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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 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 NT-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 reprogrammed 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 donor (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 to develop 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, but 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 ectopically overexpressing 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 (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 to blastocyst without the help of KDM4A overexpression. Thus, our study expands the usability of oocyte donors and establishes the histone demethylase-assisted SCNT as a general method for improving mammalian SCNT for reproductive and therapeutic cloning.
RESULTS

Identification of reprogramming resistant regions in 8-cell human SCNT embryos

Human zygotic genome activation (ZGA) takes place during the late 4-cell to the late 8-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 20-160 kb in sizes (Table S1) that were activated at least 5-fold at the 8-cell stage compared to the 4-cell stage (Figure 1B).

To determine whether ZGA takes place properly in human SCNT, we collected late 8-cell stage embryos (5/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 method). 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 <= 2, IVF vs 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 <= 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 8-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 that 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; The Encode Consortium Project, 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 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 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 mouse SCNT model that the H3K9me3 barrier could be removed by injecting mRNAs encoding the mouse H3K9me3 demethylase, Kdm4d (Matoba et al., 2014). Before moving into 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 hours post-activation (hpa) following the same procedure used in our previous study (Figure 2A) (Matoba et al., 2014). Immunostaining revealed that injection of wild-type, but not a catalytic mutant, human KDM4A mRNA greatly reduced H3K9me3 levels in the nucleus of mouse SCNT embryos (Figure 1B). Importantly, injection of
KDM4A mRNA greatly increased the developmental potential of SCNT embryos with 90.3% of them develop to the blastocyst stage, which is in contrast to the 26% blastocyst formation rate in control (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 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 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 (Bressler et al., 1988) as nuclear donors.

To reaffirm the beneficial effect of the 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. 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 2-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 8-cell stage (68.8% vs 71.4%) (Figure 2F and Table S4). However, the beneficial effect became clear at the morula stage (16.7% vs 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 nuclear transfer ESCs (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 removal of the zona pellucida, the expanded blastocysts were cultured on irradiated mouse embryonic fibroblasts (MEF) 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 similar levels as 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 the 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 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 the sixteen repeat markers located across the genome showed perfect match between donor somatic cells and their derivative NTK-ESCs (Figures 3I and S3B). Mitochondrial DNA 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 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 8-cell SCNT embryos**

The fact that KDM4A mRNA injection significantly improves SCNT embryo development post ZGA suggested that H3K9me3 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 8-cell SCNT embryos with or without KDM4A injection. Comparative transcriptome analyses indicated that as much as 50% (158) of the 318 RRRs were markedly up-regulated 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 gene(s) that might help explain the improved development of KDM4A injected SCNT embryos, we focused our analysis on genes. We identified 206 genes (Table S5) whose expression was significantly up-regulated 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., 2009).
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 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 as a general reprogramming barrier, but 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 vs. 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 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 a similar effect as Kdm4d does, 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 recipient oocyte, as we have demonstrated in this study (Figures 3H-J and S3), it provides an opportunity to treat mitochondrial DNA-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 1st 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 minutes. Then, the spindle complex was removed using a PIEZO actuator (Primetech, Japan). Dermal fibroblast cells resuspended in a drop containing HVJ-E extract (Cosmo Bio, USA) were inserted into the perivitelline space of the enucleated oocytes. The reconstructed oocytes were kept in the manipulation medium containing caffeine (1.25mM) until the cell fusion was confirmed, and then the reconstructed oocytes were transferred into Global medium 10% SPS, and incubated for 1-1.5 hours before activation. Activation was carried out by applying electropulses (2X 50µs DC pulses, 2.7 kV/cm) in 0.25M d-sorbitol buffer and 6-DMAP (2 mM, 4 hrs) as previously described (Tachibana et al., 2013). The activated embryos were transferred to Global 10% SPS medium supplemented with Trichostatin A (TSA, 10nM, Sigma) for 12 hrs, then the embryos were transferred to Global 10% FBS without TSA and cultured for up to 7 days in an incubator with atmosphere of 6% CO2 / 5% O2/ 89% N2 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 were injected into the SCNT embryos at 5 hours 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 reprogramming resistant regions
A sliding window (size 20 kb, step size 10 kb) was used to assess the genome-wide expression level of 4-cell and 8-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 8-cell relative to 4-cell IVF embryos were identified with stringent criteria (FC > 5, RPM > 5 in 8-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 8-cell embryos.

