THE ANATOMICAL RECORD 292:1229–1234 (2009)

ES Cell Extract-Induced Expression of Pluripotent Factors in Somatic Cells

YAN-NING XU, NA GUAN, ZHEN-DONG WANG, ZHI-YAN SHAN, JING-LING SHEN, QING-HUA ZHANG, LIAN-HONG JIN, AND LEI LEI*
Department of Histology and Embryology, Harbin Medical University, Nangang District, Harbin 150081, Heilongjiang Province, China

ABSTRACT

Reprogramming of somatic cells was induced by ES cell-free extract. The system relied on the transient uptake of regulatory components from a nuclear and cytoplasmic extract derived from ES cells by the nucleus of a reversibly permeabilized NIH3T3 cell. NIH3T3 cells were permeabilized by streptolysin O (SLO). Reprogramming cell-free extracts were prepared by repeatedly freezing and thawing ES cells in liquid nitrogen. After incubation in the extract for 1 hr, permeabilized NIH3T3 cells were resealed by $CaCl_2$ and continually cultured for weeks to assess expression of ES cell specific markers. As we observed using FACS and fluorescence microscope, the optimal SLO concentration for permeabilizing NIH3T3 cells was 25 U. After 2 weeks of culture, the treated NIH3T3 cells began to express *Nanog, c-Myc, Klf4*, and 6 weeks later *Oct4* was detectable. However, *Sox2* was detected only after 8 weeks of culture. Differentiated somatic cells could be reprogrammed in ES extract *in vitro*, which provides a new approach to decreasing differentiation levels in somatic cells without disturbing the DNA sequences. Anat Rec, 292:1229–1234, 2009. © 2009 Wiley-Liss, Inc.

Key words: ES cell; cell-free extract; somatic cell; reprogramming; pluripotent genes

INTRODUCTION

Recently, it was reported that overexpression of defined transcription factors can induce fibroblasts to become pluripotent stem cells (named induced pluripotent stem (iPS) cells. Four factors, *Oct4, Sox2, Klf4*, and *c-Myc,* were able to induce pluripotency in mouse embryonic and adult fibroblast cells (Takahashi K, 2006). To induce pluripotency, combinations of defined transcription factors expressed in ES cells were cotransduced in the target fibroblasts using retroviral vectors that each carried one transgene. This procedure was used in human fibroblast cells to obtain human iPS cells (Yu, 2007; Takahashi K, 2007). As reactivation of the *c-Myc* retrovirus increases tumorigenicity in chimeras and progeny mice, a modified protocol for the generation of iPS cells that does not require the *c-Myc* retrovirus has been reported (Nakagawa, et al. 2008). Recently, a micromolecular substance, BIX-01294, was used as a substitute for *Sox2* and *c-Myc* (Shi, et al., 2008). The current protocols for generating iPS cells seem simpler for clinical application, but the necessary use of lentivirus involves random genomic integration and risk of insertional mutagenesis.

The epigenetic state of a somatic nucleus can be heritably reprogrammed to become a pluripotential stem cell using hybridization of the two cell types. Following fusion with an undifferentiated ES cell, a differentiated

Grant sponsor: Innovative Fund of Harbin Medical University Graduate Student; Grant number: HCXS2008002; Grant sponsor: National Natural Science Foundation of China; Grant number: 30671025; Grant sponsor: Project of Abroad Researcher Foundation of Heilongjiang Province; Grant number: LC07C17.

*Correspondence to: Lei Lei, Department of Histology and Embryology, Harbin Medical University, China. Fax: +86 0451 86674518. E-mail: leil086@yahoo.com.cn

Yang-Ning Xu and Na Guan contributed equally to this work.

Received 20 December 2008; Accepted 22 March 2009

DOI 10.1002/ar.20919

Published online in Wiley InterScience (www.interscience.wiley.com).

somatic cell nucleus can be epigenetically reprogrammed (Tada et al., 2001; Cowan et al., 2005). As shown in this system, the hybrids display morphology, growth rate, and surface antigen expression patterns characteristic of ES cells. Whole-genome gene expression changes, allele specific gene expression, and DNA demethylation in the *Oct4* locus provides evidence for near-complete reprogramming of the fibroblast genome. The occurrence of epigenetic changes in somatic cell-ES cell hybrids indicates that fusion was likely to provide a valuable system for investigating the mechanisms underlying nuclear reprogramming. However, a major hurdle remains. Although the somatic genome is reprogrammed, the tetraploid state of the reprogrammed cell makes epigenetic analyses of the reprogrammed somatic genome challenging.

