Histone variant H3.3 is an essential maternal factor for oocyte reprogramming

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Mature oocyte cytoplasm can reprogram somatic cell nuclei to the pluripotent state through a series of sequential events including protein exchange between the donor nucleus and ooplasm, chromatin remodeling, and pluripotency gene reactivation. Maternal factors that are responsible for this reprogramming process remain largely unidentified. Here, we demonstrate that knockdown of histone variant H3.3 in mouse oocytes results in compromised reprogramming and down-regulation of key pluripotency genes; and this compromised reprogramming for developmental potentials and transcription of pluripotency genes can be rescued by injecting exogenous H3.3 mRNA, but not H3.2 mRNA, into oocytes in somatic cell nuclear transfer embryos. We show that maternal H3.3, and not H3.3 in the donor nucleus, is essential for successful reprogramming of somatic cell nucleus into the pluripotent state. Furthermore, H3.3 is involved in this reprogramming process by remodeling the donor nuclear chromatin through replacement of donor nucleus-derived H3 with de novo synthesized maternal H3.3 protein. Our study shows that H3.3 is a crucial maternal factor for oocyte reprogramming and provides a practical model to directly dissect the oocyte for its reprogramming capacity.

Pioneering nuclear transfer experiments in amphibians have revealed that the cytoplasm of the egg is able to reprogram a differentiated nucleus to the embryonic state (1, 2). The success of somatic cell nuclear transfer (SCNT) to produce cloned animals using enucleated metaphase II (MII) oocytes (3, 4), and, recently, the successful derivation of SCNT human embryonic stem cells (5), have demonstrated that maternal factors in the mature ooplasm are capable and sufficient to reprogram a differentiated cell nucleus to pluripotency. This process is known to involve a series of sequential events including protein exchange between donor nucleus and ooplasm, donor nuclear chromatin remodeling, and pluripotency gene reactivation (6–12). However, maternal factors responsible for this reprogramming process and the underlying mechanism(s) remain largely unknown.

Thousands of different maternal proteins and mRNAs have been found in mouse mature oocytes (13, 14), including variants of the core histone proteins that, along with DNA, constitute nucleosomes. Accumulating evidence suggests that histone variants play important roles in chromatin remodeling and epigenetic regulation orchestrating gene expression changes during reprogramming (12, 15, 16). In mammals, the histone variant H3.3 is encoded by two different genes (*h3f3a* and *h3f3b*), whose translation results in an identical protein product (17, 18). Unlike canonical H3 histones that are expressed and incorporated into chromatin during S phase, expression of H3.3 is not cell cycle-regulated, and the variant is expressed in quiescent cells, postmitotic cells, and proliferating cells throughout the whole cycle, enabling H3.3 deposition in a DNA synthesis-independent manner during and outside of S phase (19). It has been suggested that maternal H3.3 plays an important role in male pronucleus formation and male genome epigenetic reprogramming; indeed, maternal H3.3 is incorporated in the decondensing sperm nucleus as early as 1 h after fertilization (20–23). Considering the importance of H3.3 in oocyte fertilization, we sought to determine whether the H3.3 variant might also be a maternal "reprogramming factor" and whether it might play a specialized role during somatic cell reprogramming.

Here, we show that maternal H3.3 is critical for the development of SCNT embryos and for the reactivation of many key pluripotency genes. We demonstrate maternal H3.3 remodeling of donor nuclear chromatin through replacement of donor nucleus-derived H3 with de novo synthesized H3.3 protein, with overall replacement levels dependent on the identity of the donor nucleus.

Results

Quantitative RT-PCR (RT-qPCR) demonstrated that the cytoplasm of mouse MII oocytes is abundant with h3f3a and h3f3b transcripts (H3.3A and H3.3B; Fig. 1*A*). These maternal transcripts become largely depleted by the first embryonic cleavage (20 h after oocyte activation), whereas zygotic H3.3 is elevated after the two-cell stage (Fig. 1*B*). We microinjected siRNAs against H3.3A and H3.3B (siH3.3; 10 μM) into MII oocytes by using a fine capillary (3–5 μm in diameter), and found that maternal H3.3 mRNAs were significantly decreased and zygotic H3.3 was also suppressed up to 44 h after oocyte activation (Fig. 1 *C* and *D*). RT-qPCR analysis of H3.3A, H3.3B, H3.1, and H3.2 in the oocytes after siH3.3 injection demonstrated the specificity of H3.3 knockdown vs. the canonical histones (Fig. 1*C*). We next tested a serial dilution of siH3.3 concentrations for knockdown efficiency (the oocyte poses an upper limit to the injected volume that does not induce lysis; thus, the amount of siRNA that can be

Significance

A differentiated cell nucleus can be reprogrammed into the pluripotent state by maternal factors in ooplasm; the factors that are responsible for this reprogramming process have not yet been identified. In this paper, we show that histone variant H3.3 is one of the essential maternal factors involved in somatic nuclear reprogramming. Maternal H3.3, not H3.3 in the donor chromatin, is required for development and the reactivation of many key pluripotency genes in somatic cell nuclear transfer (SCNT) embryos. H3.3 facilitates reprogramming by remodeling the donor nuclear chromatin through replacement of donor H3 in chromatin with de novo synthesized maternal H3.3 at the beginning of reprogramming in SCNT embryos.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE56762).

