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Establishment of totipotency does not depend on Oct4A

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SUMMARY

Oct4A is a core component of the regulatory network of pluripotent cells, and by itself can reprogram neural stem cells into pluripotent cells in mouse and humans. However, its role in defining totipotency and inducing pluripotency during embryonic development is still unclear. We genetically eliminated maternal *Oct4A* using a Cre-lox approach in mouse and found that the establishment of totipotency was not affected, as shown by the generation of live pups. After complete inactivation of both maternal and zygotic *Oct4A* expression, the embryos still formed *Oct4*-GFP– and Nanog–expressing inner cell masses, albeit non-pluripotent, indicating that Oct4A is not a determinant for the pluripotent cell lineage separation. Interestingly, *Oct4A*-deficient oocytes were able to reprogram fibroblasts into pluripotent cells. Our results clearly demonstrate that, in contrast to its role in the maintenance of pluripotency, maternal *Oct4A* is crucial for neither the establishment of totipotency in embryos, nor the induction of pluripotency in somatic cells using oocytes.

Following fertilization, the genomes of the differentiated oocyte and spermatozoa are epigenetically reprogrammed to instruct the zygote to differentiate into all types of somatic cells in a highly organized manner and generate the entire organism from a single cell, a feature referred to as totipotency[1]. After 3 cell divisions, early embryos compact, and their blastomeres become polarized. Thereafter, they form blastocysts and differentiate into the

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first two lineages—the inner cell mass (ICM) and the trophectoderm (TE). The ICM gives rise to the entire embryo proper—i.e., all cell types except for the TE, an ability defined as pluripotency. *Oct4* (*Pou5f1*), a POU family transcription factor, is expressed specifically in germ cells[2], the ICM, and embryonic stem (ES) cells[3], and has been deemed a critical regulator in the pluripotency as shown by a zygotic *Oct4*-knockout study[4]. *Oct4* can activate its own expression with its partner *Sox2* through a positive autoregulatory loop in ES cells[5]. Interestingly, *Oct4* is listed as one of the 27 proven maternal-effect genes and is regarded to be functionally important for zygotic genome activation[6], which provides the first step in the establishment of totipotency-pluripotency. Functionally, Oct4 is required for the binding of two key components of the BMP and LIF signaling pathways, Smad1 and STAT3, to their respective targets, and plays a pivotal role in stabilizing the transcription factor complex[7]. Among the core components of the pluripotency circuitry formed by Oct4, Nanog, and Sox2, *Nanog* is directly regulated by Oct4 and Sox2[8], Sox2 is actually dispensable for the activation of Oct-Sox enhancers, and the forced expression of Oct4 could rescue Sox2-null ES cells[9]. Hence, *Oct4* is considered to be the genetic "master switch" in the establishment of totipotency-pluripotency during the life cycle of mammals[10], and presumed to be the most upstream gene in the molecular circuitry of pluripotency[11]. In this study, however, we provide solid evidence that challenges that viewpoint.

In the present study, we used Oct4floxed mice, in which two *LoxP* motifs had been inserted that span the proximal promoter and the Oct4A unique first exon[12] (Supplementary Fig. 1a). As the other 4 exons shared by *Oct4B* were not mutated, the *Oct4* studied is hereafter referred to as *Oct4A*. Conditional removal of maternal *Oct4A* in oocytes was done by crossing the $Oct4^{flox/flox}$ mice with ZP3Cre transgenic mice as shown in Supplementary Fig. 1b. Genotyping for *Oct4A* deletion in single germinal vesicle (GV) oocytes (47/47) (Fig. 1a), mature metaphase II (MII) oocytes (28/28), preimplantation embryos (665/666), and offspring (95/97) confirmed the efficient deletion of the floxed *Oct4A* allele by ZP3cre. Depletion of the maternal *Oct4A* mRNA was also demonstrated, without a significant reduction in the expression of the oocyte-specific genes *Sall4*, *Stella*, and *Dazl* by real-time RT-PCR with TaqMan probe-primer sets, using both the ABI PRISM 7900 Sequence Detection System (Fig. 1b) and the Fluidigm Biomark 48.48 Dynamic Array system (Supplementary Fig. 1e). Elimination of the maternal Oct4A protein was confirmed by Western blot on pools of more than 400 oocytes per sample (Supplementary Fig. 1d).

However, using the Taqman Oct4 primers spanning exon 2 and exon 3, real-time reverse transcription polymerase chain reaction (RT-PCR) data consistently showed 1–3% of the wild-type (Wt) expression level in the *Oct4A*-null oocytes and embryos (Fig. 2a). Such a low level of *Oct4* expression was also observed previously in samples with genetically inactive *Oct4* loci using the same Oct4floxed mice and was considered to be the background noise of detection methods, the expression of pseudogenes, or the expression of other POU-domain family members[13]. However, no detectable background signal was found in our *Oct4A*-null samples using *Oct4A*-specific primers spanning exons 1 and 2 (Fig. 2b). To identify the background signal, we sequenced the amplicons obtained with the Taqman primer set (Figs. 2c, d), along with another primer set spanning exons 3 and 4 (Figs. 2e, f). As shown in Supplementary Tables 1 and 2, both primer sets amplified sequences matching

to the *Oct4* mRNA. These results suggested that low levels of *Oct4B* were expressed in the *Oct4A*-null oocytes and embryos. Regardless, even though the exact function of Oct4B in mouse oocytes and embryos remains unknown, numerous studies demonstrated that the *Oct4B* exists in somatic cells[14, 15], acts as a stress response factor[16], or an antiapoptotic factor in cancer cells[17], and only the Oct4A has the ability to confer and sustain pluripotency[15]. Combined with Sox2, Klf4 and c-Myc, Oct4B was not able to reprogram somatic cell into induced pluripotent stem cells (iPSCs) unlike Oct4A[15]. Our data showed that low level of *Oct4B* expression in *Oct4A*-null embryos could not maintain pluripotency *in vitro* and *in vivo*. Therefore, we consider it highly unlikely that the low levels of *Oct4B* expression in oocytes, embryos, and ES cells can compensate for the critical roles of Oct4A in the establishment of totipotency and pluripotency.

Previous reports have speculated that maternal *Oct4* is a key regulator of oocyte developmental competence, based on microarray gene profiling on MII oocytes with minor GV morphological variation[18]. Oct4 was also claimed to be a critical regulator of the maternal-embryonic transition as embryos arrested in development after being invasively injected with Oct4-antisense morpholino oligonucleotides at the zygote stage without validation of efficiency and specificity, while ignoring the existing maternal Oct4 protein was ignored[19]. Here we investigated the functionality of *Oct4A*-null oocytes in vivo by crossing $Oct4^{flox/flox}/ZP3^{Cre/+}$ female mice with Wt male mice. To our surprise, we found that these $Oct4^{flox/flox}/ZP3^{Cre/+}$ female mice were fully fertile, with a normal litter size averaging 7.9 pups (Supplementary Fig. 1f); all these offspring had deletion of the maternal *Oct4A* allele as confirmed by PCR genotyping (Supplementary Fig. 1g), demonstrating that maternal *Oct4A* is not critical for totipotency-pluripotency.

