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Cell-Free Extracts from Mammalian Oocytes Partially Induce Nuclear Reprogramming in Somatic Cells[1]

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ABSTRACT

Nuclear transfer has been regarded as the only reliable tool for studying nuclear reprogramming of mammalian somatic cells by oocytes. However, nuclear transfer is not well suited for biochemical analyses of the molecular mechanisms of reprogramming. A cell-free system from oocytes is an attractive alternative way to mimic reprogramming in vitro, since a large number of cells can be treated and analyzed. Nevertheless, a cell-free system using oocytes has not been developed in mammals. Here, cell extracts from porcine oocytes were prepared and their ability to induce nuclear reprogramming was evaluated. Extracts from metaphase II (MII) oocytes erased the machinery for regulating gene expression in reversibly permeabilized somatic cells. For example, the extracts caused histone deacetylation and the disappearance of TATA box-binding protein from the nuclei. However, MII-extract-treated cells did not show any obvious changes after cell culture. In contrast, extracts from germinal vesicle (GV) oocytes activated pluripotent marker genes, especially *NANOG*, and induced partial dedifferentiation after cell culture. The activation of pluripotent marker genes by GV extracts was associated with histone acetylation that was induced during extract treatment. These results indicate that GV- and MII-oocyte extracts have different roles on nuclear reprogramming. Furthermore, both oocyte extracts induced site-specific demethylation in the upstream region of *NANOG*. These results indicate that cell-free extracts derived from GV- and MII-oocytes could be useful for studying the mechanisms involved in nuclear reprogramming.

cell-free extract, cell-free system, dedifferentiation, DNA methylation, extracts, gene regulation, histone acetylation, NANOG, *oocyte, pig, reprogramming*

INTRODUCTION

Oocytes are highly differentiated cells. Unlike other types of cells, oocytes have the ability to allow somatic cells to become totipotent after nuclear transfer [1]. This process is called nuclear reprogramming of differentiated cells, in which the original cell state is replaced with a totipotent state that allows the cell to develop to term [2]. However, the nuclear-transfer system has limitations for studying the molecular mechanisms of nuclear reprogramming. One limitation is that nuclear transfer requires complicated processes of embryonic manipulation so that only a small number of nuclear-transferred embryos can be produced. In addition, the experimental conditions needed for nuclear transfer are difficult to maintain in the steady state. These limitations make it difficult to analyze nuclear reprogramming using biochemical and molecular approaches.

Some biochemical and physiological events that occur in cells can be mimicked in extracts of eggs or oocytes in vitro. Such cell-free systems have been established in *Xenopus laevis* [3, 4], *Drosophila melanogaster* [5, 6], and other nonmammalian species [7, 8]. This system is superior for studying molecular mechanisms of early embryonic development [6], especially for samples whose quantities are insufficient for biochemical analysis, because a large number of cells can be treated simultaneously in the system. In the *Xenopus* cell-free system, sperm nuclei show morphological and biochemical changes that occur in fertilization, and the cell cycle of early embryonic development also proceeds [9]. This system has been used to elucidate the roles of MAP kinase [10] and linker histones [11] in early embryonic development. Recently, the *Xenopus* cell-free system has been used to induce the reprogramming of somatic cells [12–16]. Several factors that are involved in nuclear remodeling or dedifferentiation, such as ISWI [12], FRGY2a/b [13], BRG1 [14], and NPM [15] have been identified. Especially, BRG1 [14] and nucleoplasmin [15] in *Xenopus* extracts are also able to induce reprogramming events in mammalian cells, suggesting that some reprogramming processes are highly conserved among species. However, the reprogramming processes in the *Xenopus* egg cell-free system may not be comparable to those in mammals because successful reprogramming of somatic cells (resulting in development to term) has been limited to oocytes belonging to the same species. Taken together, these results suggest that a cell-free system derived from mammalian eggs or oocytes would be better for understanding the mechanisms of nuclear reprogramming in mammalian species.

A cell-free system derived from mammalian eggs or oocytes has not been established, mainly because mammalian oocytes (~100 μm) are much smaller than *Xenopus* oocytes (1.2 mm), which makes it difficult to prepare a sufficient volume of cell-free extracts. Therefore, we decided to prepare cell-free extracts from porcine oocytes, which can be easily collected in large numbers at a slaughterhouse and can be matured in vitro from the germinal vesicle stage (GV oocytes) to the metaphase II stage (MII oocytes) [17].

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Matured MII oocytes have been used as recipient cells for nuclear transfer of somatic cells in mammals, which suggests that they would be a reasonable source of extracts for inducing nuclear reprogramming. By contrast, GV oocytes are considered to be inadequate for use as recipients [18]. However, there is some evidence that components within germinal vesicles of *Xenopus* oocytes induce *Pou5f1* (also known as *Oct4*) expression in the nuclei of mouse somatic cells [19], suggesting that GV oocytes also have reprogramming potential. In this experiment, we prepared cell-free extracts from porcine GV oocytes and MII oocytes (GV extracts and MII extracts, respectively) and examined their ability to reprogram reversibly permeabilized porcine somatic cells. Several criteria were used to evaluate the reprogrammed states of somatic cells after the extract treatment. Dynamic changes of chromatin-binding proteins [20] and deacetylation of histone tails [21] are reliable markers of reprogramming, which occur soon after nuclear transfer. We first examined whether these events were reproducible in extract-treated cells (ETCs). Secondly, we examined dedifferentiation of ETCs after cell culture because we previously found that somatic cells that were treated with *Xenopus*-egg extract partially dedifferentiated after cell culture [16]. The cell-culture experiment revealed that only GV extracts could induce pluripotent gene expressions in ETCs. Finally, we showed these gene expressions might be associated with histone acetylation and DNA demethylation during extract treatment. Our results show that mammalian-oocyte extracts were able to partially reprogram somatic cells and may be useful for analyzing the mechanisms of nuclear reprogramming and dedifferentiation of mammalian somatic cells in vitro.

MATERIALS AND METHODS

In Vitro Maturation of GV Oocytes

Porcine ovaries were collected from a local slaughter house, and 700-1100 GV oocytes were recovered from ovarian follicles by aspiration. Some ovaries were used for dissecting follicles 3–6 mm in diameter with two scalpels as described by Hoshino et al. [17]. GV oocytes were divided into groups of 150–200 in 35-mm culture dish and matured in vitro by coculturing with two follicle shells for 44–45 h in TCM-199 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2.2 mg/ml $NaHCO_3$, 100 mg/ml pyruvic acid, 0.1 IU/ml human menopausal gonadotropin (ASKA Pharmaceutical, Tokyo, Japan), penicillin G, streptomycin, and 10% fetal bovine serum (FBS; JRH Biosciences, Inc., Lenexa, KS).