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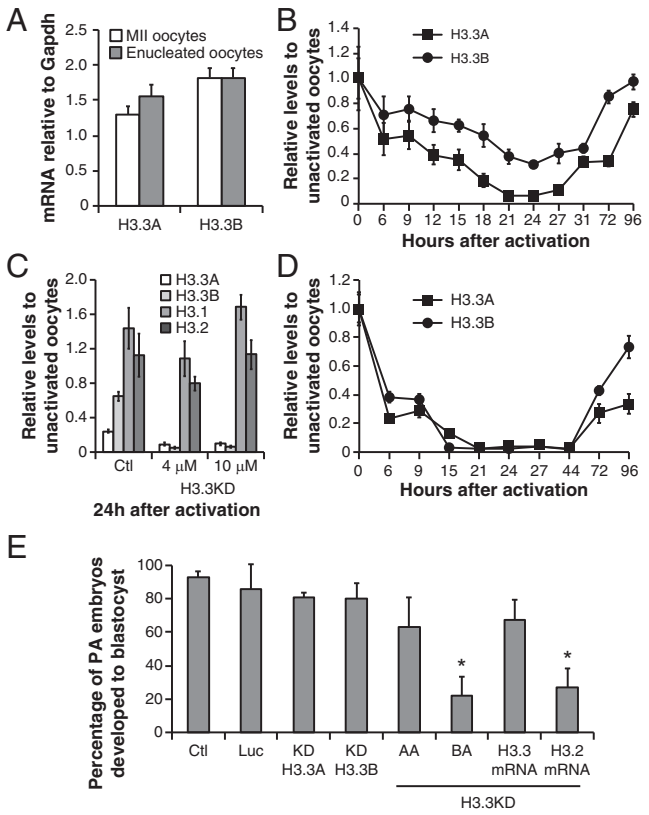

Fig. 1. H3.3 knockdown compromises embryonic development of PA embryos. (*A*) H3.3 transcript levels in MII and enucleated oocytes. For all panels, error bars indicate average ± SD ($n \geq 3$). (*B*) Dynamics of H3.3 transcript levels in PA embryos. Data are mean expression relative to Gapdh with unactivated oocytes normalized to 1. (*C*) Transcript analysis of H3.1, H3.2, and H3.3 levels after H3.3 siRNA (siH3.3) treatment and 24 h of activation. Data are mean expression relative to GAPDH with unactivated oocytes normalized to 1. H3.3KD embryos were subject to injection of 4 μM or 10 μM siH3.3 as indicated. (*D*) Dynamics of H3.3 transcript levels in oocytes treated with siH3.3 (four sets, 10 μM each) 1 h before activation. (*E*) Developmental potential of PA embryos. Control, unmanipulated PA embryos; KD-AA (after activation), siH3.3 (four sets, 4 μM each) 12 h after activation; KD-BA (before activation), siH3.3 (four sets, 4 μM each) before activation; KD+H3.2mRNA, H3.2mRNA (30 ng/μL) after siH3.3 (four sets, 4 μM each) injection; luciferase, siRNA against luciferase (16 μM); KD-H3.3A, siH3.3A only (two sets, 4 μM each); KD-H3.3B, siH3.3B only (two sets, 4 μM each); KD+H3.3mRNA, H3.3mRNA (30 ng/μL) after siH3.3 (four sets, 4 μM each) injection. Data were analyzed with χ^2 test, and statistical significance was determined compared with luciferase (*$P < 0.01$). Error bars indicate average ± SD ($n \geq 3$).

injected depends on concentration, not on volume) to determine optimal conditions for knockdown (four siRNA at 4 μM each, two against each gene; Fig. S1*A*).

As we have shown that injection of siH3.3 can specifically deplete maternal and zygotic H3.3 mRNAs, we further determined whether this decrease of H3.3 mRNAs would result in maternal mRNA-derived H3.3 protein depletion after oocyte activation. We had previously generated an ES cell line in which the endogenous *h3f3b* gene is C-terminally tagged with the HA epitope (H3.3B-HA) and an enhanced yellow fluorescent protein (EYFP) reporter of gene expression (24). We generated mice from H3.3B-HA–tagged ES cells, allowing us to track the H3.3 protein in vivo and in early embryos without the use of antibodies specific to H3.3. By using H3.3B-HA tagged oocytes for nuclear transfer with WT cumulus cells after siH3.3 injection, we validated that injection of siH3.3 resulted in depletion of maternal mRNA-derived H3.3 protein in the embryos up to 24 h after activation in the SCNT embryos (Fig. S1 *B–E*).

Knockdown of maternal H3.3 in oocytes resulted in significantly compromised embryonic development of parthenogenetically activated (PA) embryos, with arrest at the late morula or early blastocyst stage; uninjected oocytes and oocytes subjected to microinjection of an siRNA against luciferase served as controls (Fig. 1*E*, Fig. S2*A*, and Table S1). We next tested different siH3.3 concentrations on the developmental potential of PA embryos, and found that injection of four siH3.3 at 4 μM each was the lowest concentration that significantly decreased blastocyst potential (Fig. S2*B*), whereas injection of 16 μM luciferase siRNA did not significantly decrease the developmental potential of PA embryos (Fig. S2*B*). A similar phenotype in fertilized embryos was observed after maternal H3.3 knockdown in zygotes (25).

