Synergic reprogramming of mammalian cells by combined exposure to mitotic *Xenopus* egg extracts and transcription factors

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Transfer of somatic cell nuclei to enucleated eggs and ectopic expression of specific transcription factors are two different reprogramming strategies used to generate pluripotent cells from differentiated cells. However, these methods are poorly efficient, and other unknown factors might be required to increase their success rate. Here we show that *Xenopus* egg extracts at the metaphase stage (M phase) have a strong reprogramming activity on mouse embryonic fibroblasts (MEFs). First, they reset replication properties of MEF nuclei toward a replication profile characteristic of early development, and they erase several epigenetic marks, such as trimethylation of H3K9, H3K4, and H4K20. Second, when MEFs are reversibly permeabilized in the presence of M-phase *Xenopus* egg extracts, they show a transient increase in cell proliferation, form colonies, and start to express specific pluripotency markers. Finally, transient exposure of MEF nuclei to M-phase *Xenopus* egg extracts increases the success of nuclear transfer to enucleated mouse oocytes and strongly synergizes with the production of pluripotent stem cells by ectopic expression of transcription factors. The mitotic stage of the egg extract is crucial, because none of these effects is detected when using interphasic *Xenopus* egg extracts. Our data demonstrate that mitosis is essential to make mammalian somatic nuclei prone to reprogramming and that, surprisingly, the heterologous *Xenopus* system has features that are conserved enough to remodel mammalian nuclei.


Conflict of interest statement: No conflicts reported.

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Supplementary Material

**Tables**

Table S1: Summary of the reprogramming experiments.

**Figs.**

Fig. 1: Metaphase egg extracts reprogram somatic cells.

Fig. 2: Preincubation with metaphase egg extracts improves efficiency of both Nuclear Transfer and iPS Cell Production in Mammals.

Fig. 3: Metaphase egg extracts partially reprogram somatic cells in vitro.

Fig. 4: Metaphase egg extracts improve reprogramming efficiency in vivo.

Fig. 5: Metaphase egg extracts improve reprogramming efficiency in vivo.

Fig. 6: Metaphase egg extracts improve reprogramming efficiency in vivo.

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M-phase Xenopus egg extracts improve the efficiency of nuclear transfer and iPSC production from mammalian fibroblasts. (A) Schematic representation of nuclear transfer experiments using MEFs exposed to M-phase egg extracts. (B) Percentage of early embryos resulting from nuclear transfer of MEFs exposed to M-phase extract (M-Extract) or interphase (I-Extract) Xenopus egg extracts and normalized to the number of two-cell embryos. (C) Schematic representation of iPSC generation from OCT4-GFP+ colonies by ectopic expression of OSKM followed or not followed (mock) by exposure to M-phase egg extracts. (D) Number of OCT4-GFP+ colonies relative to nonpermeabilized cells. The effect of exposure to M-phase egg extracts on the efficiency of iPSC production was assessed by measuring the production of OCT4-GFP+ colonies after exposure to M-phase egg extracts (M-phase alone), OSKM overexpression (OSKM alone) and OSKM overexpression followed by exposure to buffer alone (OSKM + mock) or to M-phase egg extracts (OSKM + M-phase), in three fully independent experiments. Error bars represent SEM. (n = 3).

Characterization of M-iPS Cells. M-iPS cells presented an ES cell-like morphology and uniform expression of the pluripotency markers alkaline phosphatase, OCT4, NANOG, and stage-specific embryonic antigen-1 (SSEA1) (Fig. 2 A–C). The levels of expression of different pluripotency markers were similar to those in ES cells (Fig. 2D). The transcriptomic profiles of M-iPS cells, MEFs, and ES cells were analyzed (Fig. 2F), and scatter plots confirmed the similarity between M-iPS and ES cells (R² = 0.9175).

Efficient reprogramming has been linked tightly to hypomethylation of DNA on promoters of key regulators of pluripotency, such as Oct4 and Nanog (5). The DNA methylation profiles of M-iPS cells and ES cells were similar (Fig. 2F), confirming the efficiency of reprogramming obtained.

When induced to differentiate, all tested M-iPS clones formed embryoid bodies (Fig. 3A), and the stem cell markers Oct4, Nanog, and Klf4 were down-regulated (Fig. 3B), whereas markers of differentiation in the three germ layers were up-regulated with levels comparable to those observed in embryoid bodies obtained from ES cells (Fig. 3C) (18–21).

Finally, the complete reprogramming of the M-iPS clones was demonstrated in vivo by the capacity of two different clones, one male and one female, to produce adult chimeras after injection into CD1 blastocysts (Fig. 3C and Table S2). Germline transmission also was successful as shown by the production of F1 black offspring (because of the B6xF1 genetic background) after mating these chimeras with CD1 albino animals (Fig. 3D).