Oocyte Extract Preparation

GV and MII oocytes were denuded with hyaluronidase (Sigma-Aldrich), and the zona pellucida was removed with actinase E (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan). The oocytes were then transferred into a 1.5-ml tube and washed with extraction buffer (50 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol tetraacetic acid (EGTA), 2 mM β-mercaptoethanol, 0.1 mM PMSF, protease inhibitor cocktail, and 50 mM HEPES, pH 7.6) containing an energy-regenerating system (2 mM ATP, 20 mM phosphocreatine, 20 U/ml creatine kinase, and 2 mM GTP). After centrifugation for 10 sec by tabletop centrifuge, the supernatants were discarded. The remaining pellets from GV and MII oocytes were resuspended in 5 μl and 4 μl, respectively, of extraction buffer containing the energy-regenerating system. Oocytes were crushed by high-speed centrifugation at 90 000 rpm (370 000 × *g*) for 20 min at 4°C by Himac CS120 (HitachiKoki Co. Ltd., Tokyo, Japan) using a fixed-angle rotor (RP100AT4). Cell extracts from the GV oocytes were transferred into a new 1.5-ml tube and centrifuged again at 370 000 × *g* for 15 min at 4°C. These extract solutions were kept on ice before use.

Cell Membrane Permeabilization and Extract Treatment

Porcine fibroblast cells were obtained from the kidney of an adult male Meishan pig that was bred and maintained in our institute. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kyoto University. The collected cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) with 10% FBS. Cultured cells that underwent less than 10 passages were used for the experiments. Cell membranes were reversibly permeabilized with streptolysin O (SLO; Sigma-Aldrich) as described previously with some modifications [16]. Briefly, collected cells were suspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), and SLO stock (2 μg/ml) was added to yield a final concentration of 500 ng/ml. Cells were incubated for 30 min at 37°C in 1.5-ml tubes with occasional tapping to mix the cells. The permeabilized cells were suspended in oocyte extracts and incubated for 30 min or 1 h at 37°C with occasional tapping. The total number of cells that were treated with oocyte extracts was in the range of 0.5–3 × 10^5 cells.

Culture of Extract-Treated Cells

After extract treatment, embryonic stem (ES) cell medium (DMEM, nonessential amino acids, leukemia inhibitory factor, 0.1 mM β-mercaptoethanol, penicillin G, streptomycin, and 20% FBS) supplemented with 2 mM $CaCl_2$ for membrane resealing was directly added to ETCs, and the ETCs were then plated to 96-well dishes. After 2-h incubation at 37°C with 5% CO_2 in air, $CaCl_2$-supplemented ES medium was changed to fresh ES medium, and the cell culture was continued in the ES medium.

Reprogramming and Gene Expression Analyses

For evaluating reprogrammed-cell states of ETCs, we observed protein exchanges between oocyte extracts and ETCs by immunofluorescence and Western blot analyses and epigenetic modifications of donor nuclei by bisulfite sequencing, chromatin immunoprecipitation (ChIP), immunofluorescence, and Western blot analyses. The changes of gene expression in cultured ETCs were examined by immunofluorescence analysis and semiquantitative reverse transcription polymerase chain reaction (RT-PCR).

Immunofluorescence Analysis

After oocyte extract treatment, cells were seeded onto poly-L-lysine-coated coverslips for immunofluorescence analysis. Cultured ETCs grew on gelatin-coated coverslips for NANOG staining. The cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. After permeabilization, samples were treated with 4 N HCl and neutralized with 100 mM Tris-HCl (pH 8.5) for 5-methylcytosine (5-MeC) and HMGA1a (also known as HMG-I) immunostaining. The samples were blocked with 10% rabbit or goat serum, and were treated with primary antibodies overnight at 4°C. The sources of the antibodies were as follows: acetylated-histone H3/K9 and acetylated-histone H4/K12 (Upstate Biotechnology, Billerica, MA), HMGA1a (HMG-I) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-5-MeC (Abcam, Cambridge, United Kingdom), and NANOG (Chemicon, Temecula, CA). After the primary antibody reaction, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or rabbit anti-goat immunoglobulins/biotinylated (DAKO, Carpinteria, CA) was used as the secondary antibody. The streptavidin-biotin reaction was conducted using streptavidin/FITC (fluorescein isothiocyanate; DAKO) in case goat primary antibodies were used. Nuclei were stained with propidium iodide for HCl-treated samples or Hoechst 33342 (Sigma-Aldrich) for nontreated ones. Signals were observed under an Olympus BX50 fluorescent microscope (Olympus, Tokyo, Japan).

Silver Staining and Western Blotting

Oocytes, oocyte extracts, and nuclei of ETCs and control cells were suspended in the SDS-lysis buffer (2% SDS, 10% glycerol, 100 μl/ml β-mercaptoethanol, bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8), and the lysed samples were subjected to SDS-PAGE. Gels were stained with Silver Stain II Kit (Wako Chemical, Osaka, Japan) for comparing whole proteins between oocytes and oocyte extracts or were subjected to Western blot analysis. The gels were transferred onto polyvinylidene difluoride membranes, and the membranes were blocked with 10% skim milk in PBS-Tween solution. The blocked membranes were incubated with primary antibodies overnight at 4°C. The sources of primary antibodies were as follows: acetylated-histone H3/K9, acetylated-histone H4/K12, and histone H2B were purchased from Upstate Biotechnology, CDC2 (cell division cycle 2), cyclin B1, cyclin E, and TBP were from Santa Cruz Biotechnology Inc., and TPT1 (tumor translationally controlled protein 1) was from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (GE Healthcare/Amersham Biosciences, Piscataway, NJ), and visualized using Immobilon Western HRP Substrate (Millipore, Billerica, MA). Relative intensities of protein bands to standard histone H2B were determined using a densitograph (model 4.0A; Atto, Tokyo, Japan).