To examine whether exogenous H3 mRNAs (that are not the target of siH3.3) could rescue compromised development, we injected epitope-tagged H3.3-HA mRNA or H3.2-HA mRNA into oocytes after H3.3KD (Fig. 2*A* and Fig. S2 *C* and *D*). We found that the number of blastocyst stage embryos was significantly increased by injection of exogenous H3.3-HA mRNA, but not H3.2-HA mRNA (Fig. 1*E*, Fig. S2*A*, and Table S1), indicating that this phenotype is the result of maternal H3.3 depletion, not the reduction of total maternal histone H3 levels in H3.3KD embryos.

As the two H3.3 transcripts contain unique untranslated regions containing distinct regulatory elements, we next tested whether H3.3A and H3.3B are functionally equivalent during early embryonic development. We found no difference in embryonic developmental potential when only H3.3A or H3.3B was knocked down in oocytes (Fig. 1*E*, Fig. S2*A*, and Table S1), suggesting that H3.3A and H3.3B are functionally redundant and can compensate for each other in early mouse embryonic development.

To test whether compromised development was a result of knockdown of maternal H3.3 or zygotically transcribed H3.3, siH3.3 was injected before or 12 h after oocyte activation; injection of siH3.3 12 h after activation resulted in suppression of

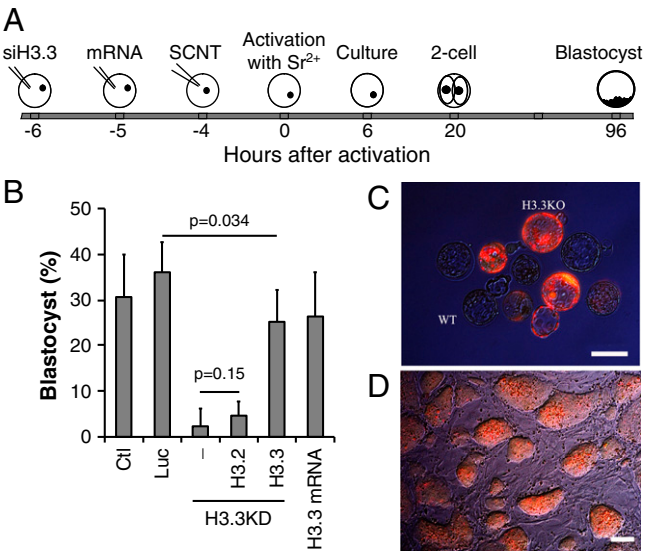

Fig. 2. Compromised reprogramming in H3.3KD SCNT embryos. (*A*) Schematic illustration of siH3.3 injection into oocytes and SCNT. (*B*) Developmental potential of control and H3.3KD cumulus SCNT embryos exogenously expressing H3.2 or H3.3. Embryos were treated as described for PA embryos in Fig. 1, with all injections occurring before nuclear transfer. Data were analyzed with the χ^2 test. Error bars indicate average ± SD ($n \geq 3$). (*C*) SCNT blastocysts produced by transfer of H3.3KO embryonic fibroblast nuclei into WT enucleated oocytes. WT MEFs from the same chimeric embryos were also used as control. (*D*) ES cell lines (ntESCs) established from H3.3KO nuclear transfer blastocysts. EYFP and mCherry indicate the deletion of H3.3B gene. (Scale bar: 100 μm.)

zygotic H3.3 with little effect on maternal H3.3 transcripts (Fig. 1*D*). A significantly higher percentage of embryos developed to the blastocyst stage when H3.3 was knocked down after activation, indicating that the maternal H3.3, rather than the zygotically transcribed H3.3, is critical for the development of PA embryos (Fig. 1*E*, Fig. S2*A*, and Table S1).

As we have shown that maternal H3.3 is critical for the development of PA embryos to blastocysts, we next investigated whether maternal H3.3 is a necessary factor for oocyte reprogramming of somatic nuclei (Fig. 2*A*). We observed significantly lower developmental potential for H3.3KD SCNT embryos compared with controls, with the majority arresting at the two- or four-cell stage and only a few reaching the morula or blastocyst stage (Fig. 2*B*, Fig. S3 *A* and *B*, and Table S2). In addition, we found that the embryonic developmental potential of the H3.3KD SCNT embryos was significantly improved when H3.3-HA mRNA, but not H3.2-HA mRNA, was injected into H3.3KD oocytes (Fig. 2*B*, Fig. S3*C*, and Table S2). Live cumulus cloned pups were also obtained by transfer of H3.3-addback morula/blastocysts into surrogate mice (Fig. S3*D*). We further tested whether overexpression of exogenous H3.3 mRNA in mature oocytes might bring any benefits to SCNT reprogramming. Unexpectedly, no significant improvement of developmental potential was observed in H3.3-HA mRNA-injected cloned embryos (Fig. 2*B* and Table S2).