Next, we analyzed gene expression using immunocytochemistry and real-time RT-PCR in *Oct4A*-null blastocysts obtained by crossing $Oct4^{flox/flox}–ZP3^{Cre/+}$ female mice with $Oct4A^{+/\Delta}$ male mice. In total, we obtained 264 maternal and zygotic-knockout embryos and 302 maternal-knockout and zygotic–Wt embryos. As maternal-knockout and zygotic–Wt embryos underwent normal full-term development and had the same genetic background, they were used as control for maternal and zygotic-knockout embryos. Although the maternal and zygotic-knockout blastocysts had a reduced size on embryonic day (E4.5) (102.4 μm ± 19.6 in diameter, n=33 vs. Control 124.7 μm ± 24.4, n=60; $P<0.01$), they exhibited normal cavitation and formed a distinct ICM (Supplementary Video 1). Notably, on E3.5, the ICM of maternal/zygotic-knockout embryos did not express the TE markers Cdx2 (Figs. 1c, 3a) and Troma-1 (Supplementary Fig. 2d). Instead, they expressed Nanog, another core factor involved in the regulatory network of ES cell self-renewal and pluripotency[20], at protein (Figs. 1d, 3a) and mRNA levels (Fig. 1e) comparable to those of the zygotic mutant, although the $Nanog^+$ cells were always found to scatter apart in the ICM (Figs. 1d, 3b). Activation of *Nanog* expression was observed as early as at the 8-cell stage at mRNA (Supplementary Fig. 2b) and protein levels (Supplementary Fig. 2c), like in control embryos. On E4.5, although the ICM of maternal and zygotic-knockout embryos maintained Nanog expression in 35 of the 41 blastocysts examined, coexpression of Cdx2 in some of the $Nanog^+$ ICM cells (Fig. 3b, Supplementary Fig. 2e) was observed in 73.1% (30/41) of the knockout embryos, while such co-localization was rarely seen in control littermate

embryos (3.3%, 2/61). Moreover, we examined the expression of the *Oct4*-GFP transgene in maternal and zygotic-knockout embryos by crossing $Oct4^{flox/flox}$–$ZP3^{Cre/+}$ female mice with $Oct4^{+/\Delta}$ $GOF18^{+/+}$ male mice. Green fluorescent protein (GFP) of *Oct4*-GFP transgene in GOF18 mice is expressed under the control of Oct4 regulatory elements and is active only in pluripotent cells and cells of the germ cell lineage[21], and as such it has been used as a convenient indicator for the acquisition of pluripotency. The time-lapse observation revealed that *Oct4*-GFP expression was activated in all maternal-knockout embryos (#2, 6, 7, and 10–14) and maternal and zygotic-knockout embryos (#5 and 9) in a timely fashion, as in Wt embryos (#1, 3, 4, and 8) (Fig. 3c, Supplementary Video 2). The genotype of each individual embryo was determined by nested PCR (Supplementary Fig. 3c) at the end of time-lapse observation, with its position tracked in the video with bright field microscopy (Supplementary Fig. 3a, Supplementary Video 3). This result is consistent with a report showing the maintenance of β-galactosidase activity from the Oct4 promoter in the ICM of zygotic Oct4–knockout embryos[4]. While expression of *Nanog* and *Oct4-GFP* is persistent, expression of *Cdx2* in *Oct4-null* E4.5 embryos was upregulated at the mRNA level as assessed by real-time RT-PCR analysis on immunosurgically prepared ICMs (Fig. 3d), indicating a reciprocal interaction between Oct4 and Cdx2 at this stage.

Previous studies have suggested that lineage commitment is controlled by the expression level of *Oct4*[4, 22]. Repression of *Oct4* expression was shown to induce the differentiation of ES cells into TE, and a less than two-fold increase in *Oct4* expression was shown to cause differentiation of ES cells into cells of primitive endoderm and mesoderm[22]. In the absence of Oct4, embryos could not form an ICM—i.e., the inner cells of morula-stage embryos were rather driven into trophoblast differentiation[4]. Therefore, these results indicated that *Oct4* plays a critical role in sustaining stem cell self-renewal and that up- or downregulation of *Oct4* induces divergent developmental programs, suggesting that *Oct4* is the master regulator of pluripotency and that it may also control lineage commitment during early embryonic development[4]. This hypothesis was further strengthened by the finding of the reciprocal interaction between Oct4 and Cdx2 in determining TE differentiation[23] and by the interaction between Oct4 and the histone H3–specific methyltransferase ESET in restricting the extraembryonic trophoblast lineage potential of pluripotent cells[24]. However, coexpression of *Oct4* and *Cdx2* was found in normal morula-stage embryos[25] and expression of *Cdx2* in ES cells was found to be rapidly initiated by Ras activation (i.e., within 24 hours), without previous or simultaneous downregulation of *Oct4* expression[26]. Moreover, *Cdx2* deficiency was not shown to interrupt TE-ICM lineage separation[27–29]. Meanwhile, zygotic *Oct4* expression was shown not to be required for initial repression of the TE genes *Cdx2* and *Gata3*, indicating that other mechanisms must lead to the restriction of these genes to the TE[30]. Now with our maternal *Oct4A*–knockout results, we added more solid evidence against the Oct4-Cdx2 interaction model of lineage commitment in early mouse embryos[23]. Our results clearly demonstrated that ICM-TE lineages were separated in maternal and zygotic *Oct4A*–knockout embryos, suggesting that first lineage separation of the ICM-TE is not determined by the reciprocal interaction between Oct4 and Cdx2. Instead, such a reciprocal interaction between Oct4 and Cdx2 is established subsequently to maintain the ICM fate. Most importantly, we found that maternal Oct4A is not at the root of

pluripotency as a determinant in initiating the pluripotent cell lineage, contrary to previous assumptions and views.

Notably, in contrast to ES cells, wherein upstream Oct4 and Sox2 regulate *Nanog* expression[8, 31], we found that activation of *Nanog* and *Oct4-GFP* expression occurred in the absence of both maternal and zygotic *Oct4A*, further suggesting that a unique *Oct4A*-independent pluripotency-initiating regulatory network is active in early embryos.

By crossing *Oct4*$^{flox/flox}$–*ZP3*$^{Cre/+}$ female mice with *Oct4*$^{+/\Delta}$ OG2$^{+/-}$ male mice, in which the *Oct4*-GFP transgene lacks the proximal enhancer and activates GFP expression through the distal enhancer and promoter (*GOF18ΔPE*)[32], we demonstrated that GFP is still activated in *Oct4A-null* embryos (Supplementary Fig. 3b). This result indicates that in oocytes and embryos, such an unknown Oct4-independent reprogramming engine activates *Oct4* expression through the distal enhancer, probably through a completely different network than that shown by Yamanaka's 4-factor reprogramming[33–35].

In an attempt to identify the factors critical for driving Oct4 and Nanog expression, we knocked down 6 reported Oct4-regulating factors, *Sall4, Tpt1, Zscan4, Esrrb, Utf1*, and *Nr5a2*, by microinjecting gene-specific siRNA duplexes into maternal *Oct4A*–deficient zygotes—to avoid any possible effect of maternal Oct4A as a positive autoregulator. The efficient knockdown of *Sall4, Tpt1, Zscan4, Esrrb*, and *Utf1* was observed at the blastocyst stage. However, we did not observe any significant change in either *Oct4* expression (Figs. 3e, Supplementary Fig. 3e) or *Oct4*-GFP activation (Supplementary Fig. 3d). Interestingly, knockdown of *Nr5a2* showed a mild, but consistent downregulation of *Oct4* expression ($P<0.0001$) (Fig. 3f). Nr5a2, an orphan nuclear receptor, was found to maintain Oct4 expression at the epiblast stage of embryonic development, by binding to the proximal enhancer and proximal promoter regions of *Oct4*, but to play no evident role in ES cell self-renewal[36]. However, Nr5a2 can induce epiblast stem cells into ground state pluripotency[37] and replace Oct4 in the reprogramming of somatic cells into pluripotent cells[34]. Our results indicate the involvement of Nr5a2 *in vivo* in assisting Oct4 activation in preimplantation embryos. Further studies to elucidate how exactly oocytes activate the pluripotent genes *Oct4A* and *Nanog* on top of the *Oct4-Sox2* autoregulatory loop will lead to a better understanding of the establishment of totipotency in zygotes and in transplanted somatic cells, and to a marked improvement in the efficiency of iPSC techniques and eventually support the generation of Oct4-independent induced totipotent stem (iTPS) cells to achieve organized differentiation into an intact organism without the use of oocytes through fertilization or nuclear transfer (NT).