Semiquantitative RT-PCR

Total RNAs of cells and oocytes were prepared using a ToTally RNA Kit (Ambion, Austin, TX). DNAse was added to preparations to avoid genomic contamination. For reverse transcription, ReverTra Ace (Toyobo, Osaka, Japan) was added to RNA solution, which contained purified RNA and Random Primer (Invitrogen) in diethylpyrocarbonate (DEPC)-treated water. Synthesized cDNAs were subjected to PCR using the specific primers listed on Supplemental Table S1 (available online at www.biolreprod.org), and PCR was performed using ExTaq (Takara, Otsu, Japan). PCR products were visualized on agarose gel under ultraviolet light and relative band intensities were determined using a densitograph during the exponential stage of PCR amplification that was determined by three different PCR cycles.

Chromatin Immunoprecipitation Analysis

ChIP analysis was done as described previously [22]. Briefly, ETCs and control cells after extract treatment were fixed with formaldehyde, and the cross-linked cells were sonicated to produce chromatin fragments. The chromatin solution (600 μl) was precleared with 50% protein G-Sepharose (GE Healthcare) slurry preadsorbed with sonicated salmon sperm DNA (ssDNA), and incubated with 4 μl of Ac-histone H4/K12 antibody (Upstate Biotechnology) overnight at 4°C. Immunoprecipitates were recovered with 20 μl of 50% protein G-Sepharose/ssDNA for 3 h, washed, and resuspended in 200 μl of 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.3 M NaCl, and 0.5% SDS. The beads and input fractions saved before the preclear were incubated at 65°C for 4 h for cross-link reversal. DNA was purified and amplified by PCR. The primers used are listed in Supplemental Table S1 (available online at www.biolreprod.org).

Bisulfite Sequencing Analysis

DNA was isolated from the cells with a Wizard SV Genomic DNA Purification System (Promega, Southampton, United Kingdom). The DNA was treated with bisulfite with an EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the vendor's recommendations. Bisulfite-modified DNAs were amplified with primers designed with MethPrimer software [23]. The upstream sequence of the porcine *NANOG* gene was cloned at China Agricultural University (GenBank accession no. EF522119). The primers are listed in Supplemental Table S1 (available online at www.biolreprod.org). The PCR products were then cloned using a TOPO TA cloning kit (Invitrogen), and successfully inserted colonies that were confirmed by colony PCR were purified as minipreps. The percentage of successful conversion of cytosine at non-CpG sites in the amplified *NANOG* sequence was 100% in all trials.

Statistical Analysis

The proportional differences of methylated CpG sites were analyzed using the ANOVA. P values < 0.05 were defined as statistically significant.

RESULTS

Characterization of Cell Extracts from Porcine GV and MII Oocytes

We developed a system for maturating a large number of GV oocytes in vitro (see *Materials and Methods*). Matured oocytes with normal morphological appearance were selected for the preparation of cell extracts. The maturation rate of the selected oocytes was 95.5% (320/335; n = 2). Extraction of oocyte components was achieved by disruption with high-speed centrifugation. The resulting solutions had liquid droplets and cell debris, which were originally present in the oocytes (Fig. 1A). The germinal vesicles of GV oocytes remained intact after the first centrifugation (Fig. 1A), and therefore an additional spin was necessary to prepare extracts from these oocytes. In the case of MII oocytes, a single centrifugation was enough to prepare the extracts because MII oocytes do not have nuclear envelopes. The protein concentrations of the GV extracts and MII extracts from oocytes were 5.7 ± 1.7 (mean ± SEM; n = 4) and 6.1 ± 1.0 mg/ml (n = 6), respectively. The pH of both extracts was approximately 7.3.

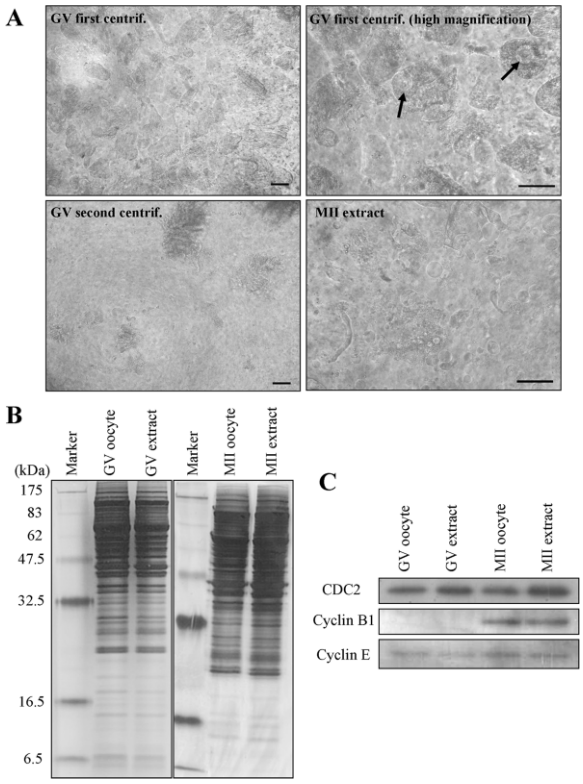

FIG. 1. Preparations of GV- and MII-oocyte extracts. **A**) Appearance of GV and MII extracts. GV first centrif. and GV first centrif. (high magnification) represent the GV extract that was recovered after the first centrifugation of GV oocytes. GV second centrif. shows the GV extract that was obtained after an additional centrifugation. Many germinal vesicles remained intact after the first centrifugation (arrows). Bars = 100 μm. **B**) Protein contents were compared between oocytes and oocyte extracts on SDS-gel electrophoresis by silver staining. The molecular weight markers are shown on the left. **C**) Western blot analyses revealed that cell cycle-related proteins were not degraded in prepared oocyte extracts.

The protein band patterns of SDS-lysed oocytes and oocyte extracts (Fig. 1B) did not show significant protein degradation by the extraction procedure. Cell cycle-related proteins are rapidly degraded or modified during the cell cycle. However, a Western blot of three of these proteins (CDC2, cyclin B1, and cyclin E) showed similar expression levels between oocytes and oocyte extracts (Fig. 1C). Cyclin B1 was not detected in GV oocytes or extracts, in agreement with the previous observation that cyclin B1 is detected from the MI stage during porcine oocyte maturation [24]. Both CDC2 and cyclin B1 are constituents of maturation-promoting factor (MPF), and cyclin B1 is quickly degraded after losing MPF activities. They were not degraded in MII extracts in this experiment. These results suggest that the protein components were successfully extracted from porcine oocytes.