Having demonstrated that maternal H3.3 in the oocytes is important for successful reprogramming of SCNT embryos, we then asked whether the presence of H3.3 protein in the donor cell nuclei is required for the cell to be reprogrammed. By using H3.3KO ES cells with fluorescent reporters (i.e., EYFP/mCherry; Table S3) (26) for blastocyst injection, we derived embryonic fibroblast cells [i.e., mouse embryonic fibroblasts (MEFs)] and isolated single cells for nuclear transfer. Embryos produced by transfer of H3.3-null MEF nuclei into WT enucleated oocytes were able to develop to the blastocyst stage and to derive ES cells, although at a reduced efficiency (Fig. 2 *C* and *D* and Table S4). This result is in agreement with our previous observation of H3.3KD PA embryos that maternal H3.3, rather than zygotically expressed H3.3, is important for early embryonic development. We conclude from the aforementioned experiments that maternal H3.3, and not H3.3 in the donor nuclei, is crucial for successful reprogramming of somatic cell nuclei into the pluripotent state.

Reactivation of pluripotency genes is a major event for successful reprogramming of somatic cells to the pluripotent state (27). To determine whether H3.3 is involved in the reactivation of pluripotency genes during reprogramming, we injected Oct4-EGFP MEFs labeled with H2B-mCherry into the blastomeres of two-cell PA WT or H3.3KD embryos (Fig. 3 *A* and *B*); these H3.3KD PA embryos can progress to morula stage, and reactivation of the somatic Oct4 gene can be easily monitored by expression of GFP in the resulting tetraploid morula or blastocyst embryos (Fig. 3*C*). Injection of Oct4-EGFP MEFs into the blastomeres of two-cell H3.3KD PA embryos resulted in only 36% of embryos (21 of 58) reactivating somatic Oct4 at the

