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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.

histones | induced pluripotent stem cells | chromatin remodeling

Nuclear transfer (NT) experiments in frogs and then in mammalian eggs have demonstrated that somatic cells can be reprogrammed to pluripotency (1–4). More recently, pluripotency was induced in somatic cells by ectopic expression of the four transcription factors octamer-binding transcription factor 4 (Oct4), Krueppel-like factor 4 (Klf4), sex-determining region Y-box 2 (Sox2), and myelocytomatosis oncogene (c-Myc) (OKSM). Murine induced pluripotent stem (iPS) cells have a complete developmental potential as demonstrated by their capacity to form teratomas, generate chimeras, and contribute to the germline. However, the efficiency of both iPS cell production and NT remains low, and most of the reprogrammed cells appear to be reprogrammed only partially. The epigenetic memory of the cell is one key barrier that must be overcome to reprogram differentiated cells (5), and additional factors may be needed to improve reprogramming efficiency (6, 7). Several attempts have been made to reprogram somatic cells by using cellular extracts, but they failed to reproduce the range of effects obtained by NT.

In NT experiments, reprogramming is induced by exposure of transplanted nuclei to the cytoplasm of the receiving oocyte. However, NT reprogramming appears hard to study *in vitro* because of the difficulty of obtaining large quantities of mammalian oocytes. *Xenopus* eggs, which can be obtained in large amounts, can remodel the nuclear lamina of mammalian cells (8), and *Xenopus* egg extracts can up-regulate Oct4 expression in cells that

already express Oct4 (9), similar to what is observed when adult mouse nuclei are injected in *Xenopus* oocytes (10). The replication origin pattern and chromosome organization of *Xenopus* erythrocyte nuclei also could be remodeled by metaphase-arrested extracts (M-phase extracts) from *Xenopus* eggs (11). We show here that such extracts increase the efficiency of NT and iPS cell production from mouse embryonic fibroblasts (MEFs). They also engage MEFs into a stem cell program and induce a global change of MEF chromatin structure and replication properties. In particular, M-phase extracts reset the level of several epigenetic marks in MEF nuclei, independently of their role in chromatin activation. Moreover, M-phase extracts, but not interphase extracts, partially reprogrammed permeabilized MEFs to form colonies that expressed pluripotency markers. iPS cell induction by ectopic expression of OSKM was increased 45-fold when MEFs were incubated in M-phase *Xenopus* egg extracts. The resulting iPS cells were fully reprogrammed, as shown by their capacity to produce chimeras and colonize the germline.

Results

Pretreatment with M-Phase *Xenopus* Egg Extracts Improves Efficiency of both Nuclear Transfer and iPS Cell Production in Mammals. We first asked whether M-phase *Xenopus* egg extracts could improve the inefficient NT of MEFs (12). Permeabilized MEF nuclei in G1 phase were preincubated with M-phase (Fig. 1A) or interphasic *Xenopus* egg extracts or buffer, and their progression to blastocyst stage was compared. NT of G1 MEFs nuclei led to 11% blastocysts (Fig. 1B and Table S1), a value that was significantly lower than obtained after NT of metaphase embryonic stem (ES) cell nuclei (55%), which previously were described as the best donor nuclei (13). Conditioning MEF nuclei in M-phase egg extracts significantly increased the rate of blastocyst formation to a level comparable to that obtained with metaphase ES cell nuclei (45%) (Fig. 1B and Table S1). These data show that M-phase *Xenopus* egg extracts efficiently improve reprogramming of somatic cells by NT. Conversely, preincubation with interphasic egg extracts did not