On the other hand, we found low levels of *Tbx1* expression in Wt E3.5 blastocysts (Fig. 1e) and E4.5 ICM (Fig. 3d) by real-time RT-PCR with a Ct value of 33 and 28–31, respectively, but we could not detect *Tbx1* mRNA in any *Oct4A–null* samples even after 40 cycles of PCR amplification. *Tbx1* encodes a T-box transcription factor and is expressed in different components of the mesoderm or mesoderm-endoderm during gastrulation[38]. Mutation of *TBX1* causes the DiGeorge syndrome phenotype, which manifests as defects in the pharyngeal arch and in cardiovascular and craniofacial development[39]. Chip-seq data has shown that Oct4 binds to the promoter region of *Tbx1*[7]. Further study on how Oct4A

activates *Tbx1* expression might reveal another role of Oct4A in the initiation of mesoderm differentiation by promoting pluripotent cells in a “poised” state.

Next, we evaluated the pluripotency of maternal-zygotic *Oct4A*–knockout embryos *in vitro* by plating them onto mouse embryonic fibroblasts (MEFs) and attempting to derive pluripotent ES cells. None of the 10 ES cell lines derived from the 43 embryos were homozygous for the Oct4-knockout allele (Fig. 4b), indicating loss of pluripotency in *Oct4A*–null embryos. Immunocytochemical analysis of the mutant outgrowths revealed many condensed and fragmented $Cdx2^{-}$ nuclei along with $Cdx2^{+}$ TE cells (Fig. 4a). Interestingly, Nanog was still detectable in some $Cdx2^{-}$ cells, but it was only localized to the cytoplasm—instead of the nucleus.

As expression of Nanog and *Oct4*-GFP was persistent in the *Oct4A*-null embryos between E2.5 and E4.5, we postulated that these defective embryos could be rescued by Wt embryos for differentiation into certain types of somatic cells. Accordingly, we aggregated $Rosa26^{+/-}$ *Oct4A*-null 8-cell embryos with Wt embryos. After 24 hours of culture, chimeric embryos with homozygous (*Oct4A* KO) and heterozygous (control) mutants were separately transferred into pseudopregnant mice. E13.5 to E14.5 fetuses were collected and subjected to LacZ staining (Fig. 4c). Consistent with our *in vitro* study, *Oct4A*-null embryos contributed exclusively to the placental tissue—and not to the embryo proper (0/7 vs. 10/11 in control). Furthermore, after aggregating with embryos of ICR mice, *Oct4A*-null embryos also did not contribute to the formation of adult mice, as shown by the coat color, while 4 of 5 control embryos did (Fig. 4d). These results demonstrated that *Oct4A*-depleted cells lost pluripotency and could only form extraembryonic tissues, supporting the importance of *Oct4* as the gatekeeper of pluripotency[3].

Studies have shown that the nuclei of terminally differentiated somatic cells can be reverted back to a totipotent state after nuclear transplantation into enucleated oocytes[40]. To explore whether maternal *Oct4A* is critical in the reprogramming of somatic cells using oocytes, NT experiments were conducted with the *Oct4A*-null oocytes from $Oct4^{flox/flox}$–$ZP3^{Cre/+}$ female mice. By using both immunocytochemistry (Supplementary Fig. 4a) and real-time RT-PCR (Supplementary Fig. 4b), we observed that *Oct4A*-null oocytes still activated the expression of pluripotent genes (*Oct4* and *Nanog*) in the ICM and the TE marker gene (*Cdx2*) in the TE in all 28 cloned embryos analyzed.

To further assess whether maternal *Oct4A*-null oocytes can induce pluripotency in somatic cells, we derived ES cells from NT embryos using these oocytes as recipient oocytes and *CAG-mRFP* and *Oct4-GFP* double transgenic male MEFs as donor cells. Of 135 reconstructed embryos, 78 developed to the morula stage and expressed both *CAG*-mRFP and *Oct4*-GFP on day 3 of culture (Fig. 5a). These morulae were plated on irradiated MEFs (Fig. 5b) and gave rise to 9 ES cell lines (Supplementary Fig. 4c). Chromosome counts from metaphase spreads showed that 7 of the 9 NT-ES cell lines were normal, with 40 chromosomes. Four of the 7 NT-ES cell lines—RG1, RG5, RG6, and RG8—were tested with the tetraploid complementation assay, the most stringent test for pluripotency (Fig. 5c). All ES cell-tetraploid embryo aggregates developed to the blastocyst stage and had ES cells integrated into the ICM (Fig. 5d). After transfer into the uterus of pseudopregnant female

mice, the RG5 ES cell line did not support any of the 30 aggregates to full-term development. RG6 gave rise to 2 pups of 50 transferred (1 dead; 1 alive initiated breathing but died shortly thereafter). RG1 and RG8 ES cell lines did very well with the test. On E19, we recovered 17 full-term male pups of 31 aggregates transferred (54.8%) in total from RG1 (Supplementary Fig. 4e) and 18 pups of 45 aggregates transferred (40%) in total from RG8. These offspring expressed *CAG*-mRFP ubiquitously in the body (Fig. 5e) and *Oct4*-GFP specifically in the gonads (Fig. 5f). Of these 17 pups from line RG1, 11 initiated breathing and were set up with foster mothers, and 5 survived to adulthood (Fig. 5g) and were fertile. Twelve of the 18 pups from RG8 initiated breathing but were rejected by their foster mothers. The pluripotency of RG6 and RG8 ES cell lines was further confirmed by differentiation into cells of all 3 embryonic germ layers in the teratoma assay (Supplementary Fig. 4d). Our NT data unequivocally demonstrated that the reprogramming engine in oocytes could work effectively to reprogram somatic cells into cells of a pluripotent state without *Oct4A*.

In conclusion, our results demonstrated that Oct4A is not the master regulator responsible for initiating totipotency-pluripotency in natural reprogramming by maternal factors in oocytes. The maternal and zygotic *Oct4A*–null blastocysts maintained the ability to activate Nanog and *Oct4*-GFP expression, indicating that unknown pathways other than the *Oct4*-centered pluripotency-regulating network are active in embryos at both the totipotent and pluripotent stages of development and function upstream of *Oct4A* in driving pluripotency.

METHODS

Induction of Recombination in *Oct4$^{flox/flox}$* Mice by Cre recombinase

A knockin 129/svj strain containing a functional *Oct4* allele flanked by *lox* P motifs (*Oct4$^{flox/flox}$*)[12] was crossed with a *ZP3-Cre* transgenic C57BL/6J mouse[41]. The *ZP3* promoter–driven *Cre* expression supports the inactivation of maternally expressed genes in growing oocytes[41] as early as day 5 after birth[42]. DNA extraction and PCR genotyping were performed as reported previously[12]. As shown in Supplementary Fig. 1a, the intact *Oct4* allele and the *lox* P site were detected by primer pair B (forward = GOF-D1, reverse = GOF-R1; amplicon size: Wt = 415 bp, floxed = 449 bp), and *Oct4* allele was found to be deleted by primer pair C (forward = GOF-AatII, reverse = GOF-ApaL1; amplicon size = 245 bp). A representative PCR genotyping result is shown in Supplementary Fig. 1c.

Nested PCR was used to genotype individual oocytes and blastomeres as described[43] with combinations of the above primers: GOF-AatII, GOF-D1, and GOF-R1 as outer primers for the primary amplification, and GOF-AatII, GOF-D1, and GOF-ApaL1 as inner primers for the nested amplification. Three genotypes of *Oct4* alleles were distinguished by amplicons of different size: Wt (362 bp), floxed (396 bp), and deleted (245 bp). For genotyping cells with *Oct4*-GFP, another set of primers (outer: GOF-AatII, GOF-HindIII, and GOF-R1; inner: GOF-AatII, GOF-ApaL1, and Oct4R344: 5’-GAGAAGGCGAAGTCTGAAGC-3’) with amplicons of different size: Wt (345 bp), floxed (379 bp), and deleted (245 bp) were used.