Nuclear Modifications of Somatic Cells in Oocyte Extracts

The GV- and MII-oocyte extracts were used to reprogram somatic cells, and the reprogrammed cell states were evaluated with some markers of reprogramming [20, 21, 25]. The cell membranes of porcine fibroblasts were reversibly permeabilized with SLO to allow nuclei to closely interact with components of oocyte extracts [16, 26]. Treatment of somatic cells with the MII extracts for 1 h significantly changed the cell nuclei. A Western blot analysis revealed that TBP disappeared

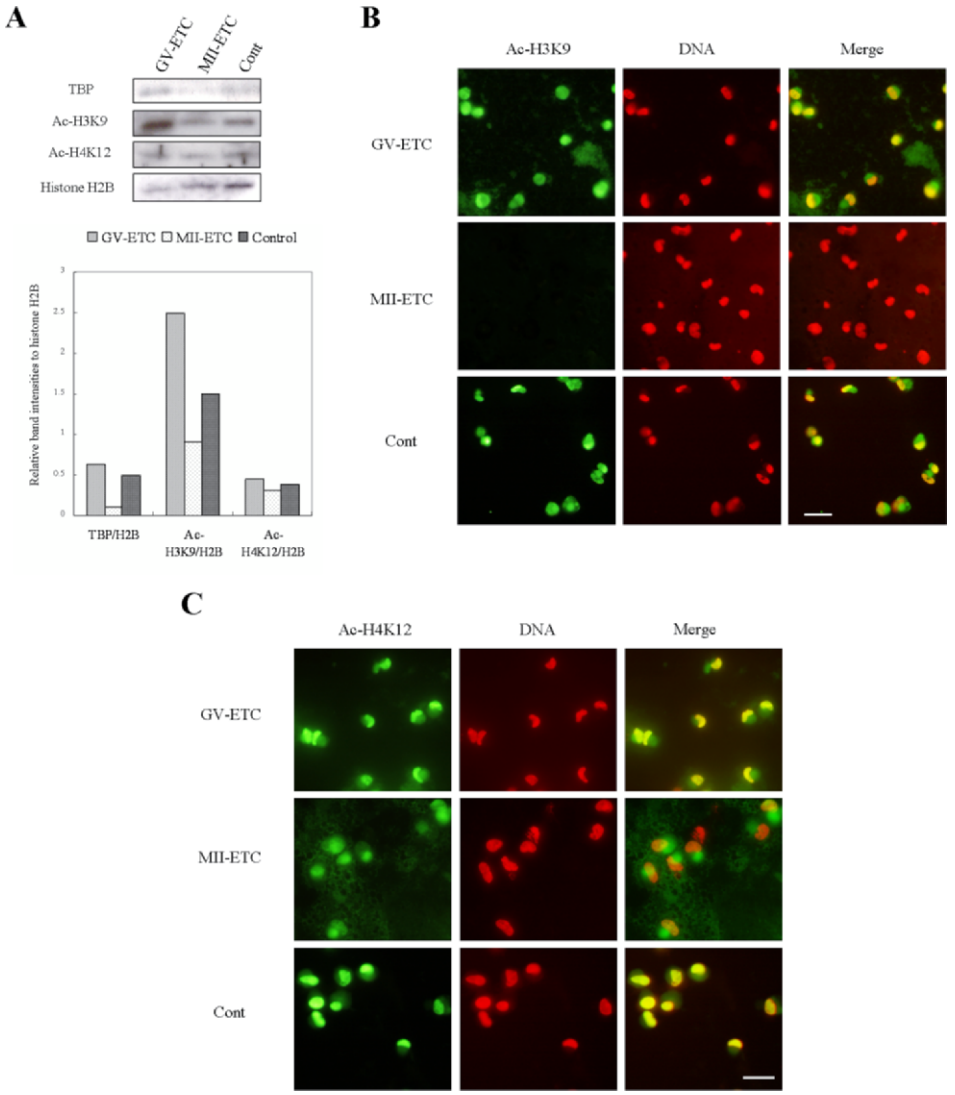

FIG. 2. Reprogrammed nuclear states of reversibly permeabilized somatic cells after treatment with oocyte extracts. **A**) Somatic cell nuclei were isolated after extract treatment and subjected to Western blot analysis. The antibodies against TBP, acetylated-histone H3 lysine 9 (Ac-H3K9), acetylated-histone H4 lysine 12 (Ac-H4K12), and histone H2B were used to evaluate modified states. As a control, permeabilized somatic cells were incubated in extraction buffer containing 5 mg/ml BSA (Cont) instead of GV extracts (GV-ETC) or MII extracts (MII-ETC). The graph represents relative intensities of Western blot bands when standardized with histone H2B. **B**) Deacetylation of H3K9 was examined by immunofluorescence analysis. After treatment with MII extracts, somatic cell nuclei were deacetylated (MII-ETC). DNA was stained with Hoechst 33342, and the blue color of the Hoechst staining was changed to red for visualizing colocalization of signals in merged photos. Yellow color on merged photos (Merge) represents nuclear localization of the antibody signals. Bar = 10 μm. **C**) Deacetylation of H4K12 was examined by immunofluorescence analysis. The nuclei of MII-ETCs were deacetylated although the signals were weakly detected. Bar = 10 μm.

from the nuclei (Fig. 2A) in MII-extract-treated cells (MII-ETCs), and it was lost even when 2×10^5 donor cells were treated with MII extracts. The epigenetic states of MII-ETCs were also modified. Histone H3 lysine 9 (H3K9) in the nuclear faction was deacetylated, which was confirmed by 39% reduction of the relative band intensity of Ac-H3K9 as compared to the control, buffer-treated cells (Fig. 2A). In addition, immunofluorescence analyses showed extensive deacetylation of MII-ETCs (Fig. 2B). Histone H4 lysine 12 (H4K12), which is deacetylated only in the meiotic stage of oocytes [25], was also deacetylated in MII-ETCs (Fig. 2C). The extent of deacetylation of H4K12 was less than that of H3K9, in agreement with previous observations [27]. However, in control cells, which were treated with extraction buffer containing 5 mg/ml BSA and their own-cell extracts, TBP did not disappear and extensive deacetylation was not observed (Fig. 2, B and C). In contrast to these alterations in cell nuclei, global DNA demethylation was not observed after extract treatment at the immunofluorescence level using 5-MeC antibody (Supplemental Fig. S1A, available online at www.biolreprod.org). Moreover, the kinetics of some chromatin-associated proteins were different from that of TBP. For instance, HMGA1a, an architectural protein of chromatin [28], was not released from donor nuclei (Supplemental Fig. S1B, available online at www.biolreprod.org). Furthermore, TPT1, a recently identified reprogramming factor [29, 30], was not incorporated into donor nuclei, although a large amount of TPT1 was present in oocyte extracts (Supplemental Fig. S1C, available online at www.biolreprod.org). These results indicate that the observed nuclear modifications may be linked to nuclear reprogramming events that occur in the MII extracts. Treatment of somatic cells with GV extracts also changed the epigenetic states of cell nuclei, but the resulting states were different from those induced by MII extracts. Histone tails were acetylated and TBP did not disappear in GV-extract-treated cells (GV-ETCs; Fig. 2).