SUPPLEMENTAL INFORMATION
Supplemental Information including 3 supplemental figures, 5 supplemental tables, Supplemental Experimental Procedures, and supplemental references which can be found online at xxxx

AUTHOR CONTRIBUTIONS
YZ conceived the project. YGC, SM, KYC, DRL and YZ designed the experiments. SM performed mouse SCNT experiments. YGC, JHE and DRL performed human SCNT experiments. YL conducted bioinformatics analyses. YGC, SM, WJ, JEL and DRL analyzed NTK-ESCs. VS performed oocyte retrievals. SM and FL prepared RNA-seq libraries and FL performed RNA-seq. SM and YZ wrote the manuscript.

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REFERENCES


FIGURE LEGENDS

Figure 1. Human reprogramming resistant regions (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 4-cell to the 8-cell stage in IVF embryos. RNA-seq data sets were obtained from a previous publication (Xue et al., 2013).

(C) Heatmap illustration of the transcriptome comparing donor somatic cells, IVF and SCNT embryos at the 8-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 8-cell embryos. FRRs, PRRs, and RRRs indicate fully reprogrammed regions (FC <= 2), partially reprogrammed regions (2 < FC<=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 data sets were obtained from the ENCODE project (Bernstein et al., 2012; The Encode Consortium Project, 2011).

(E and F) Box plots 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 data sets 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, Tables S1 and S2.

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 1-cell stage SCNT embryos stained with anti-H3K9me3 and DAPI 5 at hours 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 s.d.

(D) Representative images of SCNT embryos after 120 hours 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 2-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.

**Figure 3. Establishment and characterization of NTK-ESCs from AMD patients**

(A) Summary of established NT-ESC lines using AMD patient fibroblasts as nuclear donor 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.

(D) Scatter plot 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 data sets.

(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) Mitochondrial DNA genotyping of a representative single nucleotide polymorphism (SNP) site.

See also Figures S2 and S3.

Figure 4. Partial restoration of transcription upon KDM4A mRNA injection in SCNT 8-cell embryos

(A) Heatmap comparing transcription levels of the 318 RRRs at the late 8-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) 8-cell embryos.

See also Table S5.
Figure 1

A

IVF

Donor: Dermal fibroblast

SCNT by HVJ-E activation

8-cell in caffeine(+) medium

B

C

Donor

SCNT by HVJ-E

activation by electropulse

and 6-DMAP

8-Cell

Regions

activated

at 8-Cell

200 kb

200 kb

ChIP-seq intensity

(ChIP − input, FPKM)

DNaseI-seq intensity

(FPKM)

D

H3K9me3

H3K4me3

Regions activated at 8-Cell

200 kb

200 kb

E

Nhlf

K562

F

K562

IMR90

DNaseI-seq intensity

(FPKM)
Figure 2

A. \textit{MOUSE}

-1 h \rightarrow 0 h \rightarrow 5 h \rightarrow 10 h \rightarrow 4 days

- SCNT
- activation
- mRNA injection
- blastocyst

B. H3K9me3

control \quad KDM4A WT \quad KDM4A MUT

DAPI

C. Developmental Rate

\begin{align*}
\text{Developmental Rate} & \quad \text{control} \quad \text{KDM4A WT} \quad \text{KDM4A MUT} \\
\text{2-cell} & \quad 100 \quad 100 \quad 100 \\
\text{4-cell} & \quad 87.5 \quad 89.3 \\
\text{8-cell} & \quad 68.8 \quad 71.4 \\
\text{Morula} & \quad 16.7 \quad 4.2 \quad 0 \\
\text{Blast} & \quad 16.7 \quad 4.2 \quad 0 \\
\text{ExBlast} & \quad 0 \quad 0 \quad 0 \\
\end{align*}