A protocol for animal handling and maintenance for this study was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV NRW) under the supervision of a certified veterinarian in charge of the Max Planck Institute (MPI) animal facility.

Embryo Collection, Culture, and Immunocytochemistry

Embryos from various matings were flushed out of the mouse oviducts or uteri in M2 medium as described by Hogan et al.[44] at 2.5, 3.5, or 4.5 days post coitum (dpc) to collect embryos at stages of development as required for specific experiments.

Whole-mount immunostaining was carried out as described[29]. Briefly, embryos were fixed in 4% paraformaldehyde (20 min), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Inc., St. Louis, MO, USA) for 1 hour, and blocked with 3% BSA in PBS for 1 hour. This was followed with binding of primary antibodies (overnight at 4°C) and secondary antibodies (Alexa 488–conjugated goat anti-rabbit IgG, Alexa 488–conjugated goat anti-mouse IgG, Alexa 488–conjugated donkey anti-goat IgG, Alexa 568–conjugated donkey anti-rabbit IgG, and Alexa 647–conjugated donkey anti-mouse IgG (Invitrogen, Karlsruhe, Germany), Cy3-conjugated goat anti-rat IgG, or Cy3-conjugated goat anti-mouse IgG (Jackson Laboratory, Bar Harbor, ME, USA) at a 1:400 dilution for 1 hour. The following primary antibodies were used: rabbit anti-Nanog IgG (1:200; Cosmo Bio Company Ltd., Tokyo, Japan, REC-RCAB0002PF), mouse anti-Oct4 IgG (1:100; Santa Cruz Biotech., Santa Cruz, CA, USA, Clone C-10, SC-5279), rabbit anti-Oct4 IgG (1:2,500; generated in our lab) (Palmieri et al., 1994), goat anti-Oct4 IgG (1:100; Santa Cruz Biotechnology, Inc., SC-8628), mouse anti-Cdx2 (1:100; BioGenex, San Ramon, CA, USA, Clone CDX2-88, MU392-UC), and rat anti-Troma-1 (1:100; DSHB, Iowa City, USA). Some samples were counterstained with 5 μM DRAQ5 (Biostatus Limited, Leicestershire, UK) for 30 min. Samples were examined under a laser scanning confocal microscope (UltraVIEW; PerkinElmer Life Sciences, Inc., Jügesheim, Germany) with 488 nm, 568 nm, and 647 nm lasers. Immunocytochemistry experiments were repeated 2–3 times with 30–100 embryos in each experiment.

RNA Extraction, cDNA Synthesis, and Real-Time RT-PCR

For real-time analysis of gene expression, embryos were harvested in RLT buffer (QIAGEN GmbH, Hilden, Germany) and processed as previously described[45]. Briefly, total RNA was extracted from individual blastocysts using the MicroRNeasy Kit (QIAGEN GmbH) and complementary DNA (cDNA) synthesis was performed with the High Capacity cDNA Archive Kit (Applied Biosystems GmbH, Darmstadt, Germany) according to the manufacturer's instructions.

Real-time PCR was carried out on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems GmbH) using TaqMan probes from Applied Biosystems. All primers used are listed in Table S3. Two to 6 biological replicates were used, and each sample was run with 3 technical replications; an RT-blank and a no-template blank served as negative controls. The cycle threshold (C_T) values were collected using Applied Biosystems SDS v2.0 software and transferred to a Microsoft Excel spreadsheet for further relative

quantification analysis using the $\Delta\Delta C_T$ method (User Bulletin #2, ABI Prism 7700 Sequence Detection System, 1997). Single-oocyte real-time RT-PCR using the Biomark 48.48 Dynamic Array system (Fluidigm) was performed as previously described[19]. All real-time RT-PCR source data can be found in supplementary materials.

Microinjection of siRNA into zygotes

Zygotes were collected in the morning after mating from superovulated or natural ovulated *Oct4*$^{flox/flox}$–*ZP3*$^{Cre/+}$ female mice. Microinjection of siRNA duplexes into zygotes was performed using an Eppendorf FemtoJet microinjector and Narishige micromanipulators as previously described[29]. Approximately 2 pl of siRNAs (20 μM) was injected into cytoplasm of zygotes. Scrambled siRNA duplexes or siRNA duplexes against *GFP* was used as Control. The injected zygotes were then cultured with KSOM^{AA} (potassium simplex optimized medium plus 19 natural amino acids)[46] in a humidified 5% CO_2 in air at 37°C. Expression of the target genes was analyzed at the blastocyst stage (3.5 dpc) in pools of 10–20 embryos per sample. The target sequences of siRNA are listed in Table S4.

Somatic Cell Nuclear Transfer

Seven- to 9-week–old *Oct4*$^{flox/flox}$ (control oocytes) and *Oct4*$^{flox/flox}$–*ZP3*$^{Cre/+}$ (*Oct4-null* oocytes) female mice were used to collect oocytes and cumulus cells after superovulation. Superovulation and collection of oocytes and cumulus cells, nuclear transfer using ovarian cumulus cells, and activation of reconstructed oocytes were all performed essentially as previously described[45, 47]. Six hours after the onset of activation, the constructs were washed carefully in a 4-well dish (Nunc, Roskilde, Denmark, 176749) with 0.5 ml of alpha minimal essential medium (MEM α, Invitrogen, 12571-063) supplemented with 0.4% of BSA (Sigma-Aldrich Inc, A3311) without cytochalasin B under 5% CO_2 in air for 4 days. The blastocysts obtained were subjected to immunostaining, genotyping, and real-time RT-PCR.

For derivation of ES cells from NT embryos, 8- to 12-week–old *Oct4*$^{\Delta/flox}$–*ZP3*$^{Cre/cre}$ female mice were used to collect *Oct4-null* oocytes after superovulation. MEFs of passages 2–4, derived from male E13.5 embryos by crossing *CAG-mRFP*[48] male mice with *Oct4*-GFP female mice (GOF-18)[21], were used as nuclei donors. The NT procedures were the same except that MEF cells were briefly exposed to HVJ-E Sendai virus extract (www.cosmobio.co.jp) and then placed into the perivitelline space of recipient *Oct4*-null-enucleated oocytes on the side opposite to the 1st polar body[49]. The constructs were transferred into KSOM^{AA} and cultured for 1–2 hours in an incubator with 5% CO_2 in air at 37°C to induce fusion prior to activation. The morulae obtained on day 3 of culture were subjected to the ES cell derivation procedure according to a previous report[50].

Western Blotting

Western blot was performed as described previously[51]. Briefly, 400 Wt and 400 maternal *Oct4A*-null oocytes were collected in PBS. Oocytes, 10,000 ES cells as well as cumulus cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 1% IGEPAL CA 630, 0.5% sodium deoxycholate, 1 mM EDTA, 1× protease inhibitors [Roche], and 1× phosphatase inhibitors [Sigma-Aldrich Inc.]) containing 50 units of Benzonase (Merck,

Darmstadt, Germany) on ice for 1 hour. The lysate was cleared by centrifugation and supernatants were collected. An amount of supernatant equivalent to 250 oocytes was resolved using gel electrophoresis. Primary antibody used for western blot was mouse monoclonal anti-Oct4 IgG (Santa Cruz Biotechnology, Inc., clone: C-10, SC-5279). The western bot experiment was repeated 2 times.

Sequencing

Products of qPCR were gel purified and ligated into PCR2.1-TOPO vector (Invitrogen) according to manufacture's instruction. For each sample, 6 colorless colonies were picked and sent to GATC Biotech AG (Konstanz, Germany) in 96-well format for sequencing using M13 reverse primer.