We conclude that M-phase Xenopus egg extracts have a strong positive effect on the efficiency of iPSC cell production. Importantly, this action is not additional but synergistic, because the reprogramming efficiency when the two strategies are combined is much higher than the simple addition of their respective efficiencies (Fig. 1D).

M-Phase Xenopus Egg Extracts on Their Own Partially Reprogram Mammalian Fibroblasts. MEFs have a limited proliferation potential (22). M-phase egg extracts strongly increased the proliferation rate of MEFs during at least two cell cycles (Fig. 4A) and also induced the formation of a few colonies that expanded over a few days before growth arrest (Fig. 4B). These colonies were never seen in mock-treated MEFs.

Growth stimulation was accompanied by expression of pluripotency cell markers, which were never observed in mock-treated cells. Indeed, alkaline phosphatase expression (a marker of partial reprogramming) was induced (Fig. 4C), and endogenous expression of Oct4, a stringent marker of pluripotency (2), was detected in colonies by immunofluorescence, as well as GFP expression driven by the Oct4 promoter (Fig. 4D). Interestingly, alkaline phosphatase was expressed in a high proportion of M-phase extract-treated cells, including those that did not progress further to form colonies (Fig. 4C). The presence in several independent experiments of clones that expressed OCT4, alkaline phosphatase, or both suggests that M-phase egg extracts favor the development of a heterogeneous cell population with different levels of reprogramming. This notion is in agreement with the heterogeneity observed during the production of iPSC cells, and it is likely to be the result of a stochastic process (23). These results indicate that M-phase extract alone can change the cell-cycle properties and can induce a partial reprogramming of MEFs.

Seven days after treatment with M-phase egg extracts, the expression of the pluripotency markers Oct4, Nanog, and Rex1 was confirmed by quantitative reverse transcription polymerase chain reaction (RT PCR) (Fig. 4E) in whole unslected cell populations, because we often detected pluripotency markers before colony formation. Primers used for quantitative PCR analyses were specific for mouse transcripts, and they could not amplify RNA from M-phase Xenopus extracts, confirming the induction of expression of the endogenous mouse genes. In addition, zinc finger protein multitype 2 (Zfpm2), a transcription factor expressed in...
MEFs but not in ES cells (16), was down-regulated after exposure to M-phase egg extracts (Fig. 4E).

Our data suggest that M-phase egg extracts alone are sufficient to reprogram MEFs partly. None of these effects was observed when using interphase Xenopus egg extracts.

**Treatment with M-phase Xenopus Egg Extracts Induces Mitotic Features and Modifies the Global Epigenetic Signature.** The observations that only M-phase egg extracts had a reprogramming effect on permeabilized MEFs as well as on NT efficiency indicate that the mitotic stage of the donor extract is crucial. Therefore, we investigated whether M-phase egg extracts could induce mitotic markers in the reprogrammed nuclei. Exposure to M-phase egg extracts drove G1 MEF nuclei into a mitotic-like stage, accompanied by modification of the chromatin structure (Fig. 5A), followed by the formation of condensed chromatin fibers (Fig. 5B). Phosphorylation of histone H3 on Ser-10 and dissociation of the nuclear envelope component Lamin B1 (24), a factor involved in the nuclear structure (Fig. 5 C and D), both distinctive features of entry in mitotic phase, were observed.

Exposure to M-phase egg extracts also erases heterochromatin foci visualized by DAPI staining together with the loss of heterochromatin protein 1 (HP1) (Fig. 5A–D). We further investigated whether the global epigenetic signature of MEF nuclei was modified. Histones of the donor nuclei have been shown to be deacetylated during NT (25). Western blot analysis showed that incubation of MEF nuclei with M-phase Xenopus egg extracts reduced the level of acetylation of H3 (particularly H3K9) and of H4 at Lys-8 (Fig. 5E).

Histone hypomethylation correlates with the epigenetic plasticity of somatic mammalian cells (26). A short incubation of MEF nuclei with M-phase Xenopus egg extracts globally reduced the level of H3K9me2-me3, H4K20me3, and H3K4me2-me3 (Fig. 5E). Conversely, the level of H3K27me3 did not change, suggesting that this mark is more stable. The global demethylation at H3K9 might contribute to the improvement of NT efficiency, because maintenance of H3K9 trimethylation has been associated with developmental failure during NT (27). Incubation with M-phase Xenopus egg extracts also induced a reduction of the global level of the histone variant H3.3, which recently has been implicated in cell identity memory during reprogramming by NT (28) (Fig. 5E). Therefore, M-phase Xenopus egg extracts broadly modify the epigenetic signature of mammalian somatic nuclei by resetting several, but not all, epigenetic marks.

Finally, we analyzed the DNA methylation profile, another key marker of cell memory. Bisulfite sequencing showed that incubation in M-phase Xenopus egg extracts did not modify the DNA methylation status of the pluripotency genes Oct4 and Nanog (Fig. S2).