Extract-Treated Somatic Cells in Culture

ETCs were cultured in ES medium after resealing of the cell membrane with $CaCl_2$. The amounts of gene transcripts in GV-ETCs were compared to those in control cells by semiquantitative RT-PCR analysis, which were standardized with *ACTB* transcripts. After 7 days of culture, GV-ETCs expressed a pluripotent marker gene, *NANOG* (Fig. 3A; Supplemental Table S2, available online at www.biolreprod.org). The expression was elevated over 10-fold in prolonged culture at Day 12 (Fig. 3B), and NANOG protein was localized in the nuclei (Fig. 3C). *NANOG* gene expression on Day 7 was not observed in MII-ETCs (Supplemental Table S2, available online at www.biolreprod.org) or in control cells (those treated

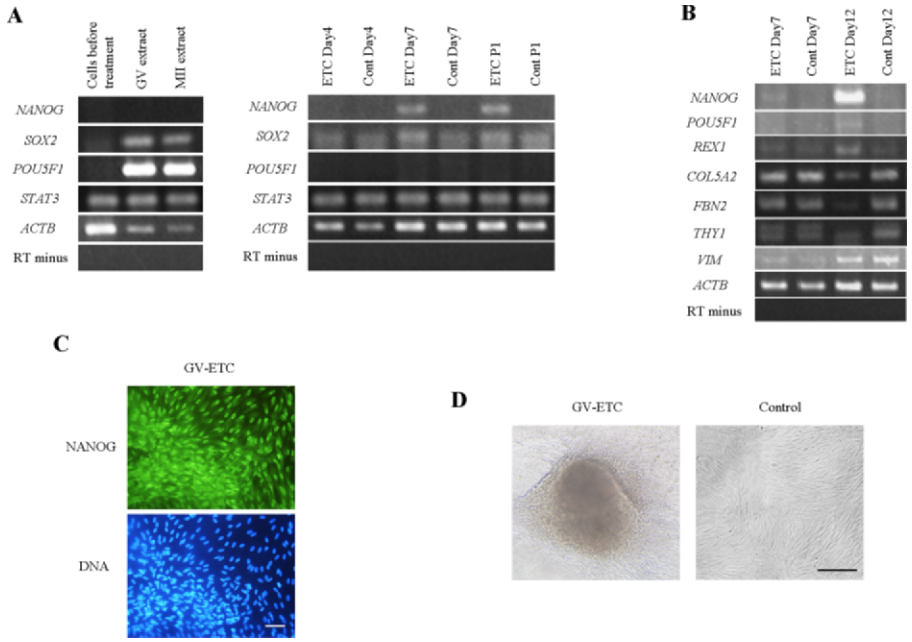

FIG. 3. Dedifferentiation of GV-extract-treated cells after cell culture. **A**) RT-PCR analysis showed that *NANOG* gene expression, which was not originally expressed in control cells and oocyte extracts (left), was observed in GV-ETCs from Day 7 after culture and even after a cell passage (ETC P1). However, cells that were treated with buffer containing 5 mg/ml BSA did not express *NANOG* (Cont). Some pluripotent-marker genes were expressed even in control cells probably because of the presence of the leukemia inhibitory factor in the culture medium, but *POU5F1* was not expressed. *ACTB* (β-actin) was used as a loading control. **B**) Semiquantitative RT-PCR analysis revealed that marked upregulation of *NANOG* gene after prolonged culture of GV-ETCs to Day 12. Faint expression of *POU5F1* and downregulation of fibroblast-marker genes such as *COL5A2*, *FBN2*, and *THY1* was also observed in GV-ETCs at Day 12. **C**) NANOG protein, which was examined by immunofluorescence analysis, was localized in nuclei of cultured GV-ETCs at Day 12. DNA was stained with Hoechst 33342. Bar = 50 μm. **D**) Cultured GV-ETCs formed colonies at Day 9. No colony formation was observed in the control group. Bar = 100 μm.

with buffer containing 5 mg/ml BSA and their own-cell extracts). Other pluripotent marker genes, such as *SOX2* (2.4 fold) and *REX1* (2.2 fold), were also activated in ETCs (Fig. 3, A and B). *POU5F1*, a key pluripotent marker gene, was slightly activated in GV-ETCs at Day 12 (Fig. 3B). Conversely, fibroblast marker genes, such as *COL5A2* (−2.8 fold), *FBN2* (fibrillin 2; −6.5 fold), and *THY1* (−12.1 fold) [31], were downregulated in GV-ETCs (Fig. 3B). Moreover, GV-ETCs formed colonies after 7 days of culture (Fig. 3D), and the colony formation was observed in about half of all the trials (7/13; Supplemental Table S2, available online at www.biolreprod.org). Colony formation was not observed in MII-ETCs or control cells. However, after 10 days of culture, the colonies were negative for alkaline phosphatase activity and did not show distinct expression of pluripotent genes such as *POU5F1* and *SOX2* (data not shown). These results demonstrate that GV extracts, but not MII extracts, could partially and transiently induce dedifferentiation in ETCs.