Generation and Analysis of Chimeric Embryos

To assess the pluripotency of *Oct4*-inactivated embryos *in vivo*, we aggregated mutated 8-cell embryos (obtained from crossing $Oct4^{flox/flox}$–$ZP3^{Cre/+}$–$ROSA26^{+/+}$ female mice with $Oct4^{+/\Delta}$–$ROSA26^{+/+}$ male mice)—after removal of the zonae pellucidae by acidic Tyrode's solution (Sigma) and biopsy for genotyping by nested PCR—with 8-cell embryos from Wt B6C3F1 mice for LacZ evaluation of chimerism in the fetus or for fur coat color evaluation in albino CFW mice. Following 24 hours of culture in $KSOM^{AA}$, successfully fused and genotyped chimeric blastocysts carrying heterozygous (69 in total as control) and homozygous mutants (60 in total as *Oct4* knockout) were transferred separately into the uterus of 10 ICR pseudopregnant recipients. Eleven fetuses carrying heterozygous mutants and 7 fetuses carrying homozygous mutants were recovered on E13.5 from 8 recipients and subjected to whole-mount LacZ staining as previously described[52]. Five full-term pups from each of the controls and the knockout mutants were obtained from 2 recipients for evaluation of coat color chimerism.

Derivation of Embryonic Stem Cell Lines

E2.5 embryos were cultured on MEFs to establish ES cell lines with a standard procedure[47] after removal of the zonae pellucidae (as described above). All ES cells lines were newly derived in the present study and tested negative for mycoplasma contamination. The complete ES cell medium composition was as follows: 4 mM L-glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 0.1 mM β-mercaptoethanol, 0.1 mM nonessential amino acids, 1,000 units ml^{-1} LIF (Chemicon), and 15% FBS (Invitrogen, 10165-185) in DMEM (GIBCO, 31885-023).

Tetraploid Embryo Complementation Assay for NT-ES Cells

The assay was conducted as described previously[53]. Briefly, 2-cell embryos were flushed 20 hours post-hCG from the oviducts of B6C3F1 mice and fused with a peak pulse of 50V for 35 μs in 0.3 M Mannitol to make tetraploid embryos. The tetraploid embryos were cultured overnight in $KSOM^{AA}$. Then clumps of 15–20 trypsin-treated ES cells were transferred into individual depressions in drops of $KSOM^{AA}$ under mineral oil. Meanwhile, batches of 30–50 embryos were briefly incubated in acidified Tyrode's solution[44] to remove zona pellucida. Two embryos were placed on each ES cell clump and cultured in an incubator

with 5% CO_2 in air at 37°C. After 24 hours of culture, 10–12 embryos at blastocyst stage were transferred into one uterine horn of a 2.5-dpc pseudopregnant recipient for full term development.

Statistical Analysis

Real-time PCR results were analyzed using a program from Applied Biosystems as recommended. For other experiments, statistical analysis was performed using the Student's t-test and Fisher's Exact Test. A *P* value of less than .05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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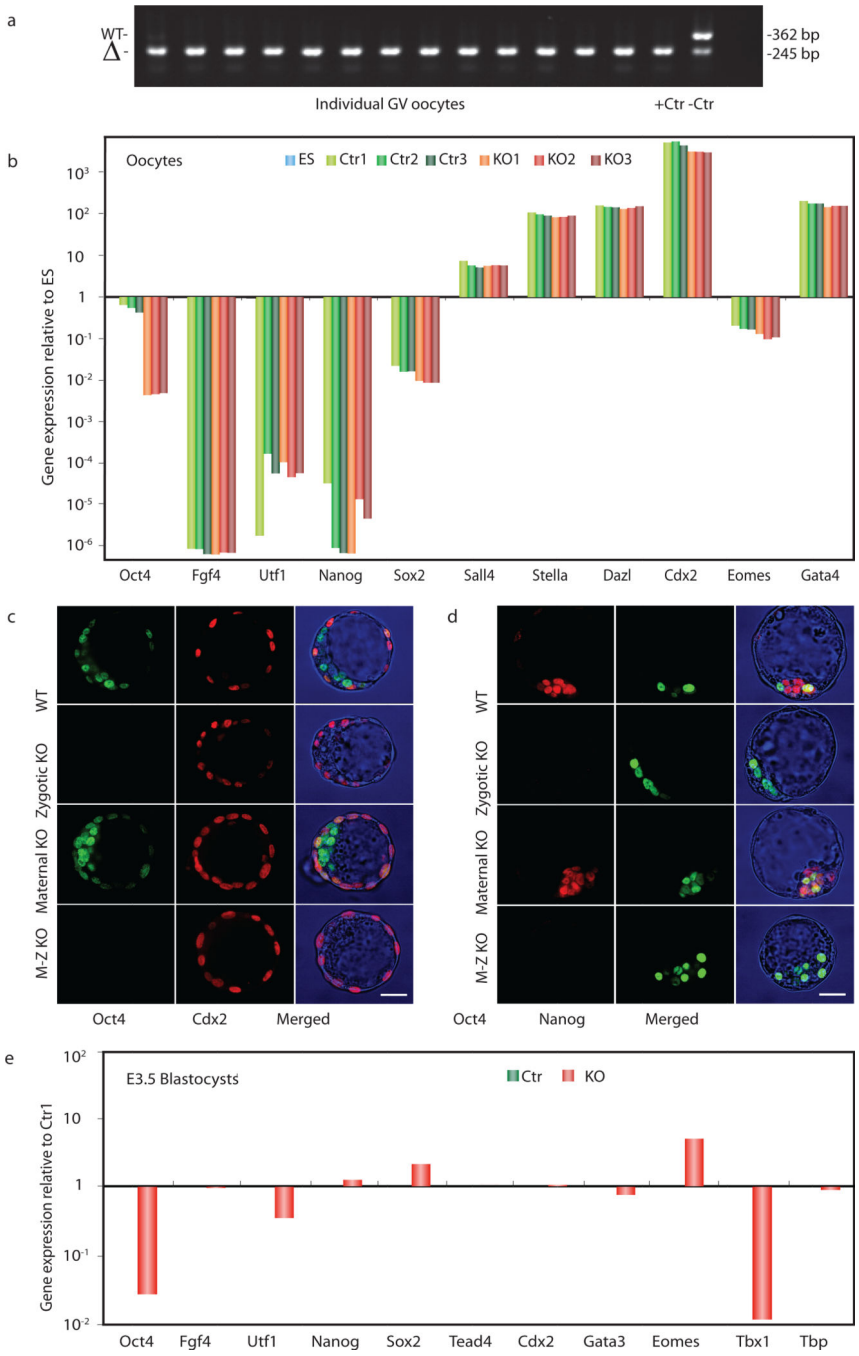
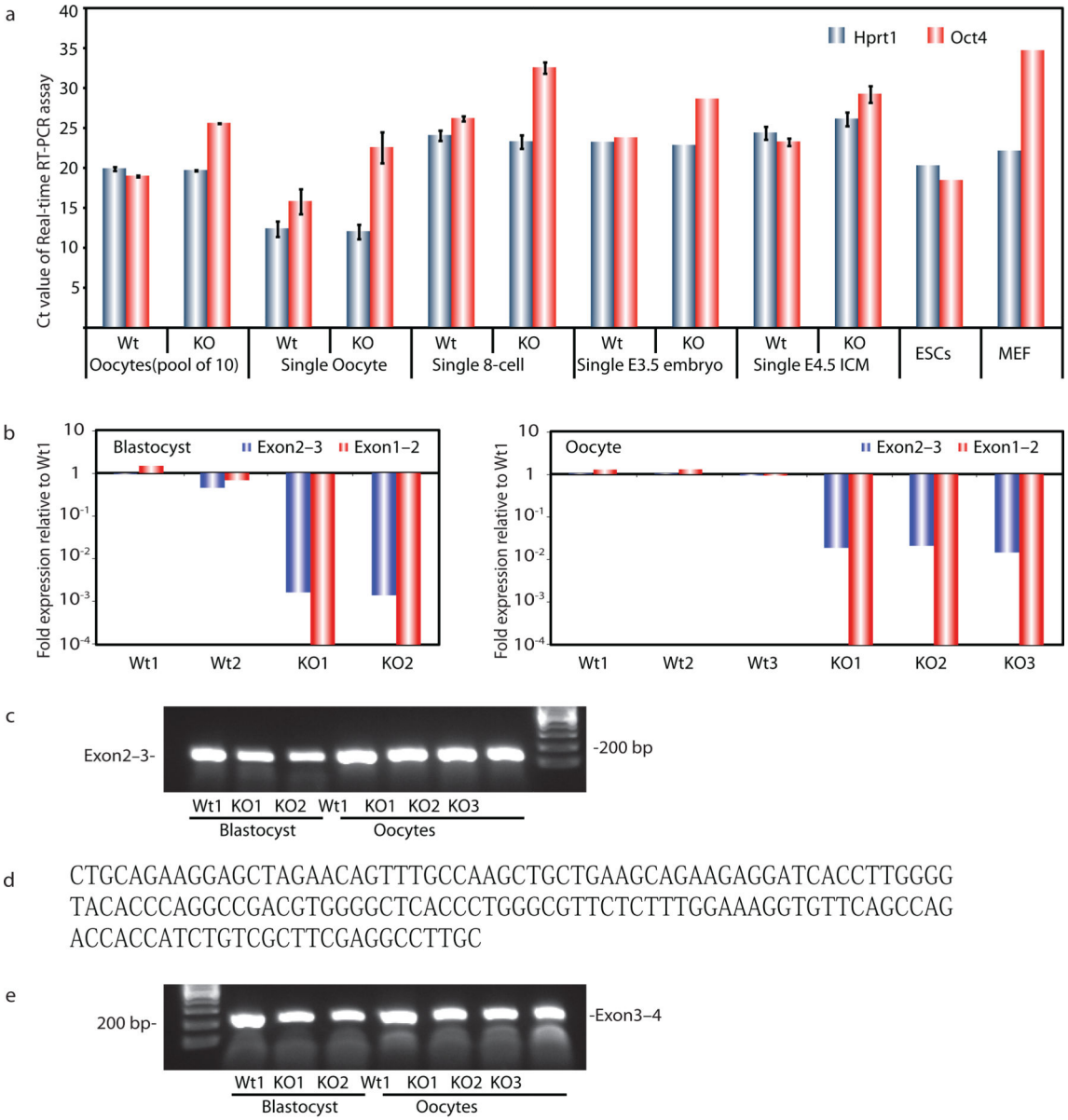