In summary, Xenopus M-phase extracts drive MEF nuclei into a mitotic state and also remodel their chromatin structure. These
ES cell-derived embryoid bodies (EBES), M-iPS, and M-iPS was performed by quantitative RT-PCR amplification of OCT4, Nanog, and Klf4 and up-regulation of the differentiation markers Sox1, Sox7, Sox17, and Brachyury (Brach) upon EB differentiation. The analysis was performed by quantitative RT-PCR amplification of RNA from ES cells, ES cell-derived embryoid bodies (EB9-L), M-iPS, and M-iPS-derived embryoid bodies (EBM-iPS) and normalized to the mean expression of GAPDH.

Fig. 3. Developmental potential of M-phase iPS cells. (A) Differentiation of embryoid bodies (EB) was induced by retinoic acid as described in Materials and Methods. EB formation was accompanied by loss of GFP expression. (Left) Early differentiating EB still expressing GFP. (Center and Right) EB bodies. (Scale bars: 200 μm.) (B) Down-regulation of the pluripotency markers Oct4, Nanog, and Klf4 and up-regulation of the differentiation markers Sox1, Sox7, Sox17, and Brachyury (Brach) upon EB differentiation. The analysis was performed by quantitative RT-PCR amplification of RNA from ES cells, ES cell-derived embryoid bodies (EB9-L), M-iPS, and M-iPS-derived embryoid bodies (EBM-iPS) and normalized to the mean expression of Actin, hypoxanthine phosphoribosyltransferase 1 (HPRT), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Histograms represent the ratio between the corresponding embryoid bodies and pluripotent cells (ES cells (blue bars) or M-iPS cells (red bars)). Error bars represent SEM (n = 3). (C) Chimeric mice produced using M-iPS cells. Two different M-iPS clones produced viable chimeras after injection into CD1 blastocysts. (D) The black color of the F1 pups (from the B6xJF1 genotype) demonstrates germline transmission.

Discussion

Reprogramming of Mouse Embryonic Fibroblasts by Xenopus Egg Extracts. The experiments described here show that a short incubation of mammalian somatic nuclei or cells with M-phase Xenopus egg extracts improves the efficiency of both NT and iPS cell production. This result suggests the existence of common barriers limiting the efficiency of reprogramming by NT and iPS cells that M-phase Xenopus egg extracts might help removing. The results presented here also emphasize that combining different strategies can improve the reprogramming of mammalian somatic cell nuclei.

Reversibly permeabilized MEFs incubated in M-phase Xenopus egg extracts acquire several features of pluripotent cells, such as induction of cell proliferation, formation of colonies, and expression of ES cell markers, including the expression of OCT4. This reprogramming activity is not stable; colonies stop growing after a couple of rapid cell cycles. However, this partial reprogramming activity is enough to increase by 45-fold the production of fully reprogrammed iPS cells by viral transduction of OKSM. This synergic effect probably is underestimated, because the proportion of efficiently reprogrammed MEFs does not exceed 30% in our hands. The resulting M-iPS clones efficiently produce chimeras.

Fig. 4. Reprogramming of permeabilized MEFs induced by M-phase Xenopus egg extracts. (A) Proliferation rate of M-phase extract-treated MEFs (blue circles) compared with mock-treated MEFs (black squares) at different days (D) after exposure. Error bars represent SEM (n = 4). (B–F) Morphology of colonies formed following treatment of MEFs with M-phase Xenopus egg extracts (phase contrast). Panel E shows boxed area in panel F at higher magnification. (Scale bars: 200 μm.) (C) Induction of alkaline phosphatase activity in MEFs after exposure to M-phase Xenopus egg extracts (M phase). (Scale bars: 200 μm.) (D) (Left) Induction of OCT4 colonies following exposure to M-phase egg extracts of wild-type MEFs (immunofluorescence analysis). (Scale bars: 100 μm.) (Right) Induction of GFP expression in OCT4-GFP MEFs after incubation with M-phase extracts. (Scale bars: 50 μm.) (E) Induction of the expression of pluripotency markers (Oct4, Nanog, and Rex1) and down-regulation of Zfpm2 (a differentiation marker) in MEFs after incubation with M-phase Xenopus egg extracts. Quantitative RT-PCR was performed using M-phase extract- and mock-treated MEFs. Error bars indicate SEM (n = 3).
M phase) or nonincubation (Mock). Samples were analyzed by Western blotting using the corresponding antibodies, and DNA was stained with DAPI. (Scale bars: 10 μm.) (C) Phosphorylation of histone H3 at Ser-10 (phospho H3) (Left) and loss of HP1α bound to chromatin (Right) after exposure of MEF nuclei to M-phase Xenopus egg extracts or nonexposure (Mock). MEF nuclei were fixed and incubated with the corresponding antibodies, and DNA was stained with DAPI. (Scale bars: 10 μm.) (D) Analysis of the expression of chromatin-bound phosphorylated histone H3 at Ser-10 (phospho H3), Lamin B1, and HP1α in MEF nuclei after incubation with M-phase egg extracts (M phase) or with buffer (mock). Chromatin was collected by centrifugation after treatment as described in Materials and Methods. Samples were analyzed by Western blotting using the corresponding antibodies. Histone H3 was probed as loading control. (E) Analysis of histone modifications in MEF nuclei after incubation in M-phase extracts (M phase) or nonincubation (mock). Samples were analyzed by Western blotting using the corresponding antibodies. Histone H3 was probed as loading control.

and colonize the germline. This synergic effect suggests that Xenopus egg extracts can modify the chromatin organization of somatic mammalian cells and facilitate reprogramming by NT or OKSM expression.