Histone Acetylation During Extract Treatment on Reprogramming

GV extracts had the ability to induce *NANOG* expression in somatic cells after culture of ETCs (Fig. 3, A–C). The cells were highly acetylated just after the GV-extract treatment (Fig. 2). In mice the 5′-upstream region of the *Nanog* gene is acetylated in *Nanog*-expressing cells [32]; therefore, histone acetylation of the region after the extract treatment was examined. Acetylation of H4K12 was examined at two *NANOG* upstream regions (Fig. 4A), and ChIP analysis revealed that the upstream regions were acetylated after permeabilized cells were treated with GV extracts (Fig. 4B). In contrast, the same region was deacetylated in MII-ETCs (Fig. 4B), in agreement with the results of the Western blot and immunofluorescence analyses (Fig. 2). Next, histone acetylation during extract treatment was inhibited by the addition of 100 μM anacardic acid (Calbiochem, Darmstadt, Germany), an inhibitor of histone acetyltransferases (HATs) [33]. The expression of the *NANOG* gene after culture was abolished in GV-ETCs by treatment with anacardic acid, although ETCs with GV extracts that were produced from oocytes collected at the same time showed *NANOG* expression (Fig. 4C). Finally, we examined whether histone deacetylation during extract treatment inhibited reprogramming. Tricostatin A (TSA; Wako Chemical), an inhibitor of histone deacetylases (HDACs) [34], was added to the extracts at a final concentration of 500 nM. This concentration inhibited histone deacetylation at the upstream region of *NANOG* by MII extracts (data not shown). The TSA treatment induced earlier expressions of *POU5F1* and *REX1* in GV-ETCs at Day 7, and *NANOG* expression was also observed (Fig. 4D). However, *NANOG* expression was not detected in MII-ETCs even after the TSA treatment. The percentage of colony formation in GV-ETCs after culture increased from 53.8% (n = 13) to 100% (n = 4), but no colonies appeared in the controls. Therefore, histone deacetylation during extract treatment may prevent the activation of pluripotent genes, while histone acetylation may accelerate reprogramming of cells to dedifferentiation.

DNA Demethylation by Extract Treatment

Because the porcine *NANOG* gene was activated in GV-ETCs after cell culture and because mouse *Nanog* gene expression is regulated by DNA methylation at the 5′-upstream region of the gene [32], we hypothesized that the oocyte-extract treatment would cause the upstream region of the

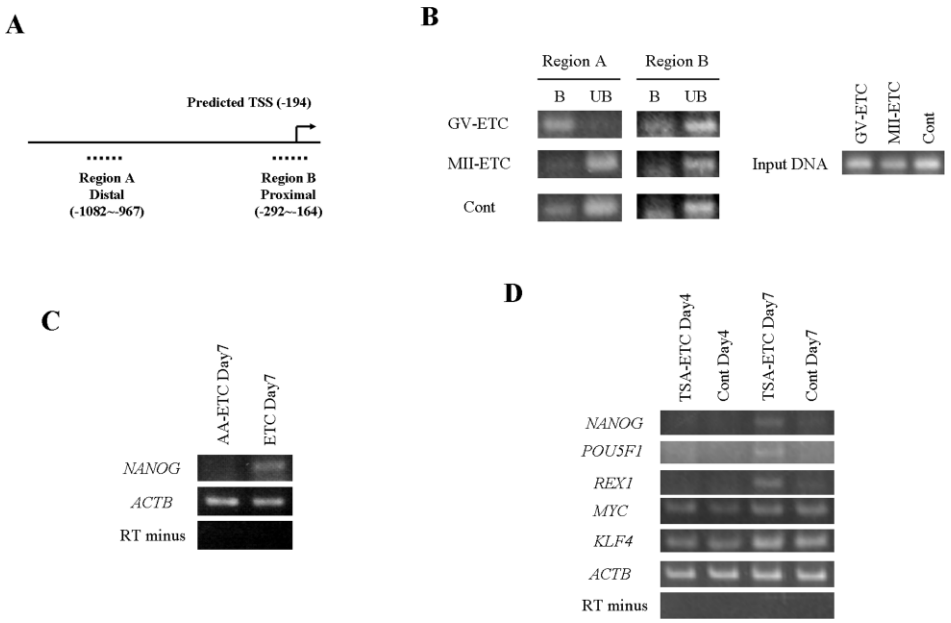

FIG. 4. Histone acetylation during extract treatment is related to pluripotent gene activations of GV-extract-treated cells after cell culture. **A**) Diagram of the upstream region of the porcine *NANOG* gene. The transcription start site (TSS) was predicted by PROSCAN Version 1.7. Two sets of primers (Region A and Region B) were designed for chromatin immunoprecipitation (ChIP) analysis. **B**) ChIP analysis was performed with acetyl-H4K12 antibody to examine acetylation of the *NANOG*-upstream region in ETCs. GV-ETCs were highly acetylated in Region A and slightly acetylated in Region B. As a control, permeabilized cells were treated with buffer containing BSA (Cont). Precipitated (B) and unprecipitated (UB) chromatin fractions and total chromatin amounts among samples were compared (Input DNA) by polymerase chain reaction. **C**) Addition of anacardic acid into GV extracts inhibited the *NANOG* expression in GV-ETCs after culture (AA-ETC), examined by RT-PCR analysis. As a control, cells were treated with GV extracts without anacardic acid and cultured (ETC). **D**) Addition of tricostatin A (TSA) into GV extracts enhanced pluripotent gene expressions in GV-ETCs after culture, examined by semiquantitative RT-PCR analysis. After 7 days of culture, GV-ETCs treated with TSA during extract treatment showed the expression of all the pluripotent genes examined (TSA-ETC).

NANOG gene to be demethylated. To test this hypothesis, the DNA methylation states at the five CpG sites near the transcription start site (TSS) were determined by bisulfite genomic sequencing. These CpG sites were slightly demethylated after the GV-extract treatment (Fig. 5A), especially the CpG at −233, which is 11 bases upstream of the TATA box, was extensively demethylated in ETCs (Fig. 5A). Unexpectedly, the same CpG sites in MII-ETCs were similarly demethylated (Fig. 5A) although MII-ETCs did not express *NANOG* on Day 7 of culture. In contrast, control cells (those treated with buffer containing 5 mg/ml BSA and their own-cell extracts) remained methylated (Fig. 5A). These results indicate that the upstream region of the *NANOG* gene in the somatic genome is slightly demethylated in cell-free extracts of GV or MII oocytes.

We further examined the methylation states of a repetitive repeat sequence to confirm that demethylation is not induced genome-wide in ETCs. We selected the porcine centromeric satellite region in heterochromatic DNA, which is gradually demethylated during early embryonic development to the blastocyst stage [35]. In contrast to the methylation state of the *NANOG* upstream region, the methylation states of the satellite region in ETCs were not significantly changed (Fig. 5B). These results agree with the results from immunofluorescence analyses with 5-methylcytosine antibody (Supplemental Fig. S1A, available online at www.biolreprod.org).