P<0.02

P<0.03

D. control \quad KDM4A WT \quad KDM4A MUT

E. \textit{HUMAN}

Dermal fibroblast of AMD patient

SCNT by HVJ-E

activation

Injection of KDM4A mRNA

expanded blastocyst

F. Developmental Rate

\begin{align*}
\text{Developmental Rate} & \quad \text{control (n=48)} \quad \text{KDM4A (n=56)} \\
\text{2-cell} & \quad 100 \quad 100 \\
\text{4-cell} & \quad 87.5 \quad 89.3 \\
\text{8-cell} & \quad 68.8 \quad 71.4 \\
\text{Morula} & \quad 16.7 \quad 4.2 \quad 0 \\
\text{Blast} & \quad 16.7 \quad 4.2 \quad 0 \\
\text{ExBlast} & \quad 0 \quad 0 \quad 0 \\
\end{align*}

P<0.02

P<0.03

G. Uninjected control

KDM4A mRNA injected

expanded blastocyst

H. Oocyte donor #49

Oocyte donor #60

Oocyte donor #58

Oocyte donor #63

Developmental Rate

\begin{align*}
\text{Developmental Rate} & \quad \text{control (n=15)} \quad \text{KDM4A (n=16)} \\
\text{2c} & \quad 0 \quad 0 \\
\text{4c} & \quad 0 \quad 0 \\
\text{8c} & \quad 0 \quad 0 \\
\text{Mo} & \quad 100 \quad 100 \\
\text{Bl} & \quad 100 \quad 100 \\
\text{EB} & \quad 100 \quad 100 \\
\text{Developmental Rate} & \quad \text{control (n=12)} \quad \text{KDM4A (n=13)} \\
\text{2c} & \quad 0 \quad 0 \\
\text{4c} & \quad 0 \quad 0 \\
\text{8c} & \quad 0 \quad 0 \\
\text{Mo} & \quad 100 \quad 100 \\
\text{Bl} & \quad 100 \quad 100 \\
\text{EB} & \quad 100 \quad 100 \\
\text{Developmental Rate} & \quad \text{control (n=13)} \quad \text{KDM4A (n=10)} \\
\text{2c} & \quad 0 \quad 0 \\
\text{4c} & \quad 0 \quad 0 \\
\text{8c} & \quad 0 \quad 0 \\
\text{Mo} & \quad 100 \quad 100 \\
\text{Bl} & \quad 100 \quad 100 \\
\text{EB} & \quad 100 \quad 100 \\
\text{Developmental Rate} & \quad \text{control (n=8)} \quad \text{KDM4A (n=17)} \\
\text{2c} & \quad 0 \quad 0 \\
\text{4c} & \quad 0 \quad 0 \\
\text{8c} & \quad 0 \quad 0 \\
\text{Mo} & \quad 100 \quad 100 \\
\text{Bl} & \quad 100 \quad 100 \\
\text{EB} & \quad 100 \quad 100 \\
\end{align*}
Figure 3

A

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<th>Oocyte donor</th>
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<th>No. of ExBlast</th>
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<th>Name of NTK-ESCs</th>
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B

NTK6

C

Expression level (FPKM)

D

ESC15 vs. NTK6

r = 0.99

E

Fibroblast

F

NTK6

G

NTK6

H

NTK6 (XX)

I

<table>
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<tr>
<th>Oocyte donor</th>
<th>Fibroblast</th>
<th>NTK6</th>
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<td>DFB-7 XX 42</td>
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<tr>
<td>#63</td>
<td>XX</td>
<td>XX</td>
<td>DFB-6 XX 52</td>
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</tbody>
</table>

J

rs2853826 (m. 10398 A>G)

rs2853826 (m. 10400 C>T)
Figure 4

**A**

[Heatmap showing expression levels of genes under different conditions]

**B**

<table>
<thead>
<tr>
<th>GO Terms</th>
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<td>4.30E-04</td>
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<tr>
<td>ribonucleoprotein complex biogenesis</td>
<td>7.80E-04</td>
</tr>
<tr>
<td>ribosome biogenesis</td>
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<td>RNA processing</td>
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**C**

[Expression level (FPKM) comparison between IVF, SCNT, control, and KDM4A for UBTFL1 and THOC5]
Figure S1. RRRs in human somatic cells possess heterochromatin features, Related to Figure 1.