Importance of Exposure to Mitotic/Meiotic Conditions for Reconditioning Differentiated Nuclei. Our experiments show that the mitotic state of the Xenopus egg extracts is crucial. Xenopus interphase egg extracts neither induced reprogramming in permeabilized MEFs nor improved NT efficiency. These results indicate that efficient reprogramming requires both an early embryonic pluripotent context and transition through mitosis. M-phase Xenopus egg extracts induced a global mitotic signature in G1 MEF nuclei, as revealed by the phosphorylation of histone H3 and remodeling of the nuclear structure. Transition through mitosis also seems important in NT experiments performed in the mouse, where zygotes temporarily arrested in mitosis support nuclear reprogramming more efficiently than interphase zygotes (29).

Incubation of somatic nuclei in mitotic egg extracts could help resynchronize the cell cycle of donor nuclei to make them compatible with an early-development context. MEF nuclei, like Xenopus somatic cell nuclei, are not competent to replicate their genome in interphase Xenopus egg extracts. The requirement of a mitotic reprogramming phase may explain why, in NT experiments, nuclei from half-cleaved embryos develop much better than nuclei from normal blastulae (30). Indeed, such nuclei were derived from embryos that failed to divide during the first cleavage, implying that they should have gone through a mitotic stage before entering S phase. In mouse, the best developmental rates are observed when activation occurs 1–3 h after nuclei transfer (31). Our observations provide an explanation for these data by showing that mitotic, but not interphase, Xenopus egg extracts can reprogram differentiated cells.

M-Phase Xenopus Egg Extracts Remodel the Global Organization of Somatic Mammalian Genomes. Conditioning nuclei in a mitotic embryonic context may facilitate reprogramming of gene expression. During mitosis, most preexisting transcription and replication factors are erased from chromatin (32). For instance, TATA-box binding protein, the main component of the transcription machinery, and transcription factor IIB are removed from the chromatin of somatic cell nuclei incubated in egg extracts, together with the disappearance of the nucleoli (33). M-phase Xenopus egg extracts induce a global mitotic signature in G1 MEF nuclei, as revealed by the loss of HP1, phosphorylation of histone H3, and remodeling of the nuclear structure. Interestingly, marks associated with transcriptional repression (H3K9me2, H3K9me3, H4K20me3) and with active chromatin (acetyl H4K8, acetyl H3K9, H3K4me3, H3K4me2) are reduced in chromatin of MEF nuclei incubated with M-phase extracts. This event is reminiscent of the atypical bivalent epigenetic signature of ES cells (34) and could promote reprogramming by resetting the memory of the somatic nuclei. Histone demethylation also is achieved by the M-phase Xenopus egg extracts.

Our results show that preincubation with M-phase Xenopus egg extracts can recapitulate reprogramming events occurring during NT. They explain the global epigenetic modifications that have been described during reprogramming of mammalian somatic nuclei injected in nonactivated, metaphase II mammalian oocytes (25). Xenopus egg extracts could provide a powerful tool to study molecular events occurring during NT because they provide all the genetic and epigenetic factors involved in mitosis as well as in pluripotency, as opposed to reprogramming through ectopic expression of a few genes. These results also emphasize the evolutionary conservation of reprogramming circuits.

Materials and Methods

Cells and Media. MEFs were derived from wild-type mouse embryos (embryonic day 13.5) or from C57BL/6/J-F1 embryos hemizygous for the OCT4-GFP transgenic allele (35). ES cells (CGR8) and M-iPS cells were grown in complete Glasgow minimum essential medium (Invitrogen) supplemented with 1,000 U/mL Leukemia inhibitory factor (ES-GRO).

Fig. 6. Preincubation with M-phase Xenopus egg extracts accelerates the rate of DNA replication of MEF nuclei in interphase egg extracts. (A) DNA replication of permeabilized MEF nuclei (blue line) and Xenopus sperm nuclei (spz) (black line) in interphase Xenopus egg extracts. The percentage of DNA replication is relative to the total DNA input in the reaction (Materials and Methods). (B) Preincubation of permeabilized MEF nuclei in M-phase egg extracts enables them to replicate DNA as efficiently as sperm nuclei in interphasic egg extracts.
Xenopus Egg Extracts. Xenopus egg extracts were prepared and used as described in refs. 11 and 36 and the protocol available at www.igh.cnrs.fr/equipimchial. MEF nuclei were prepared and incubated in the extracts as described in SI Materials and Methods.