In recent years, nuclear and cytoplasmic extracts from several cell types have been shown to elicit changes in cell fate. The procedure involves reversible permeabilization of a somatic cell, exposure of the permeabilized cells to the reprogramming extract, and resealing of the cells. Using this approach, extracts of regenerated newt limbs have been shown to induce some dedifferentiation of cultured C2C12-derived mouse myotubes into myoblasts capable of replicating DNA (McGann et al., 2001). Extracts of Xenopus eggs can also induce the expression of pluripotency gene *Oct4* and *Sox2* in mammalian somatic cells (Miyamoto et al., 2007). Transient exposure of reversibly permeabilized fibroblasts to an extract of undifferentiated mouse ES cells results in *Oct4* biphasic activation, with the first transient rise of *Oct4* upregulation being necessary for the second longterm activation of *Oct4* (Taranger et al., 2005). However, expression and precise timing of other genes involved in cell-free extract induced reprogramming have not been addressed.

Here, transient exposure of reversibly permeabilized NIH3T3 cells to extracts of mouse ES cells induced partial reprogramming of NIH3T3 cell nucleus. We found that pluripotential genes were expressed in a time order. When compared with cell fusion or iPS, our method does not introduce ES cell chromosomes or virus into somatic cells. Therefore, reprogrammed somatic cells avoided chromosome aploid or mutagenesis. The method provides a new approach to studying mechanisms of reprogramming and may be useful for therapeutic purposes.

MATERIALS AND METHODS

Cell Lines and Reagents

All reagents were purchased from Sigma Chemical Company unless stated otherwise. Mouse ES cell line, GD3, and mouse fibroblast cell line, NIH3T3, purchased from ATCC Company.

Seeding Cells

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn bovine serum (NBS). Cells were seeded to reach a confluence of 70% on the day of reprogramming. GD3 cells were cultured on mitomycin-treated mouse embryonic fibroblast feeder layers in ES cell medium (DMEM, 15% fetal bovine serum FBS, 1% nonessential amino acids, 0.1% β-mercaptoethanol, 100 U/L penicillin, and 100 U/L streptomycin) supplemented with 1,000 U/mL leukemia inhibitory factor (LIF). Before harvesting, GD3 cells were passaged and cultured on gelatin-coated plates in order to eliminate feeders. After treatment with ES cell extract, NIH3T3 cells were cultured in ES cell medium without feeder layers.

Cell Extract Preparation

To prepare GD3 and NIH3T3 extracts, cells were washed in DPBS and centrifuged at 1000 rpm for 5 min at 4°C. Cell pellet volume was estimated, and cells were resuspended into the same volume of ice-cold DPBS containing 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM protease inhibitor cocktail. Sedimented cells were resuspended in cold DPBS and incubated for 3 h on ice. Cells were repeatedly frozen and thawed in liquid nitrogen. The lysate was sedimented at 25,000 rpm for 15 min at 4°C, and the supernatant was collected in a new tube and frozen in liquid nitrogen.

Cell Permeabilization

NIH3T3 cells were washed in cold Ca^{2+}, Mg^{2+}-free phosphate buffered saline (DPBS) three times. Approximately 500,000 cells were resuspended in 500 μL DPBS, and centrifuged at 1,000 rpm for 5 min at 4°C in a swing-out rotor. Sedimented cells were suspended in cold DPBS, tubes were warmed for 2 min at 37°C, and SLO was added to a final concentration of 10 U, 25 U, and 50 U. Cells were incubated in a water bath for 50 min at 37°C with occasional agitation. 500 μL cold DPBS was added to dilute the samples, and cells were sedimented at 1,000 rpm for 5 min at 4°C. Permeabilization was assessed by fluorescent dye uptake under fluorescence microscope or by FACS. Uptake of 10,000-*M*r dextran Oregon green 488 (50 μg/mL; Invitrogen) and PI were monitored 2 hr after cell membrane resealing in 2 mM $CaCl_2$.