DISCUSSION

Functional cell-free extracts were derived from porcine GV and MII oocytes. The protein concentration of oocyte extracts, approximately 6 mg/ml, is lower than that of *Xenopus*-egg extracts [4] and NCCIT-cell (human embryonic carcinoma cell) extracts [26], but is similar to that of *Xenopus*-oocyte extracts, which are often used to study chromatin assembly [4] and which can reprogram mammalian somatic nuclei [36]. In our oocyte cell-free sytem, a 5 μl droplet of extracts derived from approximately 1000 oocytes was enough to reprogram more than 10^5 somatic cells. In other words, the components from one oocyte could reprogram more than 100 cells. This processing ability of the system imply that oocyte extracts might be used to identify reprogramming factors, as was done with the *Xenopus* system [12, 13, 29].

The putative reprogramming events in the oocyte cell-free system are summarized in Figure 6. Previous studies have reported that remodeling of somatic nuclei such as TBP disappearance and deacetylation of histone tails initiates soon after nuclear transfer to MII oocytes [20, 21]. Our results (Fig. 2) taken together with these results indicate that a part of reprogramming events that are related to the erasure of the regulatory machinery for gene expression could be induced in somatic cells in the MII extracts. Because insufficient erasure of somatic-cell memories, such as incomplete dissociation of chromatin-binding proteins or incomplete deacetylation, is thought to be one of reasons for the low efficiency of cloning [20, 37], prior exposure of donor cells to the extracts may be useful for improving cloning efficiency. In contrast to MII-ETCs, these events were not induced in GV-ETCs and histone tails were acetylated (Fig. 2). The highly acetylated states of histones in GV-ETCs correspond to the highly acetylated chromatin of GV oocytes [27, 38]. Although active states of both HATs and HDACs exist in GV oocytes [38, 39], HATs may preferentially act on somatic chromatin in this system. These results suggest that GV- and MII-oocyte extracts could reprogram somatic cell chromatin in vitro in different ways.

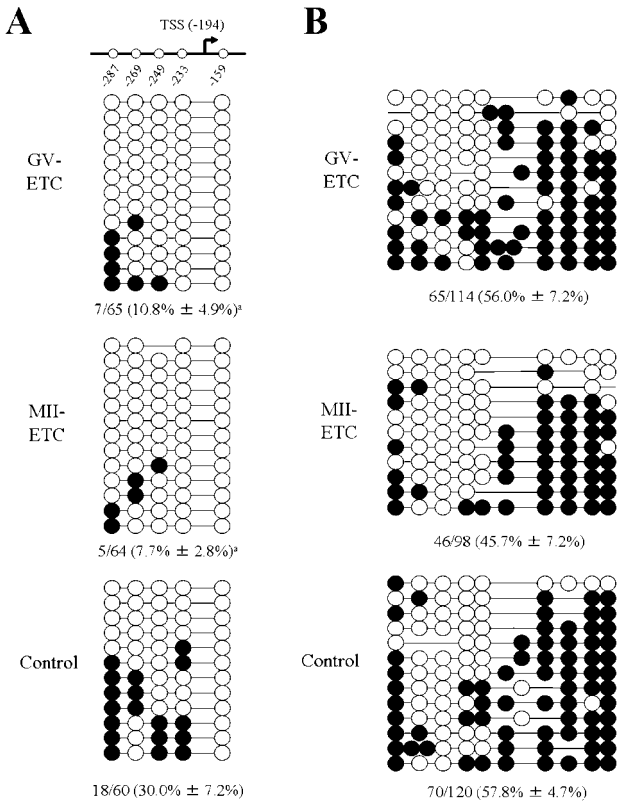

FIG. 5. Selective demethylation at the *NANOG* upstream region of somatic cells by oocyte-extract treatment. Methylation status was examined by bisulfite sequencing analysis and is marked by open (unmethylated) and closed (methylated) circles for each CpG site. The proportion of methylated CpGs to total CpGs are indicated (mean ± SEM). **A**) The upstream region from the TSS of the porcine *NANOG* gene was significantly demethylated after extract treatment in GV-ETC and MII-ETC (a, $P < 0.05$). **B**) Methylation patterns of the satellite sequence were not changed by extract treatment. Some CpG sites were absent in some clones due to mutations.

GV-ETCs were partially and transiently dedifferentiated after cell culture (Fig. 3). Introduction of exogenous pluripotent genes into somatic cells results in rapid downregulation of the somatic-cell genes in the process of producing induced pluripotent stem (iPS) cells [31]. Therefore, downregulation of the somatic-cell genes (Fig. 3B) may be due to the expression of pluripotent genes. Among pluripotent gene expressions, *NANOG* expression was highly specific in GV-ETCs and its expression preceded *POU5F1* expression (Fig. 3B). Since *POU5F1* expression is regulated by *NANOG* [40], later expression of *POU5F1* may be related to prior *NANOG* expression. Moreover, GV-ETCs formed granulated colonies after 7 days of culture. Because NIH3T3 fibroblasts where *Nanog* overexpression was forced form colonies in culture [41], colony formation of GV-ETCs may be linked to *NANOG* expression. In fact, more colonies appeared as *NANOG* gene expression increased (Supplemental Table S2, available online at www.biolreprod.org). Although these colonies themselves did not show ES-like properties, similar granulated colonies appear from virus-infected human fibroblasts during the production of iPS cells before they made their appearance [42].

One of the most interesting reprogramming events in this system is marked activation of the *NANOG* gene in GV-ETCs. To reveal the mechanism of the gene activation, we focused on histone acetylation and DNA demethylation in somatic cells during the extract treatment. Inhibition of histone acetylation during extract treatment (Fig. 4C) revealed that histone acetylation is one of essential steps for *NANOG* upregulation by GV extracts. Histone acetylation facilitates relaxed states of chromatin and promotes the binding of chromatin-binding proteins to chromatin [43]. The relaxed chromatin states induced in GV extracts may lead to permissive states for accessing of reprogramming factors in GV-ETCs. In fact, acceleration of histone acetylation by inhibiting HDACs with TSA resulted in earlier pluripotent gene expressions (Fig. 4D) and stable colony formation (100%). These results also indicate that nuclear reprogramming of somatic cells by oocytes may be somewhat inhibited by their own HDACs. This idea is supported by a previous report that TSA treatment after nuclear transfer enhances the efficiency of cloning in mice up to six fold [34]. Recently, HDAC inhibitors such as valproic acid and TSA are found to enhance dedifferentiation of somatic