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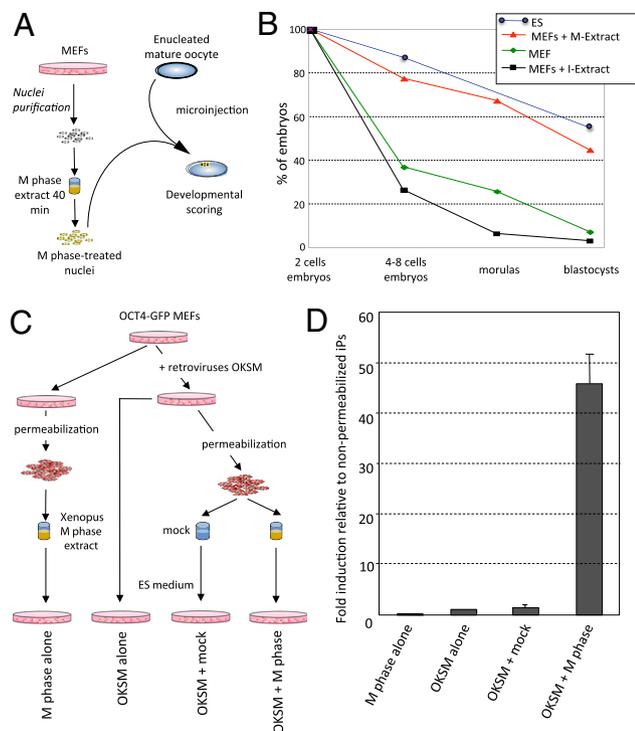


Fig. 1. M-phase *Xenopus* egg extracts improve the efficiency of nuclear transfer and iPSC production from mammalian fibroblasts. (A) Schematic representation of nuclear transfer experiments using MEFs exposed to M-phase *Xenopus* egg extracts. (B) Percentage of early embryos resulting from nuclear transfer of MEFs exposed to M-phase (M-Extract) or interphase (I-Extract) *Xenopus* egg extracts and normalized to the number of two-cell embryos. (C) Schematic representation of iPSC cell generation from OCT4-GFP⁺ MEFs by ectopic expression of OSKM followed or not followed (mock) by exposure to M-phase *Xenopus* egg extracts. (D) Number of OCT4-GFP⁺ colonies relative to nonpermeabilized cells. The effect of exposure to M-phase *Xenopus* egg extracts on the efficiency of iPSC cell 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. ($n = 3$).

improve but instead rather slightly decreased NT efficiency (3%), indicating the importance of the mitotic state of the reprogramming extract. Because both mitotic MEFs and G1 ES cell nuclei were relatively inefficient donors for NT in metaphase-blocked oocytes (summarized in Table S1), our results suggest that M-phase *Xenopus* extracts can remodel MEF nuclei toward both a mitotic and pluripotent state.

We then asked whether a similar treatment could improve the efficiency of iPSC cell production. The generation of iPSC cells by viral-mediated expression of the OSKM transcription factors, although with low efficiency, was a breakthrough in reprogramming (14–17). We therefore combined OSKM overexpression and incubation with M-phase *Xenopus* egg extracts (M-iPS cells), as described in Fig. 1C. Because OCT4 expression is a stringent reporter of reprogramming (2), OCT4-GFP MEFs were infected with retroviruses encoding the four transcription factors, permeabilized, incubated with M-phase extracts, and then resealed onto gelatin-coated plates in ES cell medium. The M-phase extract treatment did not influence the viral integration of the OSKM transgenes (Fig. S1). Fig. 1D shows that the number of GFP⁺ colonies was 45-fold higher in M-iPS cells than in cells that overexpressed only OSKM. Thus, a short incubation of mammalian somatic cells in M-phase *Xenopus* egg extracts greatly increases the yield of fully reprogrammed iPSC cells.

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. 2A–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. 2E), and scatter plots confirmed the similarity between M-iPS and ES cells ($R^2 = 0.9175$).

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. 3B) (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 B6xJF1 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 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 extracts 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 transcriptase polymerase chain reaction (RT-PCR) (Fig. 4E) in whole unselected 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 (*Zfp2*), a transcription factor expressed in