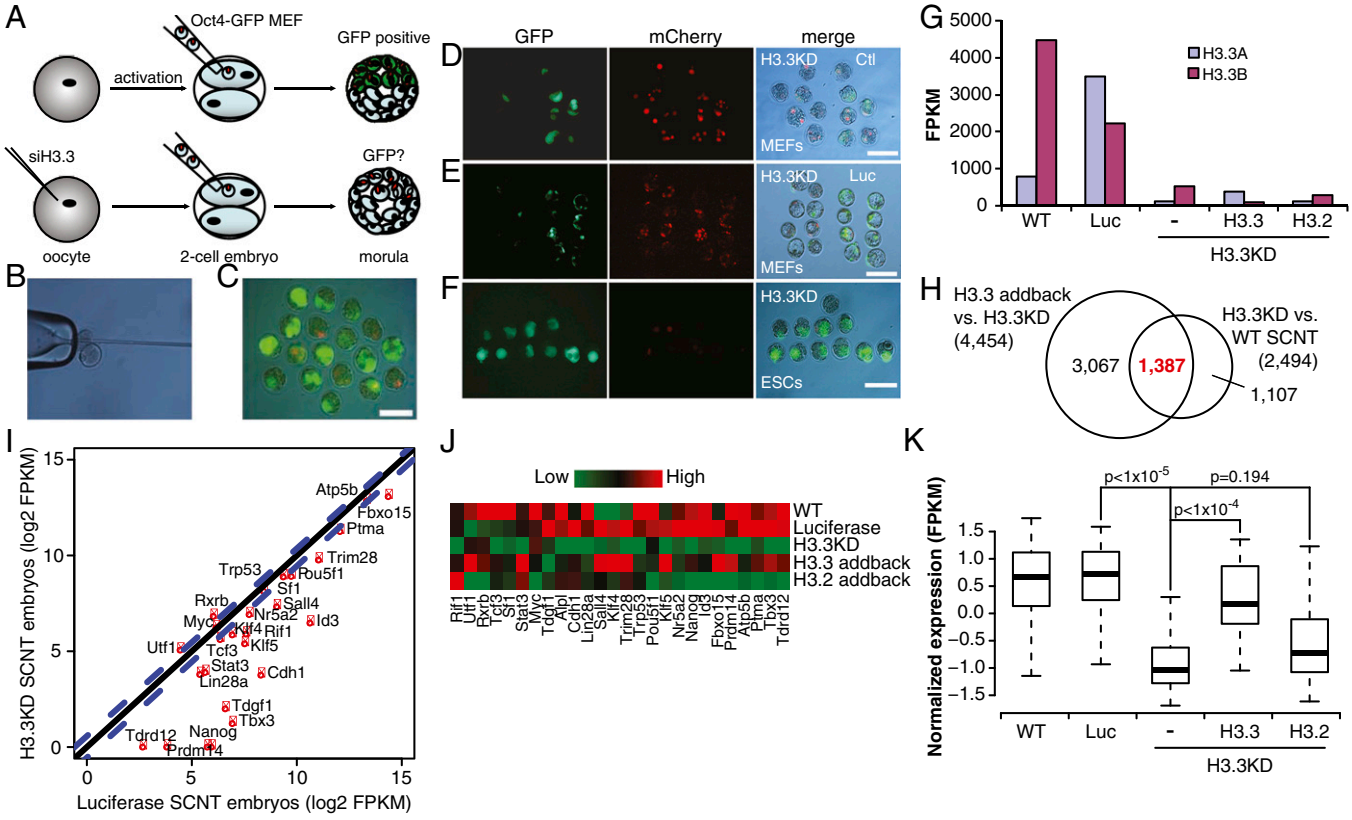

Fig. 3. Reactivation of pluripotency-associated genes is maternal H3.3-dependent in SCNT embryos. (*A*) Schematic illustration of blastomere injection and embryo culture. (*B*) Injection of Oct4-EGFP MEFs labeled with H2B-mCherry into one blastomere of a two-cell PA embryo and (*C*) culture to morula stage. (Scale bar: 100 μm.) (*D* and *E*) Injection of Oct4-EGFP MEFs into the blastomeres of H3.3KD two-cell embryos and control (*D*) or luciferase siRNA injected embryos (*E*). (*F*) Injection of Oct4-EGFP ES cells into the blastomeres of H3.3KD 2-cell embryos. (*G*) RNA-seq analysis of control and H3.3KD four-cell stage embryos exogenously expressing H3.2 or H3.3. (*H*) RNA-seq analysis of differential expression upon H3.3KD that was rescued by exogenous expression of H3.3. (*I*) RNA-seq analysis of 26 pluripotency genes (expressed in control SCNT embryos) in H3.3KD SCNT embryos. (*J*) Heat map of the 26 pluripotency genes in WT SCNT, luciferase-injected, H3.3KD, H3.3-addback, and H3.2-addback SCNT embryos at four-cell stage. (*K*) The average expression of the 26 pluripotency genes in control and H3.3KD embryos exogenously expressing H3.2 or H3.3.

morula or blastocyst stage. In contrast, 77% of control PA embryos (26 of 34) and 71% of PA embryos (12 of 17) injected with a luciferase siRNA were able to reactivate Oct4-driven EGFP expression from the somatic genome (Fig. 3 *D* and *E* and Fig. S4*A*). These results suggest that cytoplasmic expression of H3.3 can facilitate reactivation of the Oct4 gene during reprogramming. Notably, when Oct4-EGFP ES cells, in which the Oct4 gene is already active, are injected into the blastomeres of two-cell H3.3KD PA embryos, almost 90% of the injected embryos (26 of 29) are EGFP-positive at the morula stage (Fig. 3*F* and Fig. S4*A*), suggesting that cytoplasmic expression of H3.3 is not required for expression of Oct4 that is already active in early embryos. This result is consistent with our observation in ES cells that expression of pluripotent genes is not affected by H3.3 depletion (26).

We next performed high-throughput RNA sequencing (RNA-seq) of cumulus nuclear transfer (WT SCNT) embryos, Luciferase-injected SCNT, H3.3KD, H3.3KD+H3.3mRNA (H3.3-addback), and H3.3KD+H3.2mRNA (H3.2-addback) SCNT embryos at the four-cell stage, confirming H3.3A and H3.3B knockdown (Fig. 3*G*). We detected 2,494 genes that were down-regulated at least twofold in H3.3KD vs. WT SCNT, with 56% of these genes (n = 1,387) increased in expression at least 1.5-fold in H3.3-addback vs. H3.3KD SCNT embryos (Fig. 3*H*). Pathway analysis showed that many of these genes are involved in cell cycle, methylation, and gamete generation (Fig. S4*B*). Only a few pathways (mitochondrial and ribosome) were found in genes up-regulated upon H3.3KD (Fig. S4*C*).

To determine whether H3.3 played a role in pluripotency gene reactivation, we assembled a list of 26 known pluripotency-associated genes that are expressed in WT SCNT four-cell embryos based on our RNA-seq data. We found that the majority of these genes were down-regulated in H3.3KD vs. luciferase-injected SCNT embryos, with 20 of 26 genes down-regulated at least 1.5-fold upon H3.3KD (Fig. 3*I*). Sixteen of these 26 genes were increased in expression at least 1.5-fold in H3.3-addback vs. H3.3KD embryos (Fig. 3*J*). We found that the expression level of these 26 pluripotency-associated genes was overall significantly lower in H3.3KD SCNT embryos ($P < 1 \times 10^{-5}$), and H3.3 mRNA ($P < 1 \times 10^{-4}$), but not H3.2 mRNA ($P = 0.194$), rescued their expression (paired Wilcoxon test; Fig. 3*K*). Therefore, we show that the reactivation of key pluripotency genes is impaired upon H3.3KD and suggest that the reactivation of these pluripotency-associated genes is specifically dependent on maternal H3.3 during reprogramming in SCNT embryos.

To understand the mechanistic role of H3.3 in reactivation of pluripotency genes during somatic cell reprogramming, H3.3B-HA cumulus cells were used for nuclear transfer (Fig. 4*A*), and the donor nucleus-derived H3.3 protein in the nuclei of SCNT embryos was followed by using an HA antibody. We found that the donor nucleus-derived H3.3 protein was gradually lost from the transferred nuclei and became undetectable by immunofluorescence 20 h after activation (Fig. 4 *B*–*E*). To monitor incorporation of maternal H3.3 protein into reprogrammed nuclei, WT cumulus cells were transferred into H3.3B-HA enucleated oocytes (Fig. 4*F*). We found that maternal H3.3 protein accumulated in the ooplasm 3 h after activation (Fig. 4 *G* and *H*) and thereafter was incorporated into donor nuclei of SCNT embryos, with incorporation peaking at the two-cell stage (20 h after oocyte activation; Fig. 4 *I* and *J*). We next examined whether knockdown of maternal H3.3 transcripts in the oocytes could inhibit removal of donor nucleus-derived H3.3 from the nuclei of cumulus cell SCNT embryos. We found that donor nucleus-derived H3.3B-HA protein was still detectable in most of the H3.3KD SCNT embryos 20 h after activation (Fig. S5 *A* and *B*), suggesting that translation of the maternal H3.3 transcript might be required for donor-nucleus H3.3 eviction. To determine whether the loss of donor nucleus-derived H3.3 protein in SCNT embryos requires the translation of maternal mRNAs, the protein synthesis inhibitor cycloheximide was used to treat H3.3B-HA