In Vitro Reprogramming of NIH3T3 Cells

After permeabilization, cells were suspended at 1,000 cells/μL in 100 μL NIH3T3 (control) or ES extract which contained an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, 25 μg/mL creatine kinase, 100 μM GTP), and 1 mM of each nucleotide triphosphate (NTP; Roche Diagnostics, Mannheim, Germany). The tube containing cells was incubated for 1 hr at 37°C with occasional agitation. To reseal plasma membranes, cells were cultured with medium containing 2 mM $CaCl_2$ for 24 hr, and cells were seeded at 100,000 cells per well on a 48-well plate. Reprogrammed NIH3T3 cells were continually cultured in ES medium for further study.

RT-PCR

Total RNA was extracted from cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. 1 μg RNA was loaded to reverse transcribe cDNA and amplified by the RT-PCR system (Promega). The primer sequences and amplified fragment lengths are listed in Table 1.

TABLE 1. Primer sequence and annealing temperature

Gene	Primer sequence	Amplified fragment length/bp	Annealing temperature/°C
Oct4	sense5′-CTGTAGGGAGGGCTTCGGGCACTT-3′ anti5′-CTGAGGGCCAGGCAGGAGCACGAG-3′	216	58
Nanog	Sense5′-ATGAGTGTGGGTCTTCCTGGTCC-3′ anti5′-GACTCCACCAGGTGAAATATGA-3′	918	62
Sox2	sense5′-GGCAGCTACAGCATGATGCAGGAGC-3′ anti5′-CTGGTCATGGAGTTGTACTGCAGG-3′	125	55
KIf-4	sense 5′-TGCCAGACCAGATGCAGTCAC-3′ anti5′-GTAGTGCCTGGTCAGTTCATC-3′	233	55
c-Myc	sense 5′-CCCAGCGAGGACATCTGGAAGAA-3′ anti5′-GAGAAGCCGCTCCACATGCAGTC-3′	213	55
LaminA	sense5′-CTCCTCCCACTCATCCCA-3′ Anti5′-CCTCGTCGTCATCCTCATT-3′	469	55
GAPDH	sense5′-TGAAGGTCGGTGAACGGAT-3′ anti5′-CAGGGGGGCTAAGCAGTTGGT-3′	980	65

Immunofluorescence

Cells were seeded onto cover slips, fixed with 4% paraformaldehyde for 20 min at 4°C, washed with PBS containing 0.1% Triton X-100 for 45 min at room temperature, and blocked in PBS supplemented with 10% goat serum for 30 min. After washing with PBS, the cells were incubated at room temperature for 1 hr with the anti-*Oct4* monoclonal antibody (1:200, Santa Cruz Biotechnology) or anti-LaminA monoclonal antibody (1:100, Santa Cruz Biotechnology). The cells were incubated with anti-mouse IgG secondary antibody (1:200, Santa Cruz Biotechnology) for 1 hr. Nuclei of the cells were stained with Hoechst 33342 (1:20, Santa Cruz Biotechnology).

Data Analysis

Two-sample *t*-tests were used to compare the protein level of Oct4 and LaminA and analysis of variance (ANOVA) and *q*-test were used to make multiple comparison.

RESULTS

Cell-Free Extract

We tried three different methods to prepare the cell extracts. Cells were disrupted by a sonicator, sonicator with lysis buffer, or liquid nitrogen. Protein concentration and osmotic pressure of the extracts were measured. We found that the optimal method was repeated freezing and thawing of the cells in liquid nitrogen. Using this method, we obtained extracts with a high protein concentration (~46.1 mg/mL) and comparable osmotic pressure (~279 mmol/kg) (Table 2).

TABLE 2. Comparison of different methods for ES cell extract preparation

Method	Protein concentration (mg/mL)	Osmotic pressure (mmol/kg)
Sonicator	2.9	49.5
Sonicator with cell lysis buffer	3.2	55
Freezing and thawing in LN2	46.1	279

Note: Data are mean values from seven independent experiments.

Permeabilization of Somatic Cells

Here, 10 mM DTT was determined as the most suitable reagent to activate SLO. The result as determined by fluorescence microscope and FACS was that the higher the concentration of SLO the more dead cells were observed. Immediately after permeabilization, fluorescence microscope observation showed that 25 U SLO could successfully permeabilize the NIH3T3 cells (44.58%) (Fig. 1A–D). However, FACS results indicated that 10 U SLO had a better effect on permeabilization (38.32%) than that of other concentrations (Fig. 1E–I). Considering both fluorescent microscope and FACS results, we selected 25 U SLO to permeabilize NIH3T3 cells.