Streptolysin-O Permeabilization and M-Phase Extract Treatment. MEFs were permeabilized with streptolysin-O (SLO) as described by Taranger et al. (37) and detailed in SI Materials and Methods. Permeabilized cells were incubated in M-phase Xenopus egg extracts or buffer for 40 min, washed twice in Hank’s Balanced Salt Solution (HBSS), and reseeded on gelatin in complete ES cell medium supplemented with 2 mM CaCl$_2$ for 2 h and then cultured in complete ES cell medium.

M-Phase Extract-Treated iP5 Cell Production. Constructs in pMXs retroviral vectors encoding Oct4, Sox2, Klf4, and c-Myc (obtained from Addgene) were transfected in Platinum human embryonic kidney (HEK). Supernatants were collected 48 h after transfection and were supplemented with 12 μg/ml polybrene. OCT4-GFP MEFs were infected with the four virus-containing supernatants, pooled in equal amounts. Supernatants were removed 18 h later, and cells were cultured in complete ES cell medium. Five to six hours later, cells were trypsinized, permeabilized with SLO as described above, and incubated in mock buffer (HBSS) or in Xenopus M-phase egg extracts for 40 min. Medium was removed 2 h after resealing and was replaced by complete ES cell medium.

Nuclear Transfer. Nuclear transfer experiments were performed mainly as described in Zhou et al. (38), and in SI Materials and Methods.

Differentiation of ES Cells or M-Phase Extract-Treated iP5 Cells. ES cells or M-phase extract-treated iP5 cells were dissociated and plated at low density in nonadherent bacterial Petri dishes with standard ES cell culture medium (without LIF). After 2 d, medium was replaced with ES cell culture medium supplemented with 0.5 μM retinoic acid to induce differentiation of embryoid bodies.

Reprogramming Efficiency. Reprogramming efficiency after M-phase extracts treatment was analyzed 7 d after infection. Alkaline phosphatase staining was performed using the Alkaline Phosphatase Detection Kit (Sigma). Immunofluorescence and transcriptional analyses were performed as in SI Materials and Methods.

DNA Microarray Analysis. Total double-strand cDNAs from ES cells, MEFs, and M-iPS cells was hybridized on Nimblegen mouse expression arrays, and results were analyzed with the free trial ArrayStar software. Normalization was done with the RMA algorithm (39) implemented in Bioconductor. The experiments were performed in triplicate; statistical analyses are described in SI Materials and Methods.

Bisulfit Sequencing. DNA extraction and bisulfite sequencing of mock-treated and M-phase extract-treated MEF nuclei, M-iPS cells, and CGR8 ES cells were performed as described previously (40).

Generation of Chimeras. Chimeras were produced by injecting B6-JF M-iPS cells into CD1 blastocysts that subsequently were implanted into pseudopregnant CD1 females. M-phase extract-treated IPS clones were sexed by karyotyping.

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Supporting Information

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SI Materials and Methods

Cells and Media. Mouse embryonic fibroblasts (MEFs) were derived from embryonic day 13.5 (13.5E) wild-type mouse embryos or from C57BL/6-JF1 embryos hemizygous for the OCT4-GFP transgenic allele. Gonads, internal organs, and heads were removed before MEF isolation. MEFs then were expanded in high-glucose Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% embryonic stem (ES) cell-tested fetal bovine serum (FBS) (catalog no. S1810; Biowest), 2 mM L-glutamine (Invitrogen), and 1 mM sodium pyruvate (Sigma). MEFs were used up to passage 5. OCT4-GFP mice were created initially by Hans R. Schöler (Max-Planck-Institute for Molecular Biomedicine, Münster, Germany) (1) and obtained from M. A. Surani (Wellcome Trust/Cancer Research UK, Cambridge, United Kingdom). The ES cell line CGR8 was donated by C. Crozet (Institut de Génétique Humaine, Montpellier, France). ES cells were grown on 0.1% gelatin without feeders. They were cultured at 37 °C in 5% CO2 in ES cell medium: Glasgow minimum essential medium (Invitrogen) supplemented with 10% FBS, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% nonessential amino acids (Gibco), 2 mM L-glutamine, in the presence of 1,000 U/mL Leukemia inhibitory factor (LIF) (ES-GRO).