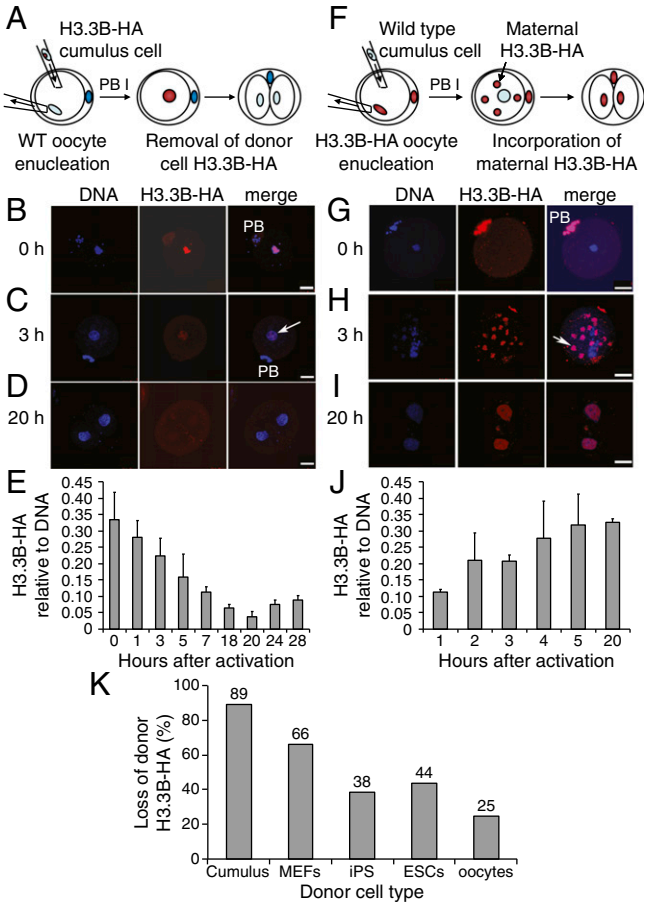

Fig. 4. Maternal H3.3 replaces donor nucleus-derived H3 in the nuclei of SCNT embryos. (*A*–*E*) Embryos were constructed by injecting an H3.3B-HA cumulus nucleus into a WT B6D2F1 enucleated oocyte (*A*), allowing us to monitor the change of donor nucleus-derived H3.3 in SCNT embryo by using immunofluorescence (*B*–*E*). Donor H3.3B-HA, indicated by white arrow, was undetectable by the two-cell stage (20 h after activation). PB, polar body. (*E*) Dynamics of H3.3B-HA removal from donor nuclei. Data represent average HA intensities relative to DNA at various time points, with error representing SD ($n \geq 5$). (*F*–*J*) Embryos were constructed by injecting a WT cumulus nucleus into an H3.3B-HA enucleated oocyte (*F*), allowing us to monitor the incorporation of maternal H3.3 into the donor nucleus using immunofluorescence (*G*–*J*). Maternal H3.3B-HA accumulated in the cytoplasm (*H*, white arrow) before incorporation into the donor nucleus at the two-cell stage (*I*). (*J*) Dynamics of maternal H3.3B-HA incorporation into SCNT embryos. Data represent average HA intensities relative to DNA at various time points, with error representing SD ($n \geq 5$). (Scale bar: 20 μm.) (*K*) H3.3B-HA cumulus, MEF, iPS, ES cell, and oocyte nuclei were used for nuclear transfer and H3.3B-HA signal in the nuclei of the NT embryos was measured at 0 h and 20 h after activation. Data represent the percentage of reduced H3.3B-HA intensities in the nuclei of the embryos 20 h after activation relative to 0 h [$(1 - HA_{20h}/HA_{0h}) \times 100\%$].

cumulus nuclear transfer embryos for 5 h during activation. Indeed, removal of donor nucleus-derived H3.3 protein was inhibited, with SCNT embryos showing strong nuclear H3.3B-HA staining 20 h after activation (Fig. S5 *A* and *B*). Together, these results suggest that loss of donor nucleus-derived H3.3 protein is dependent upon incorporation of maternal H3.3 protein into the donor cell nuclei of SCNT embryos during reprogramming.

Having established that maternal H3.3 is required for replacement of donor cell H3.3, we next wanted to determine the nature of the molecular replacement signature in the donor chromatin. We first tested whether the presence of H3.3 protein in the donor cell nucleus is required for the incorporation of maternal H3.3 in SCNT embryos. To this end, we transferred

H3.3-null nuclei (H3.3KO MEFs) into H3.3B-HA oocytes, and found that maternal H3.3B-HA protein was able to incorporate into the nuclei of NT embryos lacking H3.3 (Fig. S4*C*). Others have recently shown that all donor nucleus-derived histone H3 isoforms (i.e., H3.1, H3.2, and H3.3) are rapidly eliminated from the chromatin of nuclei transplanted into enucleated oocytes (11). Our findings, together with these published results, suggest that maternal H3.3 replaces donor nucleus-derived H3, irrespective of isoform, in the nuclei of SCNT embryos during reprogramming.