ES Cell Extract Partly Reprogrammed NIH3T3 Cells and Induced Expression of Pluripotent Factors

Some transcription factors, such as *Oct4*, *Sox2,* and *Nanog* are expressed in early stage embryos and ES cells to maintain pluripotency. Some tumor correlated genes, for instance, *stat3, E-ras, c-Myc,* and *Klf4*, could maintain the phenotype and increment characteristics of ES cells longterm. Two independent experiments revealed that 2 weeks after treatment with ES cell extract, NIH3T3 cells (named N-ES cells) started to express *Nanog, c-Myc,* and *Klf4* genes. *Oct4* was detectable after 6 weeks of culture. Moreover, after 8 weeks of culture, there was no significant difference in the expression of *c-Myc* and *Klf4* between N-ES cells and ES cells. The expression of *Sox2, Oct4,* and *Nanog* were exceptions, and were less expressed in N-ES cells at 8 weeks of culture than that in ES cells. *LaminA*, a marker for differentiated cells in mammals, showed no visible differences in expression in NIH3T3 cells, NIH3T3 cells treated with extracts of NIH3T3 cells (N-N cells) and N-ES cells 2–8 weeks after extract treatment.

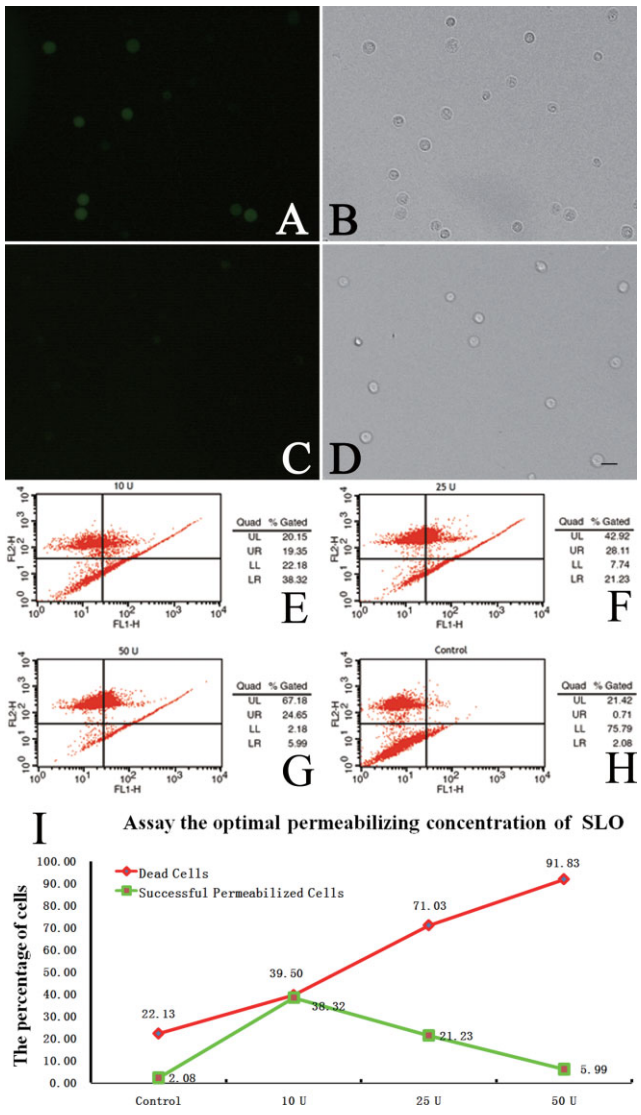

Fig. 1. Optimal permeabilization concentration of SLO. (**A–D**) The result was observed by fluorescence microscope: A partly NIH3T3 cells were successfully permeabilized by SLO with a concentration of 25U, judging by the dextran Oregon green 488, 10,000 MW uptake. (C) The control of A. (B, D) Light microscope photographs of A and C, respectively. (**E–I**) The result was detected by FACS: UL the cells died a natural death, not by SLO. UR the cells died because of SLO. LR the cells were successfully permeabilized by SLO. (E) NIH3T3 cells were permeabilized by SLO with a concentration of 10U. (F) SLO with a concentration of 25U. (G) SLO with a concentration of 50U. (H) SLO with a concentration of 0U as a control. (I) More cells were dead when treated with a higher concentration of SLO. (Bar = 50 μm).