Figure 1. Generation of oocytes lacking maternal Oct4A and their effect on embryo development

(a) Genotyping of individual oocytes at the GV stage by nested PCR showed highly efficient deletion of the floxed *Oct4A* allele. **(b)** Knockout of maternal Oct4A correlates with elimination of *Oct4A* transcript as evidenced by real-time RT-PCR on pools of 10 oocytes each. Ctr: wild-type oocytes; KO: *Oct4A*-null oocytes; ES: ES cells. **(c)** Immunocytochemistry on E3.5 blastocysts with antibody directed against Cdx2 combined with Oct4 antibody. Elimination of both maternal and zygotic Oct4A does not induce Cdx2 expression in the ICM. WT: wild-type; KO: *Oct4A*-knockout; M-Z KO: maternal- and

zygotic-*Oct4A* knockout. **(d)** Immunocytochemistry on E3.5 blastocysts with antibody directed against Nanog combined with Oct4A antibody. Elimination of maternal Oct4A does not interrupt zygotic activation of the pluripotent genes *Oct4* and *Nanog*, while loss of both maternal and zygotic Oct4A does not prevent Nanog expression. WT: wild-type; KO: *Oct4A*-knockout; M-Z KO: maternal- and zygotic-*Oct4A* knockout. **(e)** Real-time RT-PCR on single E3.5 blastocysts confirms that elimination of both maternal and zygotic *Oct4* does not affect *Nanog* and *Cdx2* expression. Ctr: $Oct4A^{+/\Delta}$; KO: maternal and zygotic knockout. The number (1, 2, and 3) right after the abbreviation refers to the biological replicate. The scale bars represent 30 μm in **c** and **d**. Each bar represents the mean from 3 technical replicates, a result representative of one (**a**) and 2 (**e**) biological replicates. The uncropped version of **a** is shown in Supplementary Fig. 5.



d

CTGCAGAAGGAGCTAGAACAGTTTGCCAAGCTGCTGAAGCAGAAGAGGATCACCTTGGGG
TACACCCAGGCCGACGTGGGGCTCACCCTGGGCGTTCTCTTTGGAAAGGTGTTCAGCCAG
ACCACCATCTGTCGCTTCGAGGCCTTGC

f

CTTGCAGCTCAGCCTTAAGAACATGTGTAAGCTGCGGCCCCTGCTGGAGAAGTGGGTGGA
GGAAGCCGACAACAATGAGAACCTTCAGGAGATATGCAAATCGGAGACCCTGGTGCAGGC
CCGGAAGAGAAAGCGAACTAGCATTGAGAACCGTGTGAGGTGGAGTCTGGAGACCATGTT
TCTGAAGTGCCCGAAGCCCTCCCTACAGCAGATCACTCACATCGCCAATCA

Figure 2. Expression of *Oct4* isoforms in *Oct4A*-knockout oocytes and embryos

(**a**) Real-time RT-PCR Ct values of *Oct4* together with the internal control *Hprt1*. The single-oocyte samples were run with preamplification for 18 cycles and tested with the Fluidigm system. The rest of the groups were tested with the ABI PRISM Sequence Detection System 7900 after preamplification for 10 cycles. The *Oct4* Taqman probe-primer set from ABI (Mm00658129_gH, spanning exon 2 and 3) consistently detected background signals in all *Oct4A*-knockout embryos of all preimplantation stages. **(b)** Further testing using equally efficient *Oct4A* specific primers (spanning exon 1 and exon 2) eliminated such

background signals in all 3 biological replicates of *Oct4A*-knockout oocytes (pool of 10 per sample) and 2 replicates of single blastocyst samples, indicating expression of truncated Oct4 transcripts. **(c)** Gel image of RT-PCR using the same Exon2–3 *Oct4* Taqman probe–primer set as **a** and using the same samples as **b**. The amplicons from the bright bands were cut out and sequenced. They were found to match the Oct4 reference sequence as showed in **d**. **(e)** The same samples were tested by PCR using different primers spanning exons 3 and 4, and again the amplicons were found to match the *Oct4* reference sequence as showed in **f**, further confirming the presence of *Oct4* isoforms in *Oct4*-knockout oocytes and embryos. Values represent the means of 2–5 biological replicates, with error bars representing S.D. in **a** and the means from 3 technical replicates, a result representative of one biological replicates in **b**. Uncropped versions of **c** and **e** is shown in Supplementary Fig. 5.