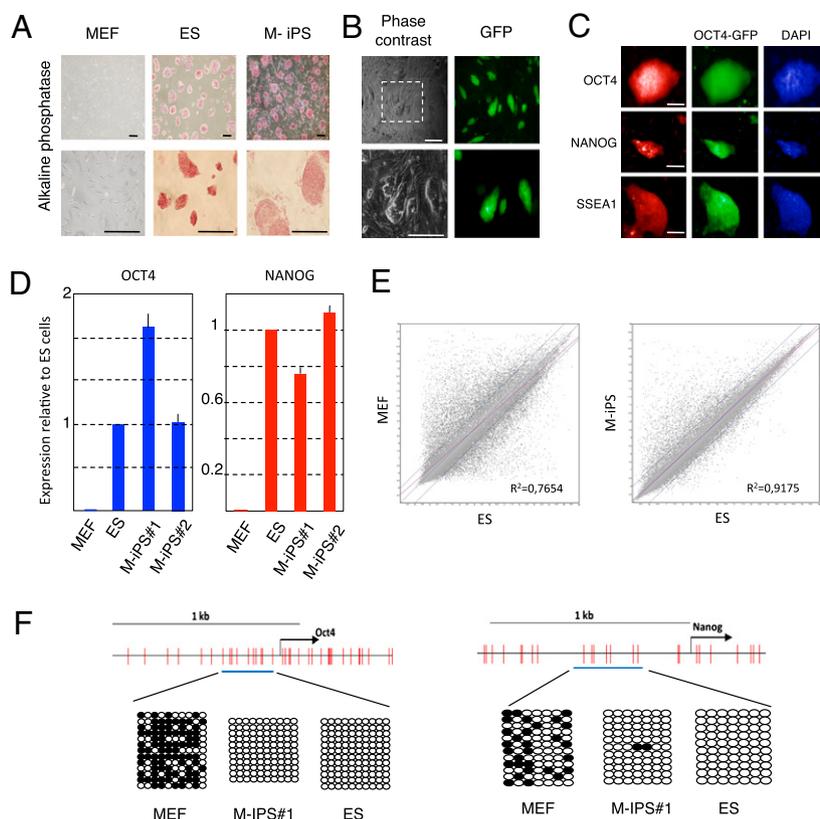


Fig. 2. Characterization of the pluripotency of iPS cells obtained by OKSM overexpression combined with exposure to M-phase *Xenopus* egg extracts. (A) Alkaline phosphatase expression in mock-treated MEFs, ES cells, and iPS cells induced by OKSM overexpression and exposure to M-phase *Xenopus* egg extracts (M-iPS). Lower panels show higher magnification of upper panels. (Scale bars: 200 μ m.) (B) Morphology and GFP expression in M-iPS cells generated from OCT4-GFP MEFs. Lower panels show higher magnification of upper panels. (Scale bars: 100 μ m.) (C) Expression of pluripotency markers assessed by immunofluorescence in M-iPS cells. OCT4, NANOG, and SSEA1 (Left) colocalized with GFP, the expression of which was driven by the *Oct4* promoter (Center). (Scale bars: 50 μ m.) (D) Expression of *Oct4* and *Nanog* measured by quantitative RT-PCR in MEFs, ES cells, and two M-iPS clones. Error bars represent SEM. ($n = 3$). (E) Scatter plots with computation of the Pearson's correlation coefficient (R^2) showing the comparisons of global gene expression between ES cells and MEFs (Left) and between ES and M-iPS cells (Right). Blue lines indicate genes that are differentially expressed in the paired cell types. (F) Bisulfite sequencing of DNA from MEFs, ES cells, and M-iPS cells. The promoter regions of *Oct4* and *Nanog* were analyzed. The 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 represent unmethylated CpGs.

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. 5C 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

