Establishment of totipotency does not depend on Oct4A

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Oct4A is a core component of the regulatory network of pluripotent cells, and by itself can reprogram neural stem cells into pluripotent cells in mice 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/loxP 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 not crucial for either the establishment of totipotency in embryos, or 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 totipotency⁵. After three cell divisions, early embryos compact, and their blastomeres become polarized. Thereafter, they form blastocysts and differentiate into the first two lineages—the inner cell mass (ICM) and the trophectoderm. The ICM gives rise to the entire embryo proper—that is, all cell types except for the trophectoderm, an ability defined as pluripotency. Oct4 (Pou5f1), a POU family transcription factor, is expressed specifically in germ cells², the ICM and embryonic stem cells³ (ESCs), and has been deemed a critical regulator of pluripotency, as shown by a zygotic Oct4-knockout study⁴. Oct4 can activate its own expression with its partner Sox2 through a positive autoregulatory loop in ESCs⁶. Interestingly, Oct4 is listed as one of the 27 proven maternal-effect genes, and is regarded as being functionally important for zygotic genome activation⁶, 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 (bone morphogenetic protein) and LIF (leukaemia inhibitory factor) signalling pathways, Smad1 and STAT3, to their respective targets, and plays a pivotal role in stabilizing the transcription factor complex⁶. Among the core components of the pluripotency circuitry formed by Oct4, Nanog and Sox2, Nanog is directly regulated by Oct4 and Sox2 (ref. 8), Sox2 is actually dispensable for the activation of Oct/Sox enhancers, and the forced expression of Oct4 could rescue Sox2-null ESCs⁹. Hence, Oct4 is considered to be the genetic ‘master switch’ in the establishment of totipotency–pluripotency during the life cycle of mammals¹⁰, and is presumed to be the most upstream gene in the molecular circuitry of pluripotency¹¹. Here, however, we provide solid evidence that challenges this viewpoint.

We used Oct4-floxed mice, in which two loxP motifs had been inserted that span the proximal promoter and the Oct4A unique first exon¹² (Supplementary Fig. S1a). As the other four exons shared by Oct4B were not mutated, the Oct4 studied is hereafter referred to as Oct4A. Conditional removal of maternal Oct4A from oocytes was done by crossing the Oct4loxP/loxP mice with Zp3Cre transgenic mice as shown in Supplementary Fig. S1b. Genotyping for Oct4A deletion in single germinal vesicle oocytes (47/47) (Fig. 1a), mature metaphase II 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 messenger RNA was also demonstrated, without a significant reduction in the expression of the oocyte-specific genes Sall4, Stella and Dazl by real-time PCR with reverse transcription (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. S1e). Elimination of

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Received 3 December 2012; accepted 2 July 2013; published online 11 August 2013; DOI: 10.1038/ncb2816
Figure 1 Generation of oocytes lacking maternal Oct4A and their effect on embryo development. (a) Genotyping of individual oocytes at the germinal vesicle stage by nested PCR showed highly efficient deletion of the floxed Oct4A allele. Δ, Oct4A-null allele; +Ctr, a germinal vesicle oocyte from a heterozygous Oct4A-knockout mouse; −Ctr, lysis buffer control. bp, base pairs. (b) Knockout of maternal Oct4A correlates with elimination of Oct4A transcript as demonstrated by real-time RT–PCR on pools of 10 oocytes each. Ctr, WT oocytes; KO, Oct4A-null oocytes. (c) Immunocytochemistry on E3.5 blastocysts using anti-Cdx2 and anti-Oct4A antibodies and confocal microscopy. Elimination of both maternal and zygotic Oct4A does not induce Cdx2 expression in the ICM. KO, Oct4A knockout; M-Z KO, maternal- and zygotic-Oct4A knockout. (d) Immunocytochemistry on E3.5 blastocysts using anti-Nanog and anti-Oct4A antibodies and confocal microscopy. Elimination of maternal Oct4A does not interrupt zygotic activation of the pluripotent genes Oct4 and Nanog, whereas loss of both maternal and zygotic Oct4A does not prevent Nanog expression. 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+/−; KO, maternal and zygotic knockout. The number (1, 2 or 3) directly after the abbreviation refers to the biological replicate. Scale bars in c,d, 30 μm. Each bar represents the mean from three technical replicates, a result representative of one (a) or two (e) biological replicates. The uncropped version of a is shown in Supplementary Fig. S5, and source data for b,e are shown in Supplementary Table S5.
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 7900 sequence detection system after preamplification for 10 cycles. The Oct4 TaqMan probe/primer set from ABI (Mm00658129_gH, spanning exons 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 exons 1 and 2) eliminated such background signals in all three biological replicates of Oct4A-knockout oocytes (pool of 10 per sample) and two 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 in a and using the same samples as in b. The amplicons from the bright bands were cut and sequenced. They were found to match the Oct4 reference sequence, as shown 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 shown in f, further confirming the presence of Oct4 isoforms in Oct4A-knockout oocytes and embryos. Values represent the means of two to five biological replicates, with error bars representing s.d., in a, and the means from three technical replicates, a result representative of one biological replicate, in b. Uncropped versions of c,e are shown in Supplementary Fig. S5, and source data for a and b are shown in Supplementary Table S5.

the maternal Oct4A protein was confirmed by western blot on pools of more than 400 oocytes per sample (Supplementary Fig. S1d).