(A) Box plots comparing the average ChIP-seq signals of six histone modifications at FRR, PRR, and RRR in human fibroblast cells (Nhlf).

(B and C) Box plots comparing the average intensities of H3K9me3-ChIP-seq (B) and DNaseI-seq (C) within FRR, PRR and RRR in different somatic cell types. ChIP-seq and DNaseI-seq data sets were obtained from ENCODE projects (ENCODE Project Consortium, 2011). Note
that H3K9me3 intensity is significantly enriched in RRRs compared to FRRs and PRRs, and DnaseI-seq intensity is significantly depleted in RRRs compared to FRRs and PRRs. *** p < 0.001, ** p < 0.01, * p < 0.05.

(D) Box plots comparing the average percentage of exonic sequences, which represents the density of protein coding genes, in FRR, PRR, and RRR in the human genome. *** p < 0.001, * p < 0.05.

(E) Box plots comparing the average percentage of repetitive sequence within FRR, PRR and RRR. *** p < 0.001, * p < 0.05. ns, not significant.
Figure S2. Human NTK-ESCs exhibit normal pluripotency, Related to Figure 3

(A) Representative immunostaining images of NTK-ESCs and IVF-derived control ESCs. ESC colonies were co-stained with anti-SOX2, anti-SSEA4 antibodies and DAPI. Scale bar, 100 μm.

(B) Scatter plot evaluation of the reproducibility of RNA-seq of different biological replicates of the control ESCs and NTK-ESCs.

(C) Scatter plots comparing global gene expression patterns between control ESCs and NTK-ESCs. Differentially expressed genes (FC > 3.0) are shown as black dots. Note that the correlation of each pair-wise comparison is extremely high (r = 0.95-0.99).

(D) Representative images of immunostained embryoid bodies (EBs) spontaneously differentiated in vitro for 2 weeks. EBs were stained with anti-TUJ1, anti-BRACHYURY or anti-AFP antibody together with DAPI. Scale bar, 100 μm.

(E) Representative histological images of teratoma derived from NTK-ESC#6 at 12 weeks after transplantation. Scale bar, 100 μm.
Figure S3. Human NTK-ESCs contain nuclear-donor derived genome and oocyte-donor derived mitochondria, Related to Figure 3

(A) Representative images of cytogenetic G-banding analysis showing normal karyotypes with expected sex chromosome compositions in the NTK-ESC lines NTK7 and NTK8.

(B) Nuclear DNA genotyping using 16 STR markers. Note that all STR markers of NTK-ESC NTK7 and NTK8 perfectly match those of the original nuclear donor fibroblast DFB-6 and DFB-8, respectively.

(C) Mitochondrial DNA genotyping of representative single nucleotide polymorphism (SNP) sites. Mitochondria of NTK-ESCs are exclusively derived from donor oocytes.
## Supplemental Tables

### Table S3. Preimplantation development of KDM4A-assisted mouse SCNT embryos, Related to Figure 2

<table>
<thead>
<tr>
<th>Donor cell-type</th>
<th>mRNA injected</th>
<th>Concentration of mRNA (ng/µl)</th>
<th>No. of replicates</th>
<th>No. of reconstructed 1-cell embryos</th>
<th>% cleaved per 1-cell ± SD</th>
<th>% 4-cell per 2-cell ± SD</th>
<th>% morula per 2-cell ± SD</th>
<th>% blast per 2-cell ± SD</th>
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</thead>
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<tr>
<td>Cumulus Water</td>
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<td>91</td>
<td>5</td>
<td>91</td>
<td>94.8 ± 2.9</td>
<td>45.6 ± 18.9</td>
<td>35.8 ± 5.6</td>
<td>26.0 ± 11.3</td>
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<tr>
<td>KDM4A WT</td>
<td>1680</td>
<td>75</td>
<td>3</td>
<td>75</td>
<td>97.0 ± 5.2</td>
<td>96.8 ± 2.7*</td>
<td>92.5 ± 3.6*</td>
<td>90.3 ± 0.3*</td>
</tr>
<tr>
<td>KDM4A MUT</td>
<td>1930</td>
<td>74</td>
<td>3</td>
<td>74</td>
<td>93.7 ± 2.7</td>
<td>43.3 ± 7.6</td>
<td>35.5 ± 13.5</td>
<td>23.7 ± 11.6</td>
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</table>