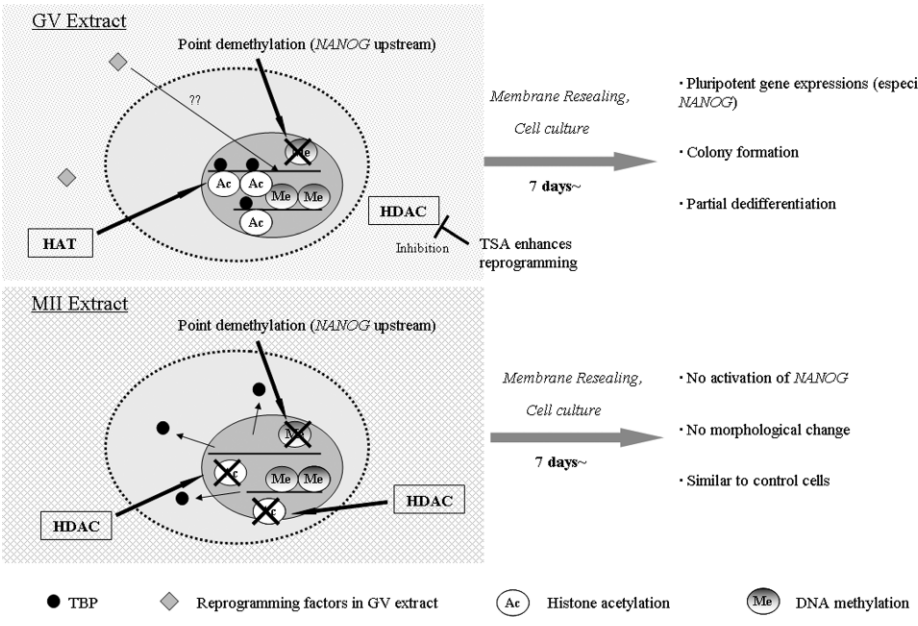

FIG. 6. Schematic diagram of nuclear reprogramming of somatic cells by oocyte extracts. In GV extracts, both HAT and HDAC are thought to exist in active states, and HAT preferentially acts on chromatin of reversibly permeabilized somatic cells, especially the *NANOG* upstream region that upon induction by GV-ETCs is acetylated and undergoes DNA demethylation. The GV-ETCs showed strong *NANOG* expression after cell culture and partial dedifferentiation. This reprogramming of gene expression is enhanced by inhibition of HDAC in GV extracts with TSA. In contrast to GV extracts, MII extracts could induce global histone deacetylation including the *NANOG* upstream region and the release of TBP from somatic chromatin. Although DNA demethylation was induced to the same region, MII-ETCs did not show *NANOG* expression. These results indicate that GV extracts have specific factors or mechanisms for activation of the *NANOG* gene. Relaxation of chromatin by histone acetylation may be important for pluripotent gene activations in GV-ETCs.

cells to iPS cells [44] and of primordial germ cells to embryonic germ cells [45]. HDACs thus exhibit inhibitory effects on reprogramming and dedifferentiation probably by preventing pluripotent gene expressions. Further investigation for an appropriate condition such as time requirement and/or concentration of HDAC inhibitors might help induce dedifferentiation of GV-ETCs.

In contrast to histone acetylation, point DNA demethylation was induced both in GV-ETCs and MII-ETCs (Fig. 5A). Porcine oocytes are thought to have an active DNA-demethylation mechanism [46], and active demethylation is thought to begin soon after fertilization and nuclear transfer [47]. Similarly, demethylation at the *NANOG* upstream region may be conducted in an active manner in oocyte extracts. Moreover, DNA demethylation was induced site-specifically at the *NANOG* upstream region and global demethylation was not observed (Fig. 5B; Supplemental Fig. S1A, available online at www.biolreprod.org). In nuclear transferred embryos, different levels of DNA demethylation has been detected depending on genomic regions [35, 48, 49]. The *NANOG* upstream region might be one of targets to be readily subjected to DNA demethylation by oocytes. This is further supported by the recent report that *Nanog* upstream region was rapidly demethylated during the one-cell stage in mouse fertilized embryos [50]. One of hot spots of demethylated CpG was located at −233 near the TATA box (Fig. 5A). A previous report [51] suggested that methylation of a CpG site close to the TATA box is associated with the silencing of gene expression. Demethylation at the CpG site of *NANOG* in GV-ETCs might be related to the expression of *NANOG*. However, the demethylation state is not crucial for activating *NANOG* because MII-ETCs were also demethylated in the same region. These results suggest that GV extracts may have reprogramming factors to activate *NANOG* gene expression, and the bindings of the factors to the regulatory region of *NANOG* may be enhanced by histone acetylation and DNA demethylation. The lack of expression of *NANOG* in cultured MII-ETCs may be attributed to the lack of histone acetylation in somatic chromatin by MII extracts and/or the lack of factors associated with *NANOG* activation in MII extracts. This is postulated from the results that TSA treatment did not induce *NANOG* expression in MII-ETCs and DNA demethylation was similarly induced in MII-ETCs. *NANOG* may be a suitable marker gene to study nuclear reprogramming and DNA demethylation in oocyte cell-free system.

Evaluation of nuclear reprogramming in many reports has focused on preimplantation development of cloned embryos to the blastocyst stage. However, some reports clearly show that reprogramming initiates soon after nuclear transfer during the one-cell stage [20, 21, 52–55], and reprogramming during that stage greatly affects developmental ability to term [55]. Our understandings for specific factors or mechanism on reprogramming are especially limited because of the lack of appropriate methods for analyzing the one-cell-stage embryos. MII-extract treatment can induce at least some parts of reprogramming soon after nuclear transfer in somatic cells and allows for the use of molecular biological assays such as ChIP (Fig. 4B), bisulfite (Fig. 5), and proteome and transcriptome analyses in a large number of cells, which might lead to the identification of reprogramming factors in oocytes. In contrast to the characteristics of MII-extract treatment, the use of a GV-extract treatment may not be able to directly provide meaningful results for ETCs because GV extracts, as prepared here, consist of artificially mixed components from both germinal vesicles and cytoplasm. However, the GV extracts activated pluripotent genes after rapid changes of histone acetylation (Figs. 3 and 4), while in the case of iPS cells histone modifications at promoter regions of pluripotent genes occur gradually [40] and expressions of endogenous *POU5F1* and *NANOG* requires more than 2 wk [56]. This result supports the idea that oocytes have distinctive and efficient mechanisms to reprogram somatic cells, which are different from reprogramming in iPS cells induced by the defined transcription factors [40, 57]. The oocyte-specific reprogramming mechanisms of gene expression might be studied using GV extracts.

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