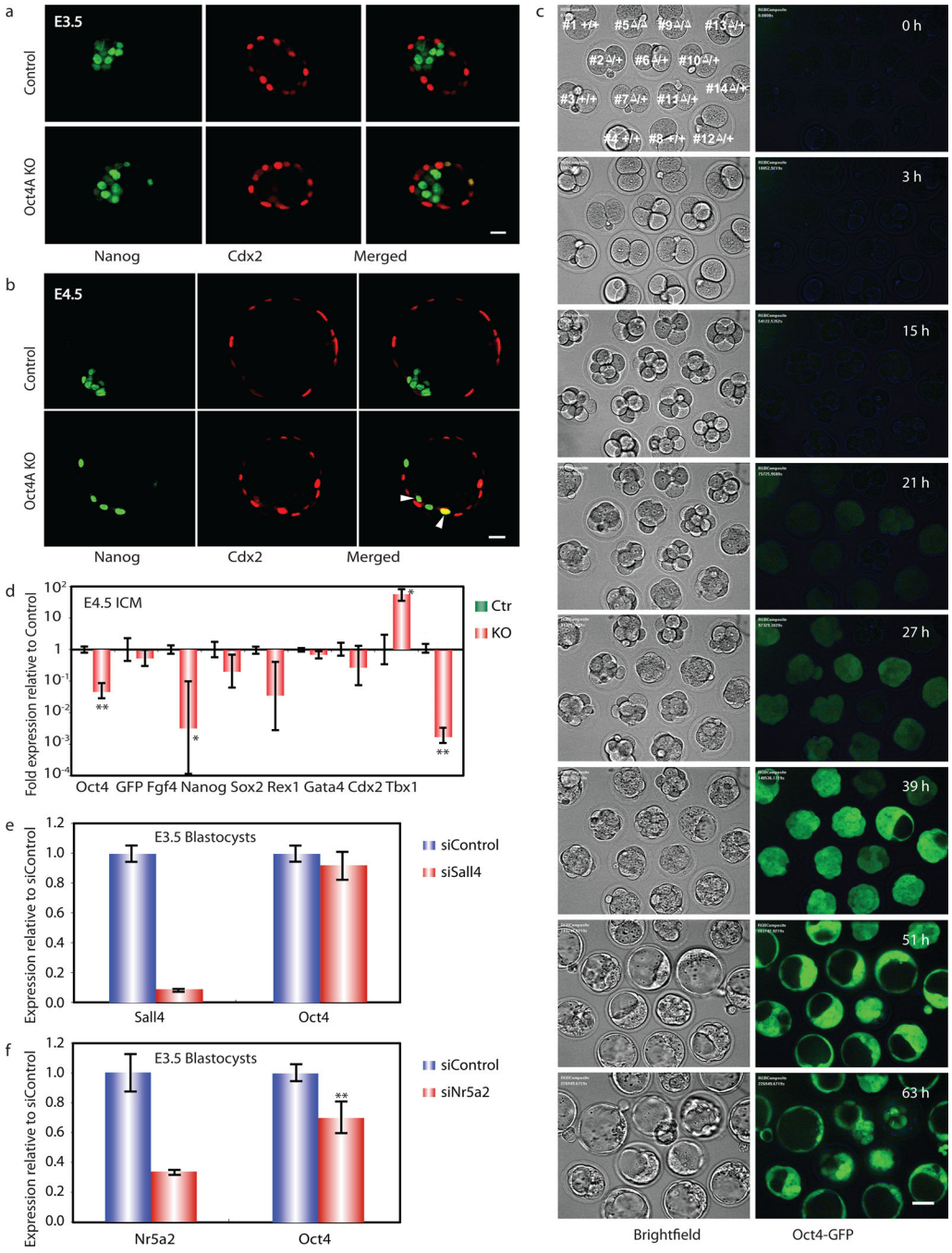


Figure 3. Gene expression of *Oct4A*-null blastocysts produced by crossing *Oct4*$^{flox/flox}$–*ZP3*$^{Cre/+}$ female mice with *Oct4A*$^{+/\Delta}$ male mice

(a) On E3.5, lineage separation in maternal and zygotic-knockout embryos (*Oct4A* KO) was clearly demonstrated by the activation of Nanog in the ICM and expression of the TE marker Cdx2 in the TE. **(b)** On E4.5, although the ICM of maternal and zygotic-knockout embryos (*Oct4A* KO) maintained Nanog expression, coexpression of Cdx2 in some of the Nanog^{+} ICM cells was observed as indicated by arrowheads, while such co-localization was rarely seen in control littermate embryos. **(c)** Snapshots of time-lapse confocal observation

showed that *Oct4*-GFP expression was activated in all maternal-knockout (Δ/+: #2, 6, 7, and 10–14) embryos and maternal and zygotic-knockout embryos (Δ/Δ: #5 and 9) in a timely fashion at about E2.5, like in Wt embryos (+/+: #1, 3, 4, and 8). **(d)** Real-time RT-PCR analysis on immunosurgically prepared ICMs (lysed TE debris was used for genotyping). The persistent expression of Nanog and *Oct4*-GFP and the upregulation of Cdx2 expression in *Oct4A*-null E4.5 embryos (KO) were confirmed at the mRNA level. **(e)** and **(f)** A comparison of *Sall4* (**e**) and Nr5a2 (**f**) mRNA levels at the blastocyst stage by real-time RT-PCR in siControl- (GFP-targeting siRNA) and siSall4- (Sall4-targeting siRNA) (**e**), or siNr5a2- (Nr5a2-targeting siRNA) (**f**) injected embryos demonstrated robust downregulation of *Sall4* without significant effect on *Oct4* expression. Expression levels of *Hprt1* were used as the internal control to normalize the data. **P*<0.05; ***P*<0.01. The scale bars represent 20 μm in **a** and **b,** and 50 μm in **c**. Values represent mean±S.D. of 5 biological replicates in **d** and 3 biological replicates in **e** and **f**.