We next asked whether the loss of donor nucleus-derived H3.3 protein is associated with the nuclear state of donor cells. We used H3.3B-HA–tagged cell nuclei from cumulus, MEFs, ES cells, induced pluripotent stem (iPS) cells, and mature oocytes for nuclear transfer. The eviction of donor nucleus-derived H3.3 was measured by H3.3B-HA immunofluorescence intensity in the nuclei of NT embryos at 0 h and 20 h after activation. We found that 89% and 66% of the donor nucleus-derived H3.3 protein was lost from the cumulus and MEF cell NT embryos, respectively, whereas 44% and 38% of donor nucleus-derived H3.3 was lost from ES cells and iPS cell NT embryos, and only 25% from oocyte nucleus NT embryos at 20 h after activation (Fig. 4*K*). This result shows that the loss of donor nucleus-derived H3.3 protein is not a global effect but is instead dependent on the identity of the donor nucleus.

Discussion

We have demonstrated that H3.3 is an essential maternal factor in oocytes for somatic cell reprogramming. Knockdown of maternal H3.3 results in compromised reprogramming and failure to reactivate many key pluripotency genes; this compromised reprogramming is rescued by injecting exogenous H3.3 mRNA into oocytes for SCNT embryos. We showed that maternal H3.3 is involved in reprogramming through replacement of donor nucleus-derived H3 (H3.3 and canonical H3) with de novo synthesized H3.3, and that replacement is not a global effect but is instead dependent on the identity of the donor nucleus.

Since the pioneering experiments of nuclear transfer in amphibians (1, 2), the mature oocyte cytoplasm has been proven to efficiently reprogram a somatic cell nucleus to totipotency or pluripotency in various mammalian species via SCNT (3–5). The most obvious first response of the donor nucleus to reprogramming is a huge increase in volume, accompanied by the exchange of proteins between nucleus and cytoplasm (27). Approximately 80% of the proteins brought in with the donor nucleus are lost within a few hours, and there is an obvious migration of maternal proteins into the donor nucleus (28). The second response of the donor nucleus is nuclear remodeling, including the nuclear structure remodeling and changes of DNA and histone modifications in chromatin (6–8, 11, 28, 29). Following nuclear remodeling, there is a switch of gene expression in the donor nucleus from somatic to embryonic, requiring reactivation of pluripotency genes such as Oct4 and Nanog (27). Reprogramming in the oocyte occurs in an ordered manner on a defined time scale, and has been proposed to be a deterministic process (30). Despite remarkable progress in our understanding of oocyte reprogramming, the oocyte is still considered mechanistically to be a "black box." In this study, we find that maternal H3.3 is essential for oocyte reprogramming, and that it plays a critical role in remodeling the donor nucleus to alter the "epigenetic landscape" for gene reactivation, providing a molecular model to link together events that have long been observed but are still poorly understood during somatic cell reprogramming.

Somatic nuclear reprogramming is characterized by a global shift in gene expression from the somatic to the embryonic state. Numerous studies suggest that histone variants are closely involved in this process through remodeling the chromatin of the donor nuclei. For example, histone B4 (also known as H1foo), an oocyte-specific linker histone variant that replaces the somatic-type linker histone H1 during reprogramming mediated by SCNT, is required for Oct4 reactivation (12, 31). MacroH2A, a histone variant of H2A, acts as a barrier for pluripotency gene reactivation during reprogramming (15, 32). A recent study showed that the HIRA-dependent incorporation of histone H3.3 into donor nuclei is an early and necessary step for global changes in transcription during reprogramming in the *Xenopus* system (9). By injection of exogenous H3.3 mRNA into the *Xenopus* egg before nuclear transfer using mouse cells, Jullien et al. (9) found that exogenous H3.3 protein could be found in the promoter of Oct4, and major satellite and rDNA, and that H3.3 enrichment was associated with increased expression of these genes. This observation is generally in agreement with the present study in the mouse developmental system. By using siRNAs and our H3.3B-HA tagged mice, we have targeted endogenous H3.3 for depletion, and demonstrate that de novo synthesized H3.3, and not H3.3 in the donor nucleus, is important to facilitate reprogramming of the somatic cell to pluripotency in oocyte reprogramming. Although canonical H3 is expressed only during S phase, showing replication-dependent incorporation by its chaperone, CAF-1, H3.3 is expressed throughout the cell cycle and shows a replication-independent incorporation mediated by at least two independent chaperone systems, with the Hira complex mainly responsible for genic deposition and the Atrx/Daxx complex responsible for H3.3 incorporation at repeat regions and telomeres (19). The availability of H3.3 outside of S phase allows this variant to participate in many replication-independent processes requiring nucleosome replacement, such as the chromatin remodeling for gene reactivation during reprogramming; this variant is particularly important for the development of mouse SCNT embryos in that the genome activation of these embryos occurs at the early two-cell stage (20 h after activation) and may require reactivation of early embryonic developmental genes before DNA replication, accounting for the compromised embryonic development of H3.3KD SCNT embryos.

In conclusion, we have identified H3.3 as an essential maternal factor in the oocyte for somatic cell reprogramming. How H3.3 replacement is initiated and factors involved in this replacement will be determined by future studies, and whether this replacement is associated with specific histone modifications should be further investigated.