* P < 0.01 as compared with water injected control.
Table S4. Preimplantation development of KDM4A-assisted human SCNT embryos, Related to Figure 2

<table>
<thead>
<tr>
<th>Oocyte donor</th>
<th>mRNA injected*</th>
<th>Somatic cell donor</th>
<th>No. of donated MII oocyte</th>
<th>No. of reconstructed 1-cell embryos</th>
<th>No. of cleaved (per 1-cell)</th>
<th>No. of 4-cell (% per 2-cell)</th>
<th>No. of 8-cell (% per 2-cell)</th>
<th>No. of morula (% per 2-cell)</th>
<th>No. of blast (% per 2-cell)</th>
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<td>13 (76)</td>
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</table>

* Concentration of injected human KDM4A mRNAs is 1500 ng/μl. Control embryos are non-injected. blast: blastocyst. ex-blast: expanded blastocyst.
Supplemental Experimental Procedures

Mice
B6D2F1/J (BDF1) mice were produced by crossing C57BL/6J females with DBA/2J males, and were used for the collection of both oocyte and somatic nuclear donor for SCNT. All animal experiments were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

In vitro transcription of human KDM4A mRNA
In vitro transcription was performed as described previously (Matoba et al., 2014). Briefly, full length human KDM4A/JHDM3A cDNA was cloned into a pcDNA3.1 plasmid containing poly(A)83 at the 3’ end of cloning site. The catalytic defective mutant form of KDM4A (H188A) was generated using PrimeSTAR mutagenesis kit (TAKARA # R045A). mRNA was synthesized using the mMESSAGE mMACHINE T7 Ultra Kit (Life technologies # AM1345). The synthesized mRNA was dissolved in nuclease-free water. The concentration of mRNA was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Aliquots of mRNA were stored at –80°C until use.

Mouse SCNT and KDM4A mRNA injection
Mouse somatic cell nuclear transfer was carried out as described previously (Matoba et al., 2014). Briefly, both recipient MII oocytes and donor cumulus cells were collected from adult BDF1 female mice through superovulation by injecting 7.5 IU of pregnant mare serum gonadotropin (PMSG; Millipore # 367222) and 7.5 IU of human chorionic gonadotropin (hCG; Millipore # 230734). Fifteen to seventeen hours after the hCG injection, cumulus-oocyte complexes (COCs) were collected from the oviducts and treated briefly with Hepes-buffered potassium simplex-optimized medium (KSOM) containing 300 U/ml bovine testicular hyaluronidase (Calbiochem # 385931) to obtain dissociated MII oocytes and cumulus cells. Isolated MII oocytes were enucleated in Hepes-buffered KSOM medium containing 7.5 μg/ml of cytochalasin B (Calbiochem # 250233) by using Piezo-driven micromanipulator (Primetech # PMM-150FU). The nuclei of donor cumulus cells were injected into the enucleated oocytes. After 1h incubation in KSOM, reconstructed SCNT oocytes were activated by incubating in Ca-free KSOM containing 2.5 mM SrCl2 and 5 μg/ml cytochalasin B for 1 h, and further cultured in KSOM.
with cytochalasin B for 4 h. Activated SCNT embryos were washed 5 hrs after the onset of SrCl₂ treatment (hours post activation, hpa) and cultured in KSOM in a humidified atmosphere with 5% CO₂ at 37.8°C. The SCNT embryos were injected with ~10 pl of water (control), 1500 ng/μl wild-type or mutant (H188A) human KDM4A mRNA at 5-6 hpa by using a Piezo-driven micromanipulator. Preimplantation developmental rates were analyzed by Student’s T-test.

