) for 12 hr, and then the embryos were transferred to Global 10% FBS without TSA and cultured for up to 7 days in an incubator with an atmosphere of 6% CO_2/5% O_2/89% N_2 at 37°C. The culture medium was changed on day 3.

For mRNA injection, the activated SCNT embryos were washed and cultured in Global 10% SPS for 1 hr before the KDM4A mRNA injection. Approximately 10 pl of KDM4A mRNA was injected into the SCNT embryos at 5 hr after activation in HEPES-HTF 10% SPS medium using a PIEZO actuator as described previously (Matoba et al., 2014). More details on donor cell preparation, mRNA preparation, RNA-seq, and other procedures can be found in the Supplemental Experimental Procedures.

Identification of Human RRRs

A sliding window (size 20 kb, step size 10 kb) was used to assess the genome-wide expression level of four-cell and eight-cell human embryos. For each window, the expression level was quantified with normalized RPM (reads per millions of uniquely mapped reads). The significantly activated regions in eight-cell relative to four-cell IVF embryos were identified with stringent criteria (FC > 5, RPM > 5 in eight-cell IVF embryos), and the overlapping regions were merged. These activated regions were classed into three groups based on their expression differences in human SCNT and IVF eight-cell embryos.

ACCESSION NUMBERS

The accession number of the RNA-seq datasets reported in this paper is GEO: GSE73362.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes three figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.10.001.

AUTHOR CONTRIBUTIONS

Y.Z. conceived the project. Y.G.C., S.M., K.Y.C., D.R.L., and Y.Z. designed the experiments. S.M. performed mouse SCNT experiments. Y.G.C., J.H.E., and D.R.L. performed human SCNT experiments. Y.L. conducted bioinformatics analyses. Y.G.C., S.M., W.J., J.E.L., and D.R.L. analyzed NTK-ESCs. V.S. performed oocyte retrievals. S.M. and F.L. prepared RNA-seq libraries and F.L. performed RNA-seq. S.M. and Y.Z. wrote the manuscript.

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