Materials and Methods

Injection of siRNA and mRNA into MII Oocytes. MII oocytes from superovulated B6D2F1 females with pregnant mare serum gonadotropin (G4527; Sigma) were recovered 14–16 h of human chorionic gonadotropin injection. siRNAs or mRNAs were injected into the oocytes with a piezo-operated micro-capillary pipette (3–5 μm inner diameter). After injection, oocytes were kept at room temperature for 30 min and then moved into the incubator for at least another 30 min before enucleation or parthenogenetic activation. siRNA and cDNA sequences are provided in *SI Materials and Methods*.

SCNT. SCNT was performed as previously described (4). B6D2F1 oocytes were transferred into a droplet of Hepes–Chatot, Ziomek, Bavister (CZB) medium containing 5 $\mu g \cdot mL^{-1}$ cytochalasin B. Oocytes undergoing micromanipulation were held with a holding pipette, the MII chromosome–spindle complex was aspirated into the pipette with a minimal volume of oocyte cytoplasm. After enucleation, oocytes were transferred into cytochalasin B-free KSOM media (Specialty Media, Cat#MR-106-D) and returned to the incubator. The donor nuclei were gently aspirated in and out of the injection pipette until their nuclei were largely devoid of visible cytoplasmic material. Each nucleus was injected into a separate enucleated oocyte. Following somatic-cell nucleus injection, oocytes were activated by culturing in Ca^{2+}-free CZB containing 10 mM Sr^{2+} and 5 $\mu g \cdot mL^{-1}$ cytochalasin B for 5 h and cultured at 37 °C under 5% (vol/vol) CO_2 in air.

Immunohistochemistry, Confocal Imaging, and Image Analyses. For immunohistochemistry staining, oocytes or embryos were fixed [4% (wt/vol) paraformaldehyde], permeabilized (0.5% Triton X-100 in PBS solution), blocked [10% (vol/vol) normal donkey serum and 0.5% Triton in PBS solution], and incubated in working dilutions of the antibodies. As primary antibodies, anti-HA goat IgG (ab9134, 1:100; Abcam) were used. As secondary antibodies, anti-rabbit and anti-goat IgG, conjugated with Alexa Fluor 546 (A-11036) and 647 (A-21245), were applied (all Invitrogen). Imaging was performed with a Zeiss 710 confocal

imaging system. *Z*-stack images with 20 consequential sections for each embryo were taken. For quantification of H3.3B-HA, the full project image was generated from the *Z*-stack files and the mean pixel intensity values for each channel were determined throughout gating individual nuclei of the embryos. The signal intensities we obtained from the full projection images are the mean intensities of each channel, which gated only the nuclei, so the size of each channel we gated in one image is the same and the intensities are all associated with DNA. We normalized the mean intensities of each channel to the mean intensities of DNA channel in the same image, so the value is not related to nucleus size. The values we obtained this way displayed an obvious pattern that was reproducible among different experiments. Data were exported and analyzed in Excel (Microsoft).

RT-qPCR. Primers were designed to span an exon–exon junction (*SI Materials and Methods*). RT-qPCR was performed by using an Applied Biosystems StepOnePlus system and Power SYBR Green PCR Master Mix. RNA from 3 to 10 oocytes or embryos was isolated by using TRIzol, and cDNA was made by using SuperScript III (Invitrogen). cDNA was treated with RNaseH and diluted 1:10 in H_2O, with 8 μL used per PCR. Gapdh was used as a control. Experiments were performed in biological triplicate and technical duplicate, with data represented as the mean and error represented as SD.

RNA Sequencing of SCNT Embryos. Five to ten four-cell-stage cumulus SCNT and H3.3KD, luciferase-injected, H3.3KD+H3.3mRNA, and H3.3KD+H3.2mRNA SCNT embryos were transferred into a PCR tube with 100 μL lysate buffer by mouth pipette, and RNA was prepared by using an Arcturus PicoPure kit (cat. no. KIT0204; Life Technologies). Each group has a biological replicate. Library preparation was done according to a published protocol (33). Briefly, purified RNA was used for first-strand synthesis, second-strand synthesis, and PCR amplification (×2). A total of 200 ng of resulting DNA was sonicated to ∼100–300 bp and then used to construct a sequencing library according to standard Illumina protocols. Libraries were sequenced by using the Illumina HiSeq2000 platform (single end, 51 bp), multiplexed at three samples per lane.

Data Analysis. All data are presented as mean ± SD. Differences between groups were tested for statistical significance by using a Student *t* test or χ^2- test. Statistical significance was set at $P < 0.05$ or $P < 0.01$. For RNA-seq analysis, RNA-seq reads were aligned to the mouse genome (mm9) by using TopHat. Expression levels were obtained using CuffLinks, by using upper-quartile normalization and by normalizing for GC content of DNA. Genes were considered expressed if their fragments per kilobase mapped (FPKM) value was greater than 5. Differentially expressed genes were identified by using a twofold cutoff, with at least one of the expression values (FPKM) equal or greater to 5. Pathway analysis was performed using an information theoretic approach (iPAGE) and mouse Gene Ontology pathways (after removal of all electronic gene function annotations). Negative log10 hypergeometric *P* values were calculated for all pathways identified by iPAGE and shown as bar plots.

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