**Preparation of human oocytes**
The protocol for human oocyte experiments (CHA001) was approved by both the CHA Regenerative Medicine Institute (CHARMI) Stem Cell Research Oversight (SCRO) Committee and the Pearl Institutional Review Board (PIRB). Initial oocyte donor recruitment was performed via web-based advertisement as described previously (Chung et al., 2014). All donors were voluntary participants that were screened on the basis of their reproductive, medical, and psychological health according to the guidelines of the American Society for Reproductive Medicine (ASRM). Oocyte donors were financially reimbursed for their time, effort, loss of wages, travel related expenses, discomfort, and other related expenses associated with the donation processes pursuant to the guidelines established by ASRM.

Ovarian stimulation was carried out as described previously (Chung et al., 2014). Briefly, a combination of human recombinant follicle-stimulating hormone (rFSH, 225-300IU, Merck) and human menopausal gonadotropin (Menopur 75IU, Ferring) were used to stimulate ovary for 9-11 days with GnRH antagonist (Ganirelix acetate, Merck) suppression. Lupron 4mg was used to mimic the LH surge when 1 or 2 follicles reached 18 mm in diameter. All medications were administered through subcutaneous injections. Transvaginal oocyte retrieval was performed approximately 36 hours after the Lupron injection. The collected COCs were denuded with 50-80 IU/ml hyaluronidase (Sigma-Aldrich) within 1-2 hours after retrieval. Then, they were kept in Global medium supplemented with 10% serum protein supplement (SPS; Cooper Surgical) (IVF Online) until use.

**Donated human IVF embryos**
The IVF embryos used for this study were obtained from the patients who had the desired number of children after standard IVF procedures, and the remaining embryos were
cryopreserved in storage for several years (2-6 years). All donors voluntarily donated their embryos (multicell cleavage stage) for researches by signing an informed consent form. The embryo donation program for the research was approved by CHA Gangnam Medical Center’s IRB.

**Human donor somatic cell preparation and characterization**

To prepare human nuclear donor somatic cells, small pieces of abdominal skin (0.5 cm x 0.3 cm) were biopsied under local anesthesia and washed 3 times in PBS supplemented with an antibiotic/antimycotic solution (anti-anti 1X, Invitrogen) to remove any possible contaminants. All the somatic cell donors used in this study were AMD patients (AMD subtype: Central Areolar Choroidal Dystrophy). DFB-6 was derived from a 52-year old female. DFB-7 was derived from a 42-year old female. DFB-8 was derived from a 59-year old male.

The procedures for somatic nuclear donor cell preparation are essentially the same as previously described (Chung et al., 2014). Briefly, the skin explant was mechanically minced and treated with collagenase (type I, 200 unit/ml, Worthington-biochem) in DMEM supplemented with 10 μg/ml penicillin-streptomycin solution to dissociate the skin tissue. After incubation overnight, the dissociated cells were collected, washed twice and seeded onto 60-mm culture dishes containing DMEM (Invitrogen, with 10% FBS, 1% non-essential amino acids and 10μg/mL penicillin-streptomycin) solution at 37°C and 5% CO2. Once the cells reached 80% confluency, 1/2 of initial outgrowths were cryopreserved, and the remaining cells were kept passaged several times, with cells from each passage being cryopreserved. Frozen cells were subsequently thawed prior to SCNT and cultured in a 4-well dish (Nunc) until they reached confluency. They were then cultured in serum-starved DMEM (0.5% FBS) for 2-3 days to synchronize the cell cycle before use.