Xenopus Egg Extract Preparation and Replication Reactions. MEF nuclei were prepared from confluent MEFs at early passages (up to passage 5) following the procedure described for Xenopus erythrocyte nuclei (2). Briefly, MEFs were trypsinized and washed twice with PBS. MEFs were incubated in hypotonic buffer [10 mM K Hepes (pH7.5); 2 mM KCl; 1 mM DTT; 2 mM MgCl2; 1 mM PMSF; and protease inhibitors] for 1 h. Swollen cells then were homogenized with 20–30 strokes and incubated in hypotonic buffer containing 0.2% Triton X-100 on ice for 3 min. Nuclei were washed twice in isotonic buffer (10 mM K Hepes, 25 mM KCl, 2 mM MgCl2, 75 mM sucrose, and protease inhibitors) for 1 h. Swollen nuclei then were centrifuged through a 0.7-M sucrose cushion and resuspended in isotonic buffer supplemented with 20% sucrose. Sperm nuclei and MEF nuclei (1,000 nuclei/μL and 500 nuclei/μL, respectively) were incubated in S-phase or M-phase extracts. DNA synthesis was measured by [3H]deoxyctydine triphosphate incorporation after incubation of sperm nuclei and MEF nuclei (1,000 nuclei/μL and 500 nuclei/μL, respectively) in Xenopus egg extracts (3). Transfer of nuclei from M-phase extracts to interphasic extracts and chromatin purification and analysis were performed as described previously (2).

Streptolysin-O Permeabilization and M-Phase Extract Treatment. MEFs were permeabilized with streptolysin-O (SLO) mainly as described by Taranter et al. (4). Briefly, MEFs were trypsinized, washed twice in PBS, and then resuspended in cold Ca2+ and Mg2+-free Hank’s Balanced Salt Solution (HBSS) at 1,000 cells/μL with 250 ng/mL SLO (SO149; Sigma). Cells were incubated at 37 °C with gentle agitation for 50 min and then washed twice with ice-cold HBSS. Permeabilized cells were incubated in M-phase Xenopus egg extract or buffer for 40 min, washed twice in HBSS, and resealed on gelatin in complete ES cell medium supplemented with 2 mM CaCl2 for 2 h and then cultured in complete ES cell medium.

M-Phase Extract-Treated Induced Pluripotent Stem (M-IPS) Cell Production. Constructs in pMXs retroviral vectors encoding octamer-binding transcription factor 4 (Oct4), sex determining region Y-box 2 (Sox2), Kruempel-like factor 4 (Klf4), and myelocytomatosis onco- gene (c-Myc) (obtained from Addgene) were transfected in Platinum HIEK cells using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s recommendations. Thirty microliters of Lipofectamine 2000 were added to 750 μL Opti-MEM (Invitrogen) and mixed with 12 μg DNA that had been diluted into 750 μL Opti-MEM and incubated for 5 min. After 20 min incubation at 20 °C, the DNA/Lipofectamine 2000 mixture was added drop by drop to Platinum HEK cells. Supernatants were collected 48 h after transfection, filtered through 0.45-μm Millex-HV (Millipore) filters, and supplemented with 12 μg/mL polybrene. OCT4-GFP MEFs were seeded on 0.1% gelatin at a density of 8 × 105 cells in 56-cm2 Petri dishes, and the four virus-containing supernatants were pooled in equal amounts and added to the MEFs. Supernatants were removed 18 h later, and cells were cultured in complete ES cell medium. Five to six hours later, cells were trypsinized and permeabilized with SLO as described above and then were incubated either in mock buffer (HBSS) or in Xenopus M-phase egg extracts for 40 min. After treatment, cells were washed twice and plated (8 × 103 cells per 56-cm2 dish) in gelatin-covered dishes with ES cell medium supplemented with 2 mM CaCl2. After 2 h, medium was removed and replaced by complete ES cell medium until the appearance of OCT4-GFP colonies. M-phase extract-treated OCT4-GFP colonies were isolated mechanically, and individual cells were dissociated and plated onto feeders for analysis that was performed after at least 15 passages on feeders.

Nuclear Transfer. Nuclear transfer experiments were performed mainly as described in Zhou et al. (5). Briefly, permeabilized MEF nuclei from confluent (B6 × 129) MEFs were prepared as described above and either were injected directly into enucleated, metaphase II mouse oocytes or were preincubated in Xenopus egg extracts for 40 min. Before injection, the efficiency of treatment and chromatin integrity were assessed by visually inspecting the nuclei with a phase-contrast microscope. Metaphase (B6 × 129) ES cells were isolated as described in Zhou et al. (5).

Viral Integration. All the cell populations (not-infected MEFs, infected MEFs, and MEFs that had been infected, permeabilized, and incubated with M-phase Xenopus egg extracts or buffer) were harvested 21 d after infection, and total DNA was extracted with the DNEasy kit (Qiagen). Quantitative PCR then was performed as described above. Quantification data were normalized to the average of two genomic regions and relative to the DNA of not-infected MEFs.