However, using the TaqMan Oct4 primers spanning exon 2 and exon 3, real-time 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 has also been observed previously in samples with genetically inactive Oct4 loci using the same Oct4-floxed mice, and was considered to be the background noise of detection methods, the expression of pseudogenes or the expression of other POU-domain family members13. 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 (Fig. 2c,d), along with another primer set spanning exons 3 and 4 (Fig. 2e,f). As shown in Supplementary Tables S1 and S2, both primer sets amplified sequences matching 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 have demonstrated that the Oct4B exists in somatic cells acts as a stress response factor, or an antiapoptotic factor in cancer cells, and only Oct4A has the ability to confer and sustain pluripotency. Combined with Sox2, Klf4 and c-Myc, Oct4B was not able to reprogram somatic cells into induced pluripotent stem cells, unlike Oct4A (ref. 15). Our data showed that the low level of Oct4B expression in Oct4A-null embryos could not maintain pluripotency in vitro or in vivo. Therefore, we consider it highly unlikely that the low levels of Oct4B expression in oocytes, embryos and ESCs 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, on the basis of microarray gene profiling on metaphase II oocytes with minor germinal vesicle morphological variation. 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, whereas the existing maternal Oct4 protein was ignored. Here we investigated the functionality of Oct4A-null oocytes in vivo by crossing Oct4flox/flox–Zp3Cre/+ female mice with WT male mice. To our surprise, we found that these Oct4flox/flox–Zp3Cre/+ female mice were fully fertile, with a normal litter size averaging 7.9 pups (Supplementary Fig. S1f); all these offspring had deletion of the maternal Oct4A allele, as confirmed by PCR genotyping (Supplementary Fig. S1g), demonstrating that maternal Oct4A is not critical for totipotency–pluripotency.