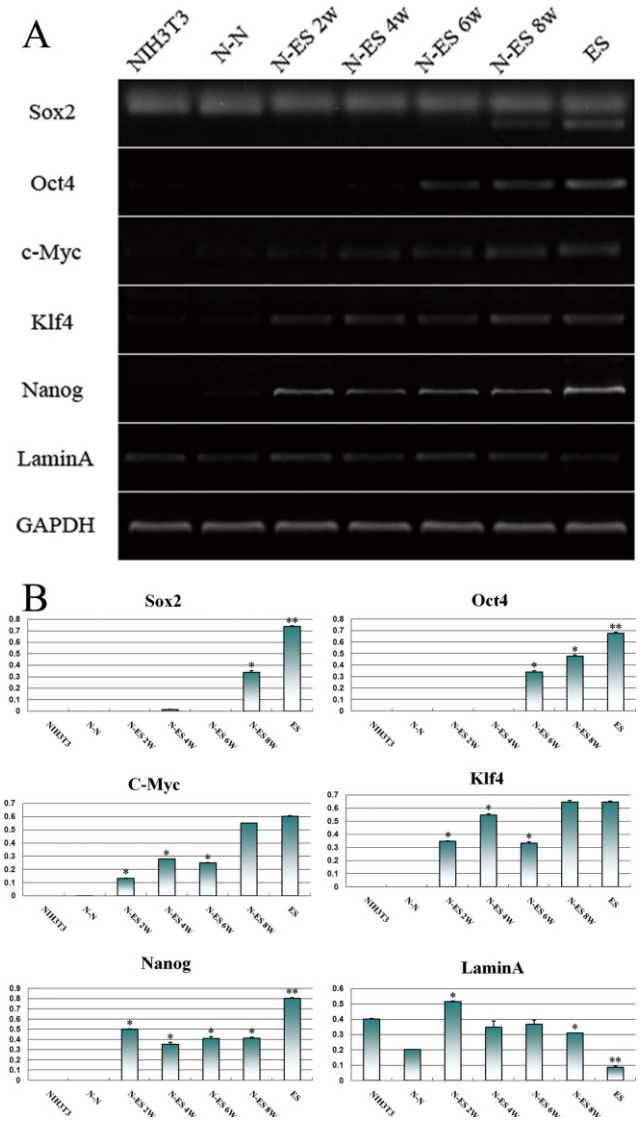

Fig. 2. RT-PCR analysis of pluripotent gene expression in N-ES cells. (**A**) After treatment with ES cell extracts, Nanog, c-Myc, and Klf4 were detected after 2 weeks of culture, and Oct4 was detected after 6–8 weeks of culture in N-ES cells. After 8 weeks, there was no significant difference in the expression of c-Myc and Klf4 between N-ES cells and ES cells. The expression of Sox2 in N-ES cells was observed after 8 weeks of culture and Sox2, Oct4, Nanog were less expressed in N-ES cells at 8 weeks of culture than that in ES cells. There were no visible differences in the expression of LaminA in NIH3T3 cells, N-N cells, N-ES cells between 2–8 weeks after culture, but in ES it was lower than the others. (**B**) Gray scale value (mean ± SD) of Nanog, c-Myc, Klf4, Sox2, Oct4, Nanog, and LaminA in NIH3T3 cells, N-N cells, N-ES cells, ES cells. $^{*}P < 0.05$ when compared with NIH3T3 cells (ANOVA, *q* test); $^{**}P < 0.05$ when compared with N-ES 8W cells (ANOVA, *q* test).

However, in ES cells it was lower than in the others (Fig. 2).

At 8 weeks after treatment, pluripotent marker OCT4 and differentiated marker LAMINA were detected in N-ES cells by immunochemistry. The results revealed that some N-ES cells were OCT4 positive, meanwhile some cells still expressed LAMINA constantly (Fig. 3). These results were confirmed by RT-PCR.