Immunofluorescence Analyses. For immunofluorescence, cells in culture were washed once in PBS and then fixed in 3% paraformaldehyde at room temperature for 15 min, washed with PBS, and permeabilized with PBS/0.2% Triton X-100 for 5 min. Cells then were washed three times in PBS with 2% BSA for 10 min, incubated with anti–OCT-3/4 (C-10) (sc-5279; Santa-Cruz), anti-NANOG (ab21603; Abcam) or anti-SSEA1 (clone 16MC480) (ab16285; Abcam) antibodies for 1 h and then with the secondary antibody for 1 h after three washes in PBS. DNA was stained with DAPI. Immunofluorescence analysis of M-phase extract–treated MEF nuclei was performed by staining the treated nuclei onto coverslips via centrifugation at 100 × g after having been diluted 10-fold in XB buffer ([100 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 10 mM KOH-Hepes (pH 7.7), 50 mM sucrose supplemented with protease inhibitors]) as described previously (3).

Quantitative RT-PCR Analysis. For transcriptional analysis, total RNA was isolated from whole-cell populations using the RNeasy
Mini Kit (Qiagen), and reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed on a Lightcycler 480 apparatus using the Lightcycler 480 SYBR Green I Master Kit from Roche. Quantification data were normalized to the average expression of the endogenous Hprt1/Gapdh and β-Actin genes within the log-linear phase of the amplification curve obtained for each primer set using the ΔΔCt method. All samples were prepared in two or three biological repeats.

Primers for quantitative RT-PCR:

Oct4  
Forward: ttcggtgcccggcttagaacaatc  
Reverse: gaggaagcgacaaatagagacccctga

Rex1  
Forward: cagctctgtcgcagaaaga  
Reverse: actatgcgcaaaaacctg

Nanog  
Forward: ttcggtgcccggcttagaacaatc  
Reverse: gaggaagcgacaaatagagacccctga

Zfpm2  
Forward: gcgaagacctgattcttt  
Reverse: gcctgggccatgacacat

β-Actin  
Forward: gcggctgtccatggtctt  
Reverse: ttcggtgcccggcttagaacaatc

Gapdh  
Forward: ttcggccatgtcttgcc  
Reverse: ttctggtcatacttgcc

Hprt1  
Forward: tctctctacagcagctaagct  
Reverse: gcatcaacagcctaaacctc

Sox1  
Forward: gtaacagctgcccct  
Reverse: gagccgacgttacctcag

Sox7  
Forward: gcggagtcgcaagagt  
Reverse: ggctcttctggaagct

Brachury  
Forward: cagccacactactgctca  
Reverse: gagctcggtggtatgta

Klf4  
Forward: gattcttcagcggccaq  
Reverse: cggaagaaggaagaacagt

Statistical Analysis. Gene-by-gene tests for differential expression between paired cell types were performed using a moderated t-statistic (6). P values were adjusted using the procedure of Benjamini and Hochberg for controlling the false-discovery rate (7). Differentially expressed genes between the paired cell types were identified using adjusted P values below 1%. Bisulfite Sequencing. Before DNA extraction, GFP+ M-iPS cells were sorted with a FACSArria cytometer (BD Biosystems) to avoid contamination by feeder cells. DNA polymorphisms between the C57BL/6J and JF1 backgrounds were used for allele discrimination in MEF and M-iPS cells.

Primers. Bis-Oct (these primers amplify only the endogenous copy of Oct-4)  
Forward: TTAGGGGATGTTGAGGTTGTTGTAAGAGT  
Reverse: CCAATCCCCACCTCTAACCCTAACCTCTA

Bis-Nanog  
Forward: TAAAATGGGATATGTTGAGTATACAATTGGG  
Reverse: TAAAAAACATCCTTCAATCTCAAAAAACAAT

Bis-Snrpn  
Forward: atgggtgagtaatgatatga  
Reverse: CAACTACTCCTACCTA

Purification and Analysis of Chromatin Fractions. Permeabilized MEF nuclei were incubated in M-phase Xenopus egg extracts for 40 min, diluted in five volumes of XB buffer, and pelleted by centrifugation at 500 × g for a 0.7-M sucrose cushion for 10 min. Nuclear pellets were resuspended in XB with 0.2% Triton X-100 and incubated on ice for 5 min. Chromatin pellets were recovered by centrifugation at 5,000 × g for 5 min, adjusted in Laemmli buffer, and analyzed by SDS/PAGE. Western blot analysis was performed using the following antibodies: anti-sr10 phosphorylated histone H3 (9701S; Ozyme), anti-histone H3 (ab1791; Abcam), anti-HPIα (MAB3584 or 2616; Millipore) anti-histone variant H3.3 (ab62642; Abcam), anti-Lamin B1 (ab16048; Abcam) anti-H3K4me2 (Ab7766; Abcam), anti-H3K4me3 (ab1012;Abcam), anti-H3K9me2 (ab7766; Abcam), anti-H3K9me3 (ab1012;Abcam), anti-H3K9me3 (Upstate), anti-H4K20me3 (ab9053; Abcam), anti-H4K8acetyl (ab1760; Abcam) and anti-acetyl H3 (ab4441; Abcam) and anti-acetyl H3 (06–599; Millipore).