Next, we analysed gene expression using immunocytochemistry and real-time RT–PCR in Oct4A-null blastocysts obtained by crossing Oct4flox/flox–Zp3Cre/+ female mice with Oct4A+/+ 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 embryos had a reduced size on embryonic day E4.5 (102.4 ± 19.6 μm in diameter, n = 33, versus control 124.7 ± 24.4 μm, n = 60, P < 0.01), they exhibited normal caviation and formed a distinct ICM (Supplementary Video S1). Notably, on E3.5, the ICM of maternal/zygotic-knockout embryos did not express the trophectoderm markers Cdx2 (Figs 1c and 3a) and Troma-1 (Supplementary Fig. S2d). Instead, they expressed Nanog, another core factor involved in the regulatory network of ESC self-renewal and pluripotency, at protein (Figs 1d and 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 and 3b). Activation of Nanog expression was observed as early as at the eight-cell stage at mRNA (Supplementary Fig. S2b) and protein levels (Supplementary Fig. S2c), as 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, co-expression of Cdx2 in some of the Nanog+ ICM cells (Fig. 3b and Supplementary Fig. S2e) was observed in 73.1% (30/41) of the knockout embryos, whereas 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 Oct4flox/flox–Zp3Cre/+ female mice with Oct4flox/flox–GOF18+/+ male mice. Green fluorescent protein (GFP) of the 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, 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 (nos 2, 6, 7 and 10–14) and maternal- and zygotic-knockout embryos (nos 5 and 9) in a timely fashion, as in WT embryos (nos 1, 3, 4 and 8 in Fig. 3c and Supplementary Video S2). The genotype of each individual embryo was determined by nested PCR (Supplementary Fig. S3c) at the end of time-lapse observation, with its position tracked in the video with bright-field microscopy (Supplementary Fig. S3a and Video S3). 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. Whereas 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 (refs 4,22). Repression of Oct4 expression was shown to induce the differentiation of ESCs into trophectoderm, and a less than twofold increase in Oct4 expression was shown to cause differentiation of ESCs into cells of primitive endoderm and mesoderm. In the absence of Oct4, embryos could not form an ICM—that is, the inner cells of morula-stage embryos were rather driven into tropheoblast differentiation. 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. This hypothesis was further strengthened by the finding of the reciprocal interaction between Oct4 and Cdx2 in determining trophectoderm differentiation and by the interaction between Oct4 and the histone H3-specific methyltransferase ESET in restricting the extraembryonic tropheoblast lineage potential of pluripotent cells. However, co-expression of Oct4 and Cdx2 was found in normal morula-stage embryos, and expression of Cdx2 in ESCs was found to be rapidly initiated by Ras activation (that is, within 24 h), without previous or simultaneous downregulation of Oct4 expression. Moreover, Cdx2 deficiency was not shown to interrupt trophectoderm–ICM lineage separation. Meanwhile, yzotic Oct4 expression was shown not to be required for initial repression of the trophectoderm genes Cdx2 and Gata3, indicating that other mechanisms must lead to the restriction of these genes to the trophectoderm (ref. 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. Our results clearly demonstrated that ICM–trophectoderm lineages were separated in maternal and yzotic Oct4A-knockout embryos, suggesting that first lineage separation of...
Figure 3 Gene expression of Oct4A-null blastocysts produced by crossing Oct4^{lox/lox-}Zp3^{Cre+} female mice with Oct4^{lox/+} 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 trophectoderm marker Cdx2 in the trophectoderm. (b) On E4.5, although the ICM of maternal- and zygotic-knockout embryos (Oct4A KO) maintained Nanog expression, co-expression of Cdx2 in some of the Nanog^{+} ICM cells was observed, as indicated by arrowheads, whereas 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/+; nos 2, 6, 7 and 10–14) embryos and maternal- and zygotic-knockout embryos (Δ/Δ; nos 5 and 9) in a timely fashion at about E2.5, as in WT embryos (+/+: nos 1, 3, 4 and 8). (d) Real-time RT–PCR analysis on immunosurgically prepared ICMs (lysed trophectoderm 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. Ctr, control. (e,f) A comparison of Sall4 (e) and Nr5a2 (f) mRNA levels at the blastocyst stage by real-time RT–PCR in embryos injected with control (GFP-targeting) siRNA and siRNA targeting Sall4 (e) or Nr5a2 (f) 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. Scale bars, 20 μm (a,b); 50 μm (c). Values represent mean ± s.d. of five biological replicates in d and three biological replicates in e and f. Source data for d-f are shown in Supplementary Table S5.
the ICM–trophectoderm 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 ESCs, wherein upstream Oct4 and Sox2 regulate Nanog expression16,17, 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\textsuperscript{fluox/flu}−Zp3\textsuperscript{Cre/−} female mice with Oct4\textsuperscript{+/-}\textsuperscript{−}/−OG2\textsuperscript{-/-} male mice, in which the Oct4–GFP transgene lacks the proximal enhancer and activates GFP expression through the distal enhancer and promoter (GOF18\textsuperscript{ΔPE}; ref. 32), we demonstrated that GFP is still activated in Oct4A-null embryos (Supplementary Fig. S3b). 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 four-factor reprogramming33–35.

In an attempt to identify the factors critical for driving Oct4 and Nanog expression, we knocked down six reported Oct4-regulating factors, Salld, Tpt1, Zscan4, Esrrb, Utf1 and Nr5a2, by microinjecting gene-specific short interfering RNA (siRNA) duplexes into maternal Oct4A-deficient zygotes, to avoid any possible effect of maternal Oct4A as a positive autoregulator. The efficient knockdown of Salld, Tpt1, Zscan4, Esrrb and Utf1 was observed at the blastocyst stage. However, we did not observe any significant change in either Oct4 expression (Fig. 3e and Supplementary Fig. S3e) or Oct4–GFP activation (Supplementary Fig. S3d). 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 ESC self-renewal36. However, Nr5a2 can induce epiblast stem cells into ground state pluripotency37 and replace Oct4 in the reprogramming of somatic cells into pluripotent cells38. 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 induced pluripotent stem cell techniques, and eventually support the generation of Oct4-independent induced totipotent stem 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 threshold cycle (C\textsubscript{T}) 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 gastrulation40. Mutation of Tbx1 causes the DiGeorge syndrome phenotype, which manifests as defects in the pharyngeal arch and in cardiovascular and craniofacial development41.

Chromatin immunoprecipitation sequencing data have shown that Oct4 binds to the promoter region of Tbx1 (ref. 7). Further study of 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 \textit{in vitro} by platting them onto mouse embryonic fibroblasts (MEFs) and attempting to derive pluripotent ESCs. None of the 10 ESC lines derived from the 43 embryos were homoygous 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\textsuperscript{-/-} nuclei along with Cdx2\textsuperscript{+/-} trophoblast cells (Fig. 4a). Interestingly, Nanog was still detectable in some Cdx2\textsuperscript{+/-} 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 cell. Accordingly, we aggregated Rosa26\textsuperscript{+/-}/− Oct4A-null eight-cell embryos with WT embryos. After 24 h of culture, chimaeric embryos with homozygous (Oct4A-knockout) and heterozygous (control) mutants were separately transferred into pseudopregnant mice. E13.5–E14.5 fetuses were collected and subjected to β-galactosidase gene (LacZ) staining (Fig. 4c). Consistent with our \textit{in vitro} study, Oct4A-null embryos contributed exclusively to the placental tissue of trophoblast origin, and not to the embryo proper (0/7 versus 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 colour, whereas four of five 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 pluripotency3.