**Derivation of human NTK-ESCs from KDM4A-assisted SCNT blastocysts**

All expanded blastocysts were treated with acid Tyrode solution to remove the zona pellucida, then the entire blastocysts (without removing trophectoderm) were plated onto mitotically-inactivated mouse fibroblasts (MEFs, Global Stem Inc.) in knockout-DMEM supplemented with Knockout Serum Replacement (10% SR, Invitrogen), FBS (10% Hyclone), bFGF (30ng/ml),
human LIF (2000 units/ml, Sigma-Aldrich), and ROCK inhibitor (1μM, Sigma-Aldrich). The derivation medium was not changed for the next 3 days, then 1/2 medium was replaced with fresh medium without the ROCK inhibitor daily as previously described (Chung et al., 2008). After 3 passages, the amount of FBS was reduced to 2%, replacing it with SR. After 5 passages, the ES cells were cultured in DMEM/F12 supplemented with FGF (8 ng/ml, Invitrogen), SR (18%, Invitrogen), and FBS (2% Hyclone). After the 10 passages, the ES cells were maintained in DMEM/F12 supplemented with FGF (8 ng/ml) and 20% SR.

**Preparation of 8-cell human embryos for ZGA analysis**

The SCNT embryos used for ZGA analysis were generated using oocytes donated by a single healthy female (#64) and dermal fibroblast cells from an AMD patient (DFB-8). SCNT and IVF embryos were cultured up to late 8-cell stage, when the compaction of blastomeres is initiated, then they were treated briefly with acid Tyrode solution to remove zona pellucida. To prepare for the 8-cell SCNT embryo, oocytes from a single oocyte donor, and skin fibroblast cells from a single somatic nuclear donor were used. All the procedures are the same as described in the “Human SCNT procedure and KMD4A mRNA injection” section. Only embryos that reached the late 8-cell stage synchronically 74 hours post activation were collected and used for this experiment.

For preparation of the control IVF embryos, several donated early 8-cell stage IVF embryos were thawed and cultured for 5-7 hours to allow them to reach late 8-cell stage before being processed. After removal of the zona pellucida, the denuded embryos were washed 3 times in PBS, loaded into RNase and DNase free PCR tubes, spin downed, and snap frozen in liquid nitrogen. Then, they were kept at -80°C until use. As controls, dermal fibroblast cells of somatic nuclear donors were also prepared. Those fibroblast cells were cultured in a 25 cm² flask in DMEM 10% FBS, and approximately 10,000 cells/donor were collected, snap frozen, and stored at -80°C until use.

**Immunostaining**

Mouse 1-cell SCNT embryos, undifferentiated human ESC colonies or differentiated embryoid bodied (EBs) were fixed by 4% paraformaldehyde (PFA) for 20 min at room temperature. After three washes with PBS containing 10 mg/ml BSA (PBS/BSA), the fixed samples were
permeabilized for 15 min by incubation with 0.5% Triton-X 100. After blocking in PBS/BSA for 1 h at room temperature, these were incubated in a mixture of primary antibodies at 4°C overnight. The primary antibodies used are as follows: anti-H3K9me3 (Abcam, ab71604, 1:500), anti-NANOG (Abcam, ab109250, 1:200), anti-OCT-4 (Santa Cruz, sc-8628, 1:100), anti-TRA 1-60 (Millipore, MAB4360, 1:100), anti-SOX2 (R&D, AF2018, 1:200), anti-SSEA4 (Millipore, MAB4304, 1:100), anti-AFP (Alpha-1-Fetoprotein; Dako A0008, 1:100), anti-BRACHYURY (Abcam ab20680, 1:100), and TUJ1 (B-Tubulin; Covance PRB-435P, rabbit, 1:100). Following three washes, the samples were incubated with secondary antibodies that include donkey anti-goat TRITC (Jackson ImmunoResearch, 705-026-147), donkey anti-mouse 488 (Jackson ImmunoResearch, 715-486-151), donkey anti-goat 649 (Jackson ImmunoResearch, 705-496-147), donkey anti-rabbit TRITC (Jackson ImmunoResearch, 711026-152) for 1 h at room temperature. The nuclei were co-stained with DAPI (Vector Laboratories).