Viral Integration. The following primers were used for viral integration:

DNA Oct4  
Forward: aagtggtgctggaactttg  
Reverse: tctggtgtctgtccactc

DNA Klf4  
Forward: gcggctgtccatggtctt  
Reverse: atcgccgactgagtagag

DNA Sox2  
Forward: tcaagggcccatgaag  
Reverse: tctggtgtctgtccactc

DNA cMye  
Forward: gcggctgtccatggtctt  
Reverse: atcgccgactgagtagag

DNA genomic1  
Forward: gcggctgtccatggtctt  
Reverse: atcgccgactgagtagag

DNA genomic2  
Forward: gcggctgtccatggtctt  
Reverse: atcgccgactgagtagag


Fig. S1. Incubation with M-phase *Xenopus* egg extracts does not affect the viral integration of the OKSM transgenes. Viral integration of each transgene in the different cell populations was assessed by quantitative PCR amplification. The different cell populations were harvested 21 d after infection, and their DNA was extracted. Blue bars represent infected, nonpermeabilized cells (OKSM); red bars represent infected, SLO-permeabilized, and mock-treated cells (OKSM+SLO+buffer); green bars represent SLO-permeabilized and M-phase extract-treated cells (OKSM+SLO+M-phase extract). Four independent experiments are shown. Error bars represent SEM ($n = 4$).

Fig. S2. Incubation with M-phase *Xenopus* egg extracts does not demethylate DNA of MEF nuclei. Bisulfite sequencing was performed in mock-treated and M-phase extract-treated MEF nuclei and ES cells. The promoter regions of (A) Oct4 and (B) Nanog were analyzed. Analysis of the promoter/imprinting control region of the imprinted *Snrpn* gene was used as a control (C). Amplified regions are indicated by a solid blue bar. Each horizontal row of circles represents the CpG dinucleotides of an individual molecule. Solid circles depict methylated CpGs; open circles indicate unmethylated CpGs. The parental allele origin (M: maternal; P: paternal) was determined in MEFs and iPS cells by using DNA polymorphisms between C57BL/6J and JF1 backgrounds. Blue triangles show individual CpGs that are absent because of polymorphisms.
Table S1. In vitro development of embryos obtained using MEF nuclei exposed to M-phase Xenopus egg extracts and injected into enucleated mouse oocytes

<table>
<thead>
<tr>
<th></th>
<th>Reconstructed</th>
<th>Activated</th>
<th>Two-cell</th>
<th>Four- to eight-cell*</th>
<th>Morula*</th>
<th>Blastocyst*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic ES cells</td>
<td>39</td>
<td>36</td>
<td>28</td>
<td>ND</td>
<td>28% (8)</td>
<td>11% (3)</td>
</tr>
<tr>
<td>G1 ES cells ** (1)</td>
<td>263</td>
<td>195</td>
<td>140</td>
<td>56% (79)</td>
<td>27% (38)</td>
<td>11% (16)</td>
</tr>
<tr>
<td>Mitotic MEFs † (2)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6%</td>
</tr>
<tr>
<td>G1 MEFs + interphasic extracts</td>
<td>178</td>
<td>84</td>
<td>30</td>
<td>27% (8)</td>
<td>7% (2)</td>
<td>3% (1)</td>
</tr>
<tr>
<td>G1 MEFs + M-phase extracts</td>
<td>148</td>
<td>87</td>
<td>49</td>
<td>78% (38)</td>
<td>67% (33)</td>
<td>45% (22)</td>
</tr>
</tbody>
</table>

Percentage of embryos relative to two-cell embryos obtained after nuclear transfer of ES cell nuclei and MEF nuclei that had been preincubated in mock buffer, interphase, or M-phase Xenopus egg extracts. Mitotic and G1 ES cell nuclei were isolated and injected as previously described (1) and mitotic MEF nuclei as in Li et al. (2). ND, not determined.

*Percent of two-cell embryos.
**Sv129/Sv cell line.
†129/SvPas cell line.


Table S2. Developmental potential of iPS cells derived from MEFs exposed to M-phase Xenopus egg extracts (M-phase iPS cells)

<table>
<thead>
<tr>
<th>Injected blastocysts</th>
<th>Born embryos</th>
<th>Number of chimeras</th>
<th>% Chimeras</th>
<th>Female chimeras</th>
<th>Male chimeras</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1 (male)</td>
<td>233</td>
<td>171</td>
<td>31</td>
<td>46</td>
<td>25</td>
</tr>
<tr>
<td>Clone 3 (female)</td>
<td>85</td>
<td>10</td>
<td>7</td>
<td>70</td>
<td>3</td>
</tr>
</tbody>
</table>

Percentage of chimeras obtained after injection of two different clones of M-phase iPS cells (one male and one female) into CD1 blastocysts and analysis of their ability to colonize the germline.