Studies have shown that the nuclei of terminally differentiated somatic cells can be reverted back to a totipotent state after nuclear transplantation into enucleated oocytes40. 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\textsuperscript{fluox/flu}/−Zp3\textsuperscript{Cre/−} female mice. By using both immunocytochemistry (Supplementary Fig. S4a) and real-time RT–PCR (Supplementary Fig. S4b), we observed that Oct4A-null oocytes still activated the expression of pluripotent genes (Oct4 and Nanog) in the ICM and the trophoblast marker gene (Cdx2) in the trophoblast in all 28 cloned embryos analysed.

To further assess whether maternal Oct4A-null oocytes can induce pluripotency in somatic cells, we derived ESCs from NT embryos using these oocytes as recipient oocytes and CAG–mRFP (monomeric red fluorescent protein) 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 nine ESC lines (Supplementary Fig. S4c). Chromosome counts from metaphase spreads showed that seven of the nine NT-ESC lines were normal, with 40 chromosomes. Four of the seven NT-ESC lines—RG1, RG5, RG6 and RG8—were tested with the tetraploid complementation assay, the most stringent test for pluripotency (Fig. 5c). All ESC–tetraploid embryo aggregates developed to the
blastocyst stage and had ESCs integrated into the ICM (Fig. 5d). After being transferred into the uterus of pseudopregnant female mice, the RG5 ESC line did not support any of the 30 aggregates to full-term development. RG6 gave rise to two pups of 50 transferred (one dead; one alive, initiated breathing but died shortly thereafter). RG1 and RG8 ESC 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. S4e) 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 five 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 ESC lines was further confirmed by differentiation into cells of all three embryonic germ layers in the teratoma assay (Supplementary Fig. S4d). 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
Figure 5 Oct4A-null oocytes reprogram somatic cells to full pluripotency as shown by generation of complete NT-ESC-derived mice through 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 ESCs were aggregated with two tetraploid embryos. (d) A blastocyst with CAG–mRFP and Oct4–GFP-expressing ESCs integrated into the ICM after overnight culture. (e) A full-term, E19.5, all ESC-derived newborn mouse with ubiquitous expression of mRFP. (f) A gonad from an all ESC-derived newborn mouse expressing mRFP in all cells and Oct4–GFP in germ cells. Inset: the Oct4–GFP-expressing germ cells in the seminiferous tubules at higher magnification. (g) Adult all ESC-derived mice. Scale bars, 50 μm in a–d, 20 μm in f.
zygotic Oct4A-null blastocysts maintained the ability to activate Nanog and Oct4-GFP expression, indicating that unknown pathways other than the Oct4-centred 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**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

We thank J. Mueller-Keuker, M. Preusser and N. Stengel for assistance in preparing the manuscript and A. Malapetsas for proofreading the manuscript. We thank K. Huebner for her technical help on immunocytochemistry and B. Schäfer for her assistance on histology work. The authors of this manuscript bear sole responsibility for the content presented, which does not necessarily represent the official views of the Eunice Kennedy Shriver National Institute of Child Health & Human Development or the National Institutes of Health. This research was supported by the Max Planck Society, DFG grants DFG SI 1695/1-2 (SPP1356) and SCHO 340/7-1, and grant NIH R01HD059946-01 from the Eunice Kennedy Shriver National Institute of Child Health & Human Development.

**AUTHOR CONTRIBUTIONS**

G.W. designed and executed experiments as well as writing the manuscript. D.H., Y.G., V.S., L.G., N.S., K.A., G.P., C.O., M.S., M.R. and A.T. executed experiments, collected data and prepared reagents. H.R.S. provided the study concept and funding, and edited the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Induction of recombination in Oct4<sup>lox/lox</sup> mice by Cre recombinase. A knock-in 129/Sv strain containing a functional Oct4 allele flanked by loxP motifs (Oct4<sup>lox/lox</sup> (ref. 12)) was crossed with a Zp3Cre transgenic C57BL/6j mouse<sup>43</sup>. The Zp3Cre promoter-driven Cre expression supports the inactivation of maternally expressed genes in growing oocytes<sup>44</sup> as early as day 5 after birth<sup>45</sup>. DNA extraction and PCR genotyping were carried out as reported previously<sup>32</sup>. As shown in Supplementary Fig. S1a, the intact Oct4 allele and the loxp site were detected by primer pair B (forward, GOF-D1, reverse, GOF-R1; amplicon size: WT, 415 bp; floxed, 449 bp), and the 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. S1c.