DISCUSSION

Cell-Free Extract Preparation

The most optimal method for preparing the cell-free extracts was repeated freezing and thawing of cells in liquid nitrogen. Using this method, we obtained ES cell-free extract with a protein concentration of 46.1 mg/mL,

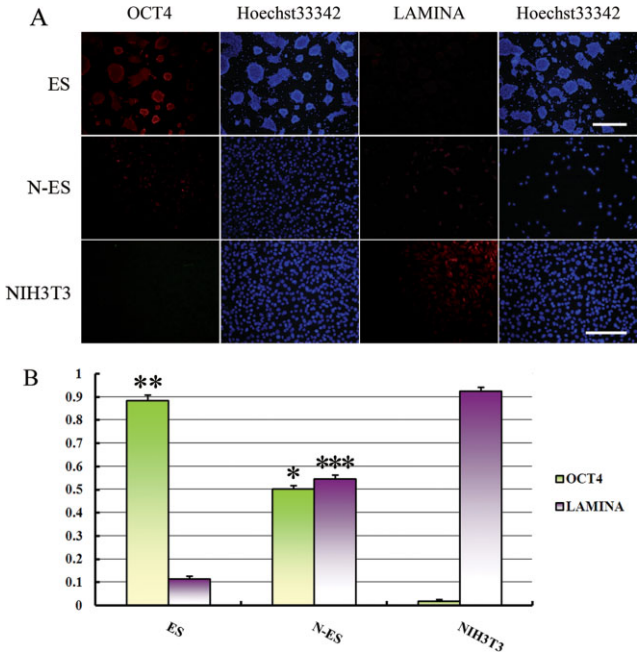

Fig. 3. Immunofluorescence analysis of pluripotent genes in N-ES cells. (**A**) 8 weeks after culture, some of the N-ES cells expressed OCT4, while some still expressed LAMINA. In N-ES cells, reprogramming of nuclear function had partly taken place in vitro. ES cells just expressed OCT4, and NIH3T3 cells just expressed LAMINA. (Bar in ES cell is 50 μm, and in NIH3T3 cells is 30 μm). (**B**) Proportions (mean ± SD) of NIH3T3 cells, N-ES cells, and ES cells expressing OCT4, LAMINA. Three sets of 200 cells were examined for each marker. $^{*}P < 0.05$ when compared with NIH3T3 cells (*t* test), $^{**}P < 0.05$ when compared with N-ES cells (*t* test), $^{***}P < 0.05$ when compared with NIH3T3 cells (*t* test).

and osmotic pressure close to physiological conditions. In other studies, high-speed centrifugation and sonication were used to prepare cell extracts from Xenopus eggs (Miyamoto et al., 2007), mitotic bovine kidney cells (Eddie et al., 2004), and cadiocytes (Gaustad et al., 2004). These methods obtained similar protein concentration and osmotic pressure in cell-free extracts. However, the freezing and thawing of cells in liquid nitrogen is much easier to perform and requires less equipment.

Permeabilization of Somatic Cells

Although some cell types, such as adipose tissue stem cells, readily endocytose (Gaustad et al., 2004), most cell types require efficient permeabilization for components from the extract to be taken up. Permeabilization should be reversible as cells are expected to be cultured after exposure to the extract. Permeabilization could be accomplished with SLO, a cholesterol-binding cytolysin, forming pores of about 30 nm in the surface membrane (Walev et al., 2002). This permeabilization could be reversed by subsequent incubation with fetal bovine serum or Ca^{2+}. Although the result obtained by fluorescence microscope and FACS was not concordant, we decided to use 25 U SLO to permeabilize NIH3T3 cells. The reason was that in FACS, we found some cells which were successfully permeabilized with 25 U SLO but not fully recovered after resealing for a short time. These cells were stained by PI and show up as dead cells but were able to survive after overnight culture. The sensitivity to SLO appears to vary in many species and cell types because of the different cholesterol content of the cell membrane (Bhakdi et al., 1996). It was recommended to test a range of SLO concentrations varying between 3.57–35.7 U/mL (100–1,000 ng/mL) (Collas, www.collaslab.com) on any given cell type. In our study, the SLO concentration used to permeabilize NIH3T3 cells was 25 U/mL, which was in the recommended range.