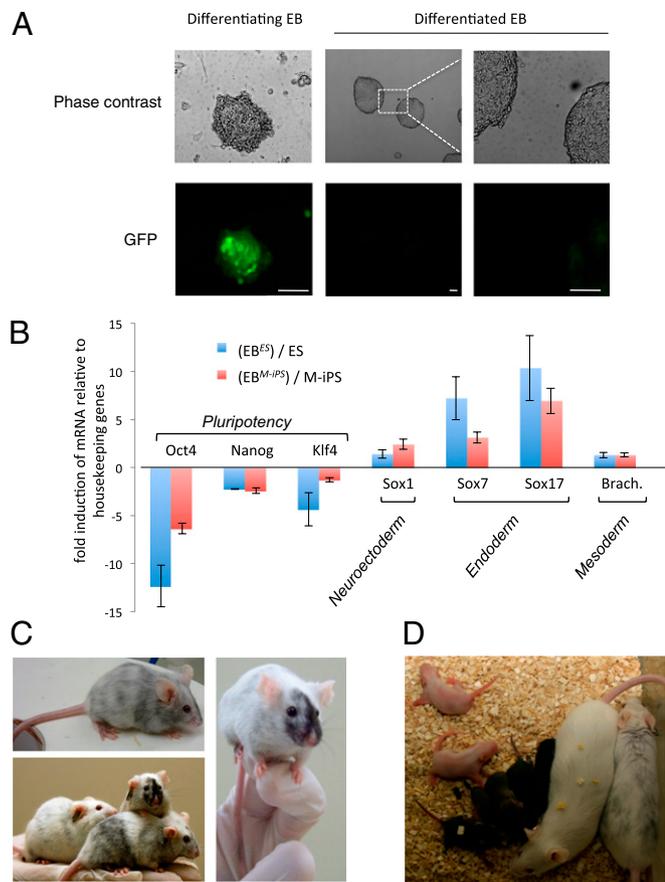


Fig. 3. Developmental potential of M-phase iPS cells. (A) Differentiation of embryoid bodies (EB) was induced by retinoic acid as described in *Materials and Methods*. EB formation was accompanied by loss of GFP expression. (Left) Early differentiating EB still expressing GFP. (Center and Right) EB bodies. (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 (EB^{ES}), M-iPS, and M-iPS-derived embryoid bodies (EB^{M-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 B6JF1) genotype) demonstrates germline transmission.

results could explain the strong synergistic effect of the treatment with M-phase *Xenopus* extract on NT and iPS cells production.

MEF Nuclei Are Adapted to an Embryonic Replication Program When Preincubated in M-Phase *Xenopus* Egg Extracts. We previously showed that M-phase egg extracts could reset the replication program of nuclei from differentiated *Xenopus* cells to an embryonic profile of DNA replication (11). We asked whether MEF nuclei could be reprogrammed similarly (schematized in Fig. S3). G1 MEF nuclei exposed only to interphase egg extracts did not replicate DNA or replicated DNA very poorly (Fig. 6A). Conversely, preincubation of MEF nuclei in M-phase egg extracts induced DNA replication with kinetics nearly similar to that of *Xenopus* sperm nuclei (Fig. 6B). We conclude that mouse somatic nuclei passing through mitosis in *Xenopus* egg extracts are reprogrammed partially and acquire the accelerated rate of DNA replication characteristic of *Xenopus* early embryos.

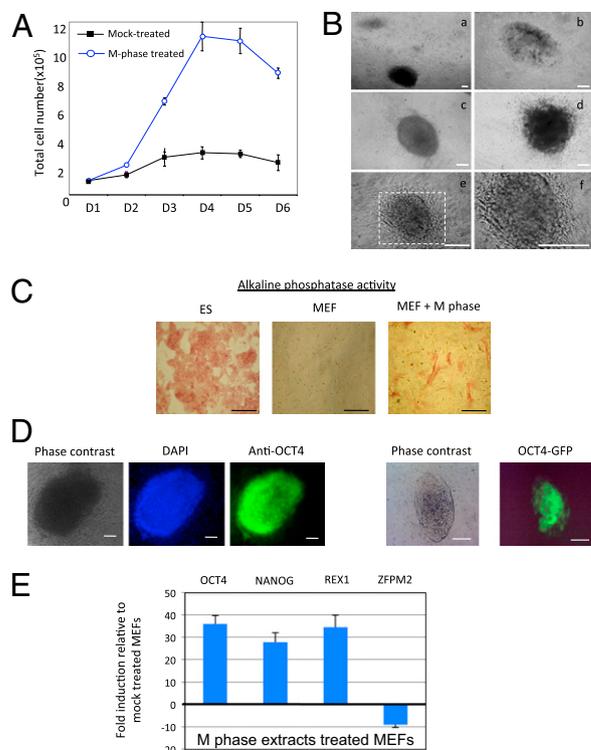


Fig. 4. Reprogramming of permeabilized MEFs induced by M-phase *Xenopus* egg extracts. (A) Proliferation rate of M-phase extract-treated MEFs (blue circles) compared with mock-treated MEFs (black squares) at different days (D) after exposure. Error bars represent SEM ($n = 4$). (B–f) Morphology of colonies formed following treatment of MEFs with M-phase *Xenopus* egg extracts (phase contrast). Panel f shows boxed area in e 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 *Zfp2* (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 ($n = 3$).