Nested PCR was used to genotype individual oocytes and blastomeres as described<sup>46</sup> 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 sizes: WT (362 bp), floxed (396 bp) and deleted (245 bp). For genotyping cells with Oct4<sup>-/-</sup>, 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 sizes, 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 under the supervision of a certified veterinarian in charge of the Max Planck Institute 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.<sup>47</sup> 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<sup>48</sup>. Briefly, embryos were fixed in 4% paraformaldehyde (20 min), permeabilized with 0.1% Triton X-100, and stained with primary antibodies (rabbit anti-Nanog IgG (1:200; Cosmo Bio Company, Japan); goat anti-mouse IgG (Jackson Laboratory) at a 1:400 dilution for 1 h. The following secondary antibodies were used: rabbit anti-Nanog (Jackson ImmunoResearch, West Grove, PA, USA) at 1:200; goat anti-CD2 (Santa Cruz Biotechnology, clone C-10, SC-5279); rabbit anti-Oct4 IgG (1:2;500; generated in our laboratory); goat anti-Oct4 IgG (1:100; Santa Cruz Biotechnology, SC-8628); mouse anti-Cdx2 (1:100; BioGenex, clone CDX2-88, MU392-U2); rat anti-Troma-1 (1:100; DSHB). Some samples were counterstained with 5 μM DRAQ5 (Biostatus) for 30 min. Samples were imaged using a laser scanning confocal microscope (UltraVIEW; PerkinElmer Life Sciences) with 488 nm, 568 nm and 647 nm lasers. Immunocytochemistry experiments were repeated two or three times with 30–100 embryos in each experiment.

RNA extraction, complementary DNA synthesis and real-time RT–PCR. For real-time analysis of gene expression, embryos were harvested in RLT buffer (Qiagen) and processed as previously described<sup>49</sup>. Briefly, total RNA was extracted from individual blastocysts using a MicroNeasy kit (Qiagen) and cDNA synthesis was carried out with a High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s instructions. Real-time PCR was carried out on the ABI PRISM 7900HT sequence detection system (Applied Biosystems) using TaqMan probes from Applied Biosystems. All primers used are listed in Supplementary Tables S3. Two to six biological replicates were used, and each sample was run with three technical replications; an RT blank and a no-template blank served as negative controls. The C<sub>T</sub> values were collected using Applied Biosystems SDS v2.0 software and transferred to a Microsoft Excel spreadsheet for further relative quantification analysis using the ΔΔC<sub>T</sub> method (User Bulletin no. 2, ABI PRISM 7700 sequence detection system, 1997). Single oocyte real-time RT–PCR using the Biomark 48.48 Dynamic Array System (Fluidigm) was carried out as previously described<sup>50</sup>. All real-time RT–PCR source data can be found in Supplementary Informations.

Microinjection of siRNA duplexes into zygotes. Zygotes were collected in the morning after mating from superovulated or natural ovulated Oct4<sup>lox/lox</sup>–Zp3<sup>cre</sup>/+ female mice. Microinjection of siRNA duplexes into zygotes was carried out using an Eppendorf Femtotjet microinjector and Narishige micromanipulators as previously described<sup>46</sup>. Approximately 2 pl of siRNAs (20 μM) was injected into cytoplasm of zygotes. Scrambled siRNA duplexes or siRNA duplexes against GFP were used as control. The injected zygotes were then cultured with KSO<sup>45</sup> (potassium simplex optimized medium plus 19 natural amino acids<sup>47</sup>) in a humidified 5% CO₂ in air at 37 °C. Expression of the target genes was analysed at the blastocyst stage (3.5 dpc) in pools of 10–20 embryos per sample. The target sequences of siRNA are listed in Supplementary Table S4.

Somatic cell nuclear transfer. Seven- to 9-week-old Oct4<sup>lox/lox</sup> (control oocytes) and Oct4<sup>lox/lox</sup>–Zp3<sup>cre</sup>/+ (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 carried out essentially as previously described<sup>51</sup>. Six hours after the onset of activation, the constructs were washed carefully in a four-well dish (Nunc, 176749) with 0.5 ml of alpha minimal essential medium (Invitrogen, 12571-063) supplemented with 0.4% of bovine serum albumin (Sigma-Aldrich, A3311) without cytochalasin B under 5% CO₂ in air for 4 days. The blastocysts obtained were subjected to immunostaining, genotyping and real-time RT–PCR.