In vitro differentiation and teratoma assays of ESCs

For in vitro differentiation assay, ESCs were culture in low-attachment dishes in ESC medium without bFGF for 1 week until they formed embryoid bodies (EBs). Thereafter, EBs were transferred to four-well dishes (Nunc) coated with matrigel (BD Biosciences) and cultured for an additional week. After washing, blocking and permeabilization in PBS containing 1% BSA and 0.1% Triton-X, EBs were incubated with the primary antibodies overnight. After three washes with PBS containing 1% BSA, EBs were stained with secondary antibody and DAPI for 1 h and observed under fluorescent microscopy. For teratoma assay, approximately 1 x 10^5 of undifferentiated NTK-ESCs were injected into the testicle of a NOD/SCID mouse. For each NTK-ESC line, at least 3 animals were used. After 12 weeks, teratomas were excised, fixed in PFA, embedded in paraffin, sectioned and then analyzed histologically after staining as described previously (Chung et al., 2014).

Chromosome analysis

Chromosome analyses for both NTK-ESC lines were performed by a standard protocol as previously described (Chung et al., 2014). Metaphase spreads were stained by GTG (G-bands by trypsin using Giemsa)-banding technique and 20 metaphases were analyzed and karyotyped by
two cytogenetics experts. The ideogram was produced by the Ikaros karyotyping system (MetaSystems, Germany).

**RNA-sequencing analysis**

Five 8-cell embryos for each group were directly lysed and used for cDNA synthesis using SMART-Seq v4 Ultra Low Input RNA Kit (Clontech). For MEF donor, 10 ng total RNA was used for cDNA synthesis using SMART-Seq v4 Ultra Low Input RNA Kit. After amplification, the cDNA samples were fragmented using Covaris sonicator M220 to an average size of 150 bp (Covaris). Sequencing libraries were made with the fragmented DNA using NEBNext Ultra DNA Library Prep Kit for Illumina according to manufacturer’s instruction (New England Biolabs) with different barcodes. For each RNA-seq analysis of hESCs, 1 μg total RNA was used for mRNA purification. Barcoded RNA-seq libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). Single end 50 bp sequencing was performed on a HiSeq 2500 sequencer (Illumina). Sequencing reads were mapped to the human genome (hg19) with Tophat2. All programs were performed with default settings (unless otherwise specified). At least 22 million uniquely mapped reads were obtained for each sequencing library, and subsequently assembled into transcripts guided by the reference annotation (Refseq gene models) with Cufflinks v2.0.2. Expression level of each gene was quantified with normalized FPKM (fragments per kilobase of exon per million mapped fragments). Statistical analyses were performed with R (http://www.r-project.org/). Independent 2 group Wilcoxon rank sum tests were used to compare distributions using the wilcox.test function in R. Pearson’s r coefficient was calculated using the cor function with default parameters. The hierarchical clustering analysis of the global gene expression pattern in different samples was carried out using heatmap.2 function (gplots package) in R.

**Analyses of published ChIP-seq and DNA methylation data sets**

To perform the histone modification enrichment analyses in Figures 1, S1 we used the following published ChIP-seq and DNaseI-seq data sets: H3K9me3, H3K4me1, H3K4me2, H3K4me3, H3K27me3, H3K36me3, H3K27ac and H4K20me1 ChIP-seq in Nhlf fibroblast cells (ENCODE/Broad Histone project), H3K9me3 ChIP-seq in Hsmm and K562 cells (ENCODE/Broad Histone project), H3K9me3 ChIP-seq in Mcf7 cells (ENCODE/Sydh Histone
project), DnaseI-seq in IMR90, Hsmm, K562 and Mcf7 cells (ENCODE/OpenChromDnase project). We also used whole genome bisulfite sequence data sets of IMR90 cells from Roadmap Epigenomics project for DNA methylation analysis (Roadmap Epigenomics et al., 2015). The processed DNA methylation data in IMR90 was downloaded from http://egg2.wustl.edu/roadmap/web_portal/. ChIP-seq intensity was quantified with normalized FPKM. Position wise coverage of the genome by sequencing reads was determined and visualized as custom tracks in the UCSC genome browser. Independent 2-group Wilcoxon rank sum tests were used to compare the ChIP-seq distributions between each group using the wilcox.test function in R.
Supplemental References


Supplemental Movies & Spreadsheets
Click here to download Supplemental Movies & Spreadsheets: Tables S1,2,5.xlsx