Pluripotent Factors Expressed in a Particular Order in NIH3T3 Cells After Reprogramming by ES Cell-Free Extract

RT-PCR results showed that *Nanog, c-Myc,* and *Klf4* expressed earlier than *Oct4* and *Sox2* in N-ES cells. The precise mechanism for this expression order needs further study. In the mammalian genome, there may be up to 25,000 *c-Myc* binding sites (Cawley et al., 2004), and C-MYC protein may induce global histone acetylation (Fernandez et al., 2003), thus allowing *Oct4* and *Sox2* to bind to their specific target loci. *Klf4* has been shown to repress *p53* directly (Rowland et al., 2005), and P53 protein has been explored as a suppressor of *Nanog* during ES cell differentiation (Lin et al., 2004). Therefore, *Klf4* might contribute to the activation of *Nanog* and other ES cell-specific genes through *p53* repression (Takahashi and Yamaka, 2006). This might partly explain why *Nanog, c-Myc,* and *Klf4* express earlier than *Oct4* and *Sox2* in N-ES cells.

Oct4 and *Sox2* both showed up late in N-ES cell development, but were not expressed simultaneously. *Oct4* expression started after 6 weeks of reprogramming while *Sox2* reactivated 2 weeks later. Both transcription factors have been shown to cooperate in the transcriptional activation of target genes in maintaining pluripotent state. However, *Oct4* is expressed in the inner cell mass (ICM), epiblast, and later in germ cells (Pesce and Scholer, 2000). It is essential for establishing and maintaining pluripotency of the ICM. *Sox2* also marks the pluripotent lineage of the early mouse embryo, but more specifically in the multipotential cells of the extraembryonic ectoderm (ExE). It is possible that *Sox2* and *Oct4* function together to maintain a pluripotent state throughout early development, but *Oct4* functions earlier than *Sox2* (Avilion et al., 2003). This finding is consistent with our results that *Oct4* is expressed earlier than *Sox2* in N-ES cells, and *Oct4* might be a prerequisite for *Sox2* expression.

Collas et al. reported that ES cell extract induced a biphasic wave of *Oct4* transcription and translation in fibroblast cells. In their study, *Oct4* was first detected as early as 1 hr after recovery from ES cell extract treatment, the level peaked at 24 hr, and then *Oct4* expression decreased and was barely detected at 48 hr. However, by 72 hr, a second wave of *Oct4*, similar or even higher than the first wave, showed up and lasted for 120 hr (Taranger, 2005). We did not check *Oct4* transcription soon after treatment, because we presumed the early *Oct4* expression in somatic cells might have derived from the ES extract. We started *Oct4* detection from the 2nd week, and found that *Oct4* was detectable 6 weeks after treatment. This late expression suggests

Oct4 reactivation in reprogrammed NIH3T3 cells. Whether *Oct4* expression persists at a stable level or varies at different time points needs further study.

There were no visible differences in the expression of *LaminA* in N-ES cells between 2–8 weeks after extract treatment, but *LaminA* was lower in ES cells. The reason might be that after treatment with ES cell extract, not all NIH3T3 cells were reprogrammed, and some non-reprogrammed cells remained, which contributed to *LaminA* expression after PCR amplification. In ES cells, there was still weak *LaminA* expression, which might have derived from a few feeder layer cells (MEF) mixing in ES cells.

Extracts of nondifferentiated cells contain regulatory factors that induce pluripotency of differentiated cells. *Sox2* was detected in permeabilized and nonpermeabilized pig fibroblasts after treatment with Xenopus egg extracts, but *Nanog* was not detected (Miyamoto et al., 2007). These results suggest that the nuclei of pig somatic cells could be partially reprogrammed by Xenopus egg extracts but not to a pluripotent state as ES cells. So, there are variations in the extent of transcriptional reprogramming which might reflect incomplete reprogramming or progressive deregulation of the new program. Functional reprogramming *in vivo* also remains to be demonstrated. Ways to enhance stability of extract-induced gene expression may include repeated extract exposure, destabilization of the epigenome with chromatin modifying agents, or induction of a dedifferentiation step before triggering a new target cell-specific program.

ACKNOWLEDGMENTS

The authors are grateful to Xi-Mei Zhang and Qiu-Ming Li at Harbin Medical University for their valuable discussions.

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