For derivation of ESCs from NT embryos, 8- to 12-week-old Oct4<sup>lox/lox</sup>–Zp3<sup>cre</sup>/+ 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 (ref. 49) male mice with Oct4-GFP female mice (GOF-18) (ref. 21), were used as nucleus donors. The NT procedures were the same except that MEF cells were briefly exposed to HVJ-E Sendai virus extract (www.cosmобio.co.jp) and then placed into the perivitelline space of recipient Oct4-null-enucleated oocytes on the side opposite the first polar body<sup>51</sup>. The constructs were transferred into KSO<sup>45</sup> and cultured for 1–2 h in an incubator with 5% CO₂ in air at 37 °C to induce fusion before activation. The morulae obtained on day 3 of culture were subjected to the ESC derivation procedure according to a previous report<sup>51</sup>.

Western blotting. Western blot was carried out as described previously<sup>46</sup>. Briefly, 400 WT and 400 maternal Oct4<sup>-/-</sup> oocytes were collected in PBS. Oocytes, 10,000 ESCs as well as cumulus cells were lysed in RIPA buffer (50 mM Tris at pH 8.0, 300 mM NaCl, 1% IGEPA CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 1 × protease inhibitors (Roche) and 1 × phosphatase inhibitors (Sigma-Aldrich)) containing 50 units of Benzonase (Merck) on ice for 1 h. 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, clone C-10, SC-5279). The western blot experiment was repeated twice.

Sequencing. Products of quantitative PCR were gel purified and ligated into PCR2.1-TOPO vector (Invitrogen) according to the manufacturer’s instructions. For each sample, six colourless colonies were picked and sent to GATC Biotech AG (Konstanz) in 96-well format for sequencing using M13 reverse primer.

Generation and analysis of chimaeric embryos. To assess the pluripotency of Oct4-inactivated embryos in vivo, we aggregated mutated eight-cell embryos (obtained from crossing Oct4<sup>lox/lox</sup>–Zp3<sup>cre</sup>/+–ROSA26<sup>+/-</sup> female mice with Oct4<sup>lox/lox</sup>–ROSA<sup>26</sup>/+ male mice)—after removal of the zona pellucidae by acidic Tyrode’s solution (Sigma) and biopsy for genotyping by nested PCR—with eight-cell embryos from WT B6C3F1 mice for LacZ evaluation of chimaerism in the fetus or for coat colour evaluation in albino CFW mice. Following 24 h of culture in KSO<sup>45</sup>, successfully fused and genotyped chimaeric blastocysts carrying heterozygous (69 in total as control) and homozygous mutants (60 in total as Oct4 knockout) were transferred separately into the uteri of 10 ICR pseudopregnant recipients. Eleven fetuses carrying heterozygous mutants and seven fetuses carrying homozygous mutants were recovered on E13.5 from eight recipients and subjected to whole-mount LacZ staining as previously described<sup>52</sup>. Five full-term pups from each of the controls and the knockout mutants were obtained from two recipients for evaluation of coat colour chimaerism.

Derivation of ESC lines. E2.5 embryos were cultured on MEFs to establish ESC lines with a standard procedure<sup>46</sup> after removal of the zona pellucidae (as described above). All ESC lines were newly derived in the present study and tested negative for mycoplasma contamination. The complete ESC medium composition was as follows: 4 mM l-glutamine, 100 units ml<sup>-1</sup> penicillin, 100 μg ml<sup>-1</sup> streptomycin,
0.1 mM β-mercaptoethanol, 0.1 mM non-essential amino acids, 1,000 units ml⁻¹ LIF (Chemicon) and 15% fetal bovine serum (Invitrogen, 10165-185) in DMEM (Gibco, 31885-023).

**Tetraploid embryo complementation assay for NT-ESCs.** The assay was conducted as described previously. Briefly, two-cell embryos were flushed 20 h post-hCG (human chorionic gonadotrophin) from the oviducts of B6C3F1 mice and fused with a peak pulse of 50 V 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 ESCs 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 to remove the zona pellucida. Two embryos were placed on each ESC clump and cultured in an incubator with 5% CO₂ in air at 37°C. After 24 h 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 analysed using a program from Applied Biosystems as recommended. For other experiments, statistical analysis was carried out using Student’s t-test and Fisher’s exact test. A P value of less than 0.05 was considered significant.