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 permeabilized MEFs does not exceed 30% in our hands. The resulting M-iPS clones efficiently produce chimeras

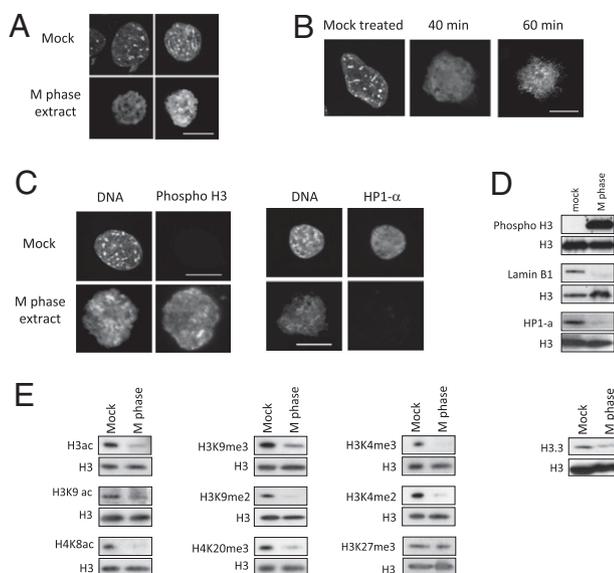


Fig. 5. Remodeling of the chromatin structure of MEF nuclei incubated with M-phase *Xenopus* egg extracts. (A) Morphology of MEF nuclei incubated with buffer alone (Mock) or with M-phase *Xenopus* egg extracts for 40 min. Nuclei were stained with DAPI. (Scale bar: 10 μ m.) (B) Morphology of MEF nuclei treated with buffer alone (Mock treated) or M-phase egg extracts for 40 or 60 min. Nuclei (stained with DAPI) show different degrees of chromatin compaction. (Scale bar: 10 μ m.) (C) Phosphorylation of histone H3 at Ser-10 (phospho H3) (Left) and loss of HP1- α 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 μ m.) (D) Analysis of the expression of chromatin-bound phosphorylated histone H3 at Ser-10 (phospho H3), Lamin B1, and HP1- α 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* interphasic 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 temporally 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 interphasic *Xenopus* egg extracts. The requirement of a mitotic reprogramming phase may explain why, in NT experi-

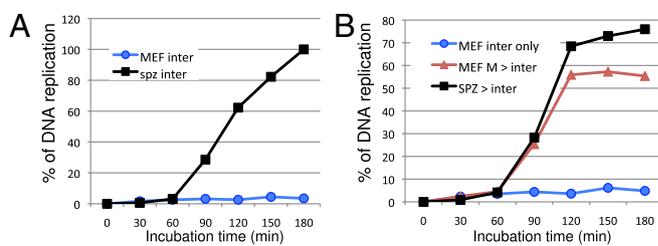


Fig. 6. Preincubation with M-phase *Xenopus* egg extracts accelerates the rate of DNA replication of MEF nuclei in interphasic egg extracts. (A) DNA replication of permeabilized MEF nuclei (blue line) and *Xenopus* sperm nuclei (spz) (black line) in interphasic *Xenopus* egg extracts. The percentage 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.

ments, 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 nuclear transfer (31). Our observations provide an explanation for these data by showing that mitotic, but not interphasic, *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/6J-JF1 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).

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/equip/mechali/. 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 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 iPS 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 iPS Cells. ES cells or M-phase extract-treated iPS 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 calculated with the RMA algorithm (39) implemented in Bioconductor. The experiments were performed in triplicate; statistical analyses are described in *SI Materials and Methods*.