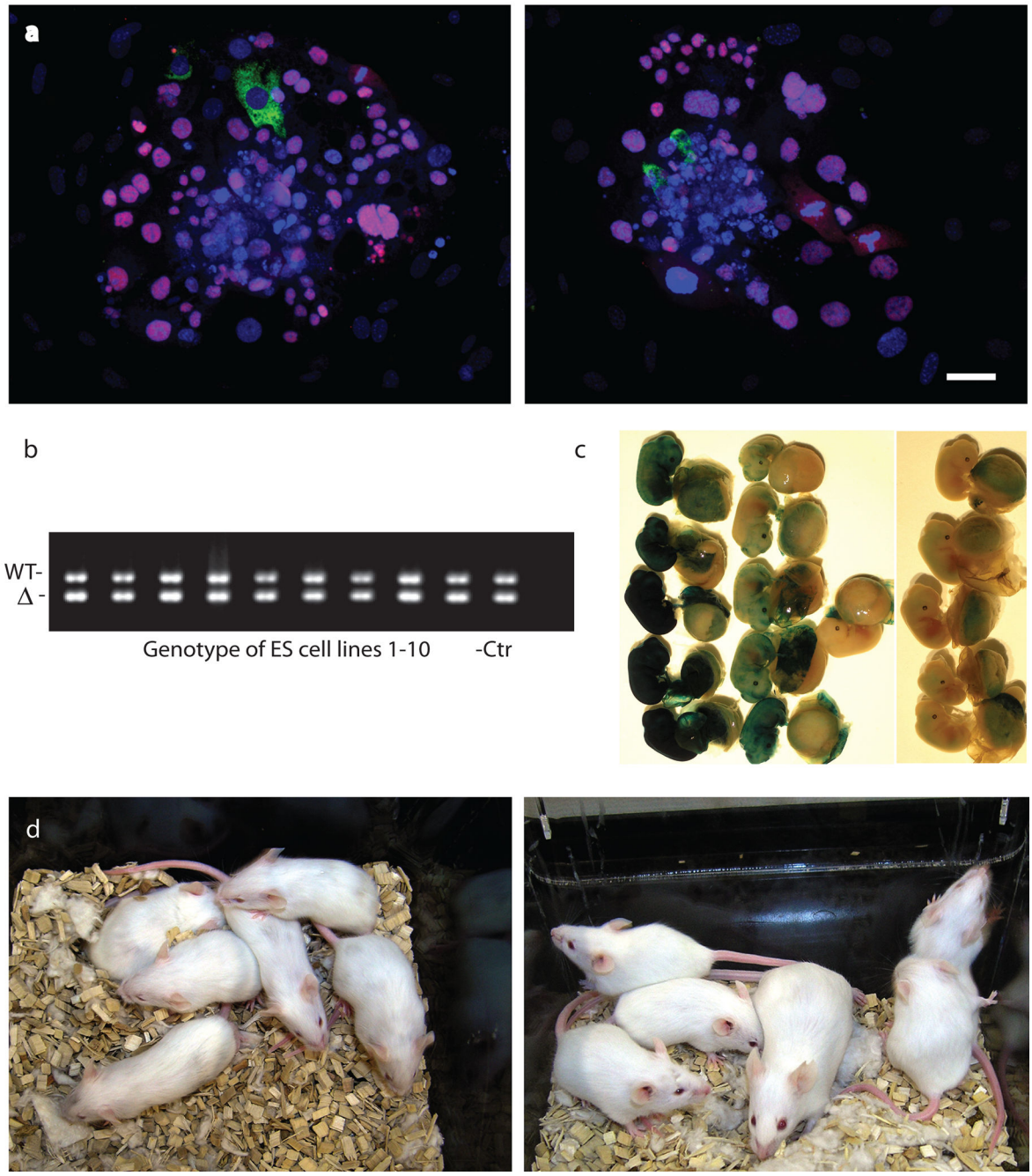


Figure 4. Fate of *Oct4A-null* embryos *in vitro* and *in vivo*

(a) Confocal image of 2 typical *Oct4A-null* outgrowths with immunostaining for Nanog (green) and Cdx2 (red). Nuclear counterstaining with DAPI (blue). Note the cytoplasmic localization of Nanog. **(b)** Genotyping of 10 established ES cell lines from embryos obtained by crossing $Oct4^{flox/flox}$–$ZP3^{Cre/+}$ female mice with $Oct4A^{+/\Delta}$ male mice shows all lines had the *Oct4* Wt allele (415 bp) and no ES cells were derived from $Oct4A^{\Delta/\Delta}$ (245 bp) embryos, confirming that the pluripotent phenotype of cells is dependent on the presence of Oct4. **(c)** *Oct4A*-null embryos marked by *ROSA26* transgene (right) contribute to the

formation of only extraembryonic tissues in chimeric embryos, whereas the majority of control embryos (left) contribute to both embryonic and placental tissues. Blue: LacZ staining. **(d)** After aggregating with CFW embryos (albino), *Oct4A*-null embryos (agouti) did not contribute to the formation of the adult chimeric mice as shown by coat color, whereas 4 of 5 control embryos did contribute to adult chimeric mice. The scale bar represents 20 μm in **a**. The uncropped version of **b** is shown in Supplementary Fig. 5.

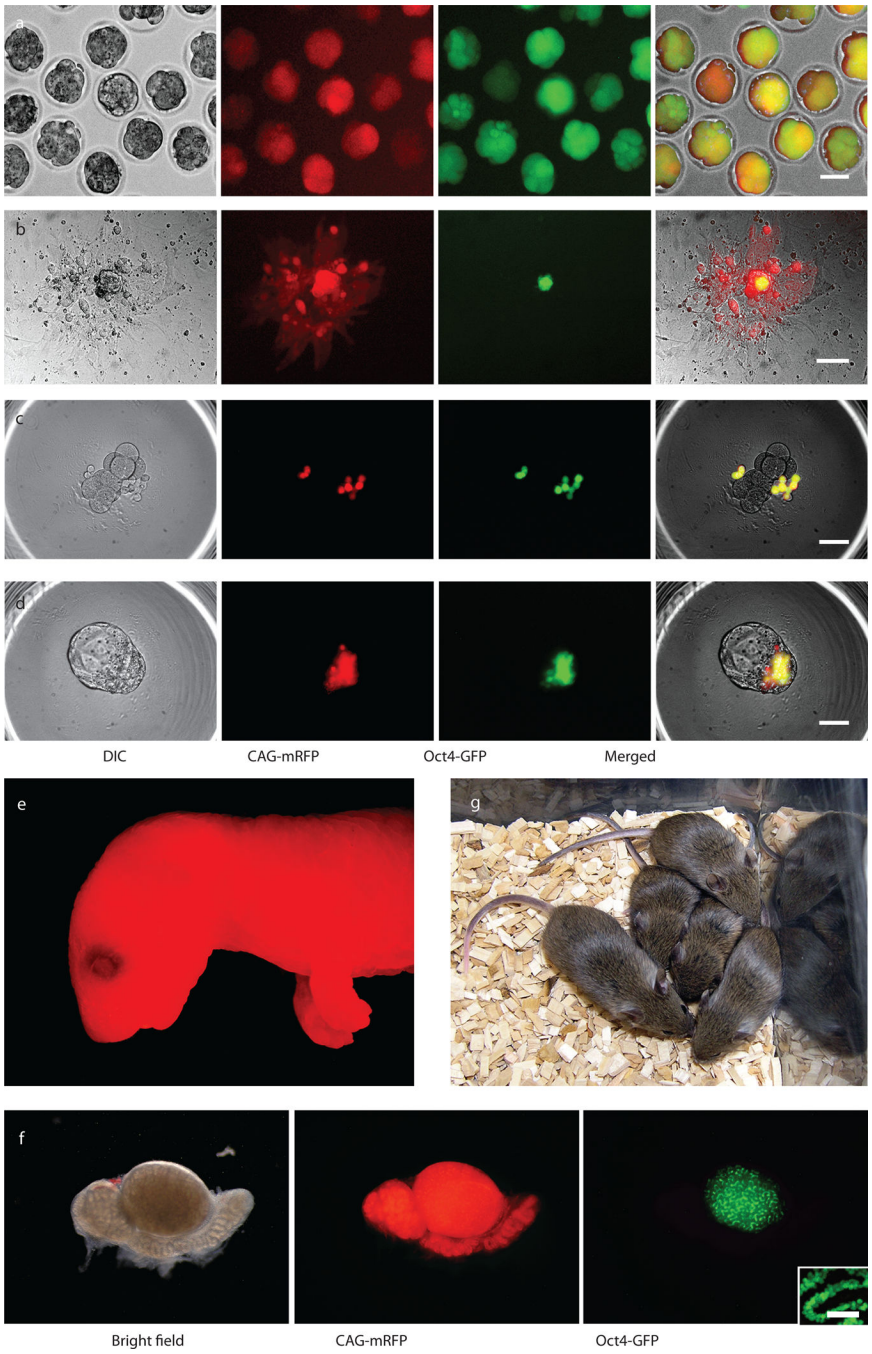


Figure 5. *Oct4A*-null oocytes reprogram somatic cells to full pluripotency as shown by generation of complete NT–ES cell–derived mice via tetraploid complementation

(a) Morulae from NT with *Oct4A*-null oocytes expressed *CAG*-mRFP and *Oct4*-GFP on day 3. **(b)** An outgrowth from NT embryos showed a small group of cells expressing *Oct4*-GFP along with ubiquitous expression of mRFP. **(c)** *CAG*-mRFP and *Oct4*-GFP–expressing ES cells were aggregated with 2 tetraploid embryos. **(d)** A blastocyst with *CAG*-mRFP and *Oct4*-GFP–expressing ES cells integrated into the ICM after overnight culture. **(e)** A full-term, E19.5, all ES cell–derived newborn mouse with ubiquitous expression of mRFP. **(f)** A

gonad from an all ES cell–derived newborn mouse expressing mRFP in all cells and *Oct4*-GFP in germ cells. The insert shows the *Oct4*-GFP–expressing germ cells in the seminiferous tubules at higher magnification. **(g)** Adult all ES cell–derived mice. The scale bars represent 50 μm in **a, b, c,** and **d,** and 20 μm in **f**.