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Figure S1 Elimination of maternal Oct4A did not show any effect on the oocyte’s developmental competence. (a) Schematic representation of the Oct4A targeting DNA construct and position of the genotyping primer sets modified from Kehler et al. (2004). Filled arrowheads: lox P sites; Oval box: Oct4 promoter; filled rectangles: Oct4 exons 1–5. (b) Mating strategy to generate maternal Oct4A-null oocytes. The Oct4floX/floX mice were mated with ZP3Cre/Cre mice to produce offspring with the Oct4floX/+ZP3Cre/+ genotype. Then the Oct4floX/+ZP3Cre/+ male mice were backcrossed with Oct4floX/floX female mice to obtain Oct4floX/floX/ZP3Cre/+ mice. The male mice of this genotype were backcrossed with Oct4floX/floX females to obtain Oct4floX/floX/ZP3Cre/+ male mice. (c) The tails of offspring were cut and genotyped with primer pair B to detect for the presence of the Oct4 floxed allele (449 bp), Wt allele (415 bp), and a primer pair for Cre (373 bp). Oct4 floxed/floxed male mice were mated with ZP3Cre/Cre mice to produce offspring with the Oct4floX/+ZP3Cre/+ genotype. Then the Oct4floX/+ZP3Cre/+ male mice were backcrossed with Oct4floX/floX female mice to obtain Oct4floX/floX/ZP3Cre/+ mice. The male mice of this genotype were backcrossed with Oct4floX/floX females to obtain Oct4floX/floX/ZP3Cre/+ male mice. (d) Validation of elimination of maternal Oct4A at the protein level by Western blot analysis. Oocyte samples comprised extracts from 400 or more GV oocytes. A monoclonal Oct4A antibody detected a weak band of Oct4 protein in wild-type oocytes (WT) and very strong band in the ES cell sample (ES) of approximately 45 kDa, but not in the Oct4-knockout oocytes (KO) and cumulus cells (CM). (e) Gene expression of single oocytes at the GV stage, analyzed by Fluidigm qRT-PCR using the Biomark 48.48 Dynamic Array system (Fluidigm) further confirmed the elimination of the Oct4A transcript without a significant impact on the expression of oocyte- and lineage-specific genes examined. The number (1, 2, 3, or 4) right after the abbreviation (Ctr, for wild-type control, or KO, for knockout) refers to the biological replicates. (f) Oct4A-knockout oocytes can support establishment of totipotency, which is necessary for full-term development, as shown by the normal litter size from the crossing of Oct4floX/floX/ZP3Cre/+ female mice with CD1 wild-type male mice. (g) PCR genotyping of the offspring from the above crossing confirmed that the Oct4A allele had been deleted. The bars represent the means from 3 technical replicates, a result representative of each biological replicate in e. The uncropped version of d is shown in Supplementary Fig. 5 and source data for e are shown in Supplementary Table 5.
Figure S2 Phenotype of Oct4A-null embryos (a) Genotyping with nested PCR on single biopsied blastomeres from embryos obtained by crossing Oct4flox/flox/ZP3Cre/+ female mice with Oct4flox/+ male mice. (b) Quantitative RT-PCR on single genotyped 8-cell embryos with triplicates shows that Oct4 elimination does not delay activation of Nanog gene transcription. Ctr: Oct4A+/−, KO: maternal and zygotic Oct4A-knockout. (c) Immunocytochemistry of E2.5 embryos for Nanog (green) and Oct4 (red), M-Z KO: maternal and zygotic Oct4A knockout. (d) Immunocytochemistry of E3.5 blastocysts for Troma-1 (red), another TE marker, and Oct4 (green) localized the protein to the TE, which further confirmed the lineage separation of ICM/TE in Oct4A-null embryos. (e) Average cell numbers of Nanog- and Cdx2-positive cells per E4.5 embryo were counted on confocal images of immunostained embryos. KO: Oct4A-knockout; M-Z KO: maternal and zygotic Oct4A knockout. The scale bars represent 25 μm in c and d. Value represents mean±S.D. of 3 biological replicates in b and mean±S.D. of 61 and 41 embryo samples for wildtype and Oct4A KO, respectively in e. Source data for b are shown in Supplementary Table 5.
Figure S3 Oct4-GFP expression is activated in Oct4A-null embryos. (a) At the end of the time-lapse observation on Oct4-GFP expression, each individual embryo was marked by a number with its genotype (maternal/zygotic) as determined by c. (b) Generated by crossing Oct4flox/flox/ZP3Cre/+ female mice with OG2-GFP+/- Oct4+/- male mice, GFP-expressing E4.5 embryos were selected as shown. Genotyping of these embryos revealed that half (17/36) were maternal/zygotic knockout and suggested that OG2-GFP was still activated in Oct4A-null embryos. (c) Genotype was determined by nested PCR with corresponding numbers to a. (d) Oct4-GFP expression was not affected in E2.5 (left) and E3.5 (right) embryos following injection of siSal4 into zygotes obtained by crossing Oct4flox/flox/ZP3Cre/+ female mice with GOF18-GFP male mice. Embryos without the Oct4-GFP transgene were used as negative control and siRNA targeting GFP (siGFP) was used as positive control. Fluorescence intensity was quantified by ImageJ software. (e) Efficient knockdown of Tpt1, Zscan4, Esrrb and Utf1 at the mRNA levels by injection of siRNA duplexes as assessed by real-time RT-PCR. No significant effect on Oct4 expression was observed. Expression levels of Hprt1 were used as the internal control to normalize the data and siGFP-injected embryos were used as calibrators. Scale bars represent 50 μm in a and b. The error bars represent mean±S.E.M. of 8-16 biological replicates in d and mean±S.D. of 3 biological replicates in e. The uncropped version of c is shown in Supplementary Fig. 5 and source data for e are shown in Supplementary Table 5.
Figure S4 Oct4A-null oocytes reprogrammed somatic cell nuclei to pluripotent status. (a) Immunocytochemistry of E4.0-NT blastocysts show activation of Nanog and Oct4 expression by Oct4A-knockout (Oct4A KO) oocytes. WT: wild type; NT: Nuclear transferred; PA: parthenogenic. (b) Gene expression profiling of NT blastocysts revealed activation of expression of the pluripotent genes Oct4 and Nanog without maternal Oct4A expression. The gene expression levels were obtained with pools of 3 blastocysts with triplicates and presented in comparison with ES cells (ES). Ctr: NT embryos using wild-type oocytes; KO: NT embryos using Oct4A-knockout oocytes; PA: parthenogenic embryos using Oct4-knockout oocytes. The number (1, 2, or 3) right after the abbreviation (Ctr and KO) refers to the biological replicates. (c) Morphology of NT-ES cells grown on MEFs expressing CAG-mRFP and Oct4-GFP. (d) Histology of teratoma from NT-ES cell line RG6 4 weeks after injection into SCID mice as assessed by haematoxylin and eosin staining. The teratoma contained cells of all 3 embryonic germ layers. Upper left panel: keratinized stratified squamous epithelial cells (ectodermal); upper right panel: neural rosettes (ectodermal); lower left panel: striated muscle (mesodermal); lower right: ciliated columnar epithelial cells adjacent to pancreatic acinar cells (both endodermal). (e) A litter of neonatal NT-ES cell–derived pups delivered by cesarean section on E19.0. In this particular litter, 8 pups showed normal full-term development, of which one was dead and one failed to initiate breathing (*). The scale bars represent 30 μm in a, 100 μm in c and 50 μm in d. Values represent means±S.D. of 3 biological replicates. Source data for b are shown in Supplementary Table 5.
Figure S5 Uncropped figures for Fig. 1a, 2c, 2e, 4b and Supplementary Fig. 1d and 3c.
Supplementary Table 1 Sequencing results of RT-PCR amplicon using primers spanning exon 2 and exon 3 of the Oct4 gene in Oct4A-null oocytes and embryos.