Bisulfite 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-JF1 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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1. Yamanaka S, Blau HM (2010) Nuclear reprogramming to a pluripotent state by three approaches. *Nature* 465:704–712.
2. Jaenisch R, Young R (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* 132:567–582.
3. Hochedlinger K, Jaenisch R (2006) Nuclear reprogramming and pluripotency. *Nature* 441:1061–1067.
4. Gurdon JB, Melton DA (2008) Nuclear reprogramming in cells. *Science* 322:1811–1815.
5. Maherali N, et al. (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* 1:55–70.
6. Feng B, Ng JH, Heng JC, Ng HH (2009) Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. *Cell Stem Cell* 4:301–312.
7. Huangfu D, et al. (2008) Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol* 26:795–797.
8. Alberio R, Johnson AD, Stick R, Campbell KH (2005) Differential nuclear remodeling of mammalian somatic cells by *Xenopus laevis* oocyte and egg cytoplasm. *Exp Cell Res* 307:131–141.
9. Hansas C, Barreto G, Malry N, Niehrs C (2004) Nuclear reprogramming of human somatic cells by *Xenopus* egg extract requires BRG1. *Curr Biol* 14:1475–1480.
10. Byrne JA, Simonsson S, Western PS, Gurdon JB (2003) Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes. *Curr Biol* 13:1206–1213.
11. Lemaître JM, Danis E, Pasero P, Vassetzky Y, Mechali M (2005) Mitotic remodeling of the replicon and chromosome structure. *Cell* 123:1–15.
12. Yang X, et al. (2007) Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat Genet* 39:295–302.
13. Li X, Li Z, Jouneau A, Zhou Q, Renard JP (2003) Nuclear transfer: Progress and quandaries. *Reprod Biol Endocrinol* 1:84.
14. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
15. Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. *Development* 136:509–523.
16. Wernig M, et al. (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448:318–324.
17. Park IH, et al. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141–146.
18. Kanai-Azuma M, et al. (2002) Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* 129:2367–2379.
19. Beddington RS, Rashbass P, Wilson V (1992) Brachyury—a gene affecting mouse gastrulation and early organogenesis. *Dev Suppl* 157–165.
20. Niimi T, Hayashi Y, Futaki S, Sekiguchi K (2004) SOX7 and SOX17 regulate the parietal endoderm-specific enhancer activity of mouse laminin alpha1 gene. *J Biol Chem* 279:38055–38061.
21. Pevny LH, Sockanathan S, Placzek M, Lovell-Badge R (1998) A role for SOX1 in neural determination. *Development* 125:1967–1978.
22. Todaro GJ, Green H (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol* 17:299–313.
23. Hanna J, et al. (2009) Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature* 462:595–601.
24. Moir RD, Yoon M, Khuon S, Goldman RD (2000) Nuclear lamins A and B1: Different pathways of assembly during nuclear envelope formation in living cells. *J Cell Biol* 151:1155–1168.
25. Wang F, Kou Z, Zhang Y, Gao S (2007) Dynamic reprogramming of histone acetylation and methylation in the first cell cycle of cloned mouse embryos. *Biol Reprod* 77:1007–1016.
26. Baxter J, et al. (2004) Histone hypomethylation is an indicator of epigenetic plasticity in quiescent lymphocytes. *EMBO J* 23:4462–4472.
27. Santos F, et al. (2003) Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr Biol* 13:1116–1121.
28. Ng RK, Gurdon JB (2008) Epigenetic memory of an active gene state depends on histone H3.3 incorporation into chromatin in the absence of transcription. *Nat Cell Biol* 10:102–109.
29. Egli D, Rosains J, Birkhoff G, Eggan K (2007) Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. *Nature* 447:679–685.
30. Gurdon JB, Laskey RA (1970) The transplantation of nuclei from single cultured cells into enucleated frogs' eggs. *J Embryol Exp Morphol* 24:227–248.
31. Wakayama T, Tateno H, Mombaerts P, Yanagimachi R (2000) Nuclear transfer into mouse zygotes. *Nat Genet* 24:108–109.
32. Egli D, Birkhoff G, Eggan K (2008) Mediators of reprogramming: Transcription factors and transitions through mitosis. *Nat Rev Mol Cell Biol* 9:505–516.
33. Kikyo N, Wade PA, Guschin D, Ge H, Wolffe AP (2000) Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. *Science* 289:2360–2362.
34. Bernstein BE, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125:315–326.
35. Yeom YI, et al. (1996) Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* 122:881–894.
36. Menu S, Lemaître JM, Hair A, Méchali M (1999) *DNA Replication and Chromatin assembly Using Xenopus Egg Extracts. Advances in Molecular Biology: A comparative Methods Approach to the Study of Oocytes and Embryos*, ed Richter JD (Oxford Univ Press, Oxford, UK), pp 196–226.
37. Taranger CK, et al. (2005) Induction of dedifferentiation, genomewide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. *Mol Biol Cell* 16:5719–5735.
38. Zhou Q, Jouneau A, Brochard V, Adenot P, Renard JP (2001) Developmental potential of mouse embryos reconstructed from metaphase embryonic stem cell nuclei. *Biol Reprod* 65:412–419.
39. Irizarry RA, et al. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4:249–264.
40. Arnaud P, et al. (2006) Stochastic imprinting in the progeny of Dnmt3L^{-/-} females. *Hum Mol Genet* 15:589–598.