Supplementary Table 2 Sequencing results of RT-PCR amplicon using primers spanning exon 3 and exon 4 of Oct4 gene in Oct4A-null oocytes and embryos.

Supplementary Table 3 Primers for gene expression study.

Supplementary Table 4 siRNA target sequences

Supplementary Table 5 Source data file. This file includes the original source data for real-time RT-PCR assay.

Legends to Supplementary Videos

Supplementary Video 1 Time-lapse recording of in vitro development of Oct4A-null 8-cell embryo A biopsied and genotyped morula with maternal/zygotic Oct4A-null was cultured on MEFs in ES cell medium and observed on the stage of a microscope with an incubation chamber (TOKAI HIT, Japan) filled with 5% CO$_2$ in air and maintained at 37°C. Brightfield pictures were taken every 5 min for 4 days and were compiled into a movie with 24 frames per second. The video demonstrates that Oct4A-null embryos initiated cavitation and formed grossly normal-looking blastocysts with distinct ICM. However, immunostaining of the outgrowth (Fig. 3a) showed cytoplasmic localization of Nanog as well as fragmentation of nuclei.

Supplementary Video 2 Time-lapse confocal recording revealed activation of Oct4-GFP expression in maternal-knockout and maternal/zygotic-knockout embryos Twelve 2-cell embryos from the mating of Oct4$^{floxed}$/ZP3$^{Cre+}$ female mice with Oct4$^{A+/+}$/Oct4-GFP$^{+/+}$ male mice and 4 embryos (#1, 3, 4 and 8) from the mating of Oct4$^{floxed}$/ male mice with Oct4$^{A+/+}$/Oct4-GFP$^{+/+}$ male mice were placed in KSOMAA in a glass bottom dish with the same condition as Supplementary Video 1 for confocal examination with 488 nm laser. A confocal picture had been taken every 10 min for 3 days and was compiled into a movie with 24 frames per second. The video demonstrated that regardless of the genotype, all embryos activated Oct4-GFP at around E2.5 in a timely fashion, as did wild-type embryos. The genotype of each embryo is shown in Fig. S3a.

Supplementary Video 3 Brightfield time-lapse recording of the same embryos at the same time point as Supplementary Video 2 This video was used to monitor the developmental stage of the embryos and to trace the position of individual embryos for genotype determination.