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The cytoplasm of mouse germinal vesicle stage oocytes can enhance somatic cell nuclear reprogramming

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In mammalian cloning, evidence suggests that genomic reprogramming factors are located in the nucleus rather than the cytoplasm of oocytes or zygotes. However, little is known about the mechanisms of reprogramming, and new methods using nuclear factors have not succeeded in producing cloned mice from differentiated somatic cell nuclei. We aimed to determine whether there are functional reprogramming factors present in the cytoplasm of germinal vesicle stage (GV) oocytes. We found that the GV oocyte cytoplasm could remodel somatic cell nuclei, completely demethylate histone H3 at lysine 9 and partially deacetylate histone H3 at lysines 9 and 14. Moreover, cytoplasmic lysates of GV oocytes promoted somatic cell reprogramming and cloned embryo development, when assessed by measuring histone H3-K9 hypomethylation, Oct4 and Cdx2 expression in blastocysts, and the production of cloned offspring. Thus, genomic reprogramming factors are present in the cytoplasm of the GV oocyte and could facilitate cloning technology. This finding is also useful for research on the mechanisms involved in histone deacetylation and demethylation, even though histone methylation is thought to be epigenetically stable.

KEY WORDS: Nuclear transfer, Chromatin remodeling, Histone acetylation, Histone methylation, Oocyte cytoplasm

INTRODUCTION

The success of somatic cell cloning gives hope to programs such as species conservation, livestock propagation and cell therapy for regenerative medicine (Wilmut et al., 1997; Wilmut et al., 2002). To date, almost all animals cloned from somatic cell nuclei have been produced using enucleated meiosis stage II (MII) oocytes as recipient cytoplasts (Greda et al., 2006). However, enucleated germinal vesicle stage (GV) oocytes, which are thought to be better recipients than MII oocytes (Bao et al., 2000), or enucleated zygotes have failed to support cloned embryo development to term (McGrath and Solter, 1984). We previously reported that the use of zygotes as recipients damaged the transferred donor nuclei and concluded that there was no firm reason for using this approach for mouse cloning (McGrath and Solter, 1984; Wakayama et al., 2000). The cytoplasm of GV oocytes and zygotes was therefore though to be unsuitable for nuclear transfer. However, Greda et al. and Egli et al. then suggested that one or more factors crucial for embryonic development or reprogramming might be localized to the pronuclei during interphase and that enucleation must remove them (Egli et al., 2007; Greda et al., 2006). They developed alternative methods of enucleation, which left the reprogramming factors in the zygote cytoplasm. They removed chromatin either by rupturing the pronuclear envelope or by extracting the chromosomes during the first zygotic mitosis. They showed support for this by generating cloned mice from eight-cell blastomeres and from embryonic stem (ES) cells using enucleated zygotes as recipients. However, the GV oocyte is though to be a superior nuclear recipient for cloning. In the mouse, immature oocytes can reprogram the immature nucleus, whereas MII oocytes cannot (Bao et al., 2000; Obata et al., 2002). Moreover, passing the donor nucleus through the cytoplasm of the oocyte at these earlier stages of oogenesis might further improve reprogramming (Obata et al., 2002). Yet no cloned mice have been produced using GV oocytes. Gao et al. (Gao et al., 2002) concluded that when the GV is removed before nuclear envelope breakdown and meiotic metaphase arrest, the oocytes become unsuitable as recipients for nuclear transfer, leading to abnormal cell division (Polanski et al., 2005). Moreover, Ogushi et al. (Ogushi et al., 2008) report that the nucleolus of the GV stage nucleus is not necessary for oocyte maturation but is essential for further embryonic development. Therefore, crucial nuclear factors must be extracted in the karyoplasts from GV oocytes or zygotes during enucleation, so that the remaining cytoplasm can no longer support cloned embryo development. Reprogramming factors in embryonic stem (ES) cells (Tada et al., 2001; Cowan et al., 2005) might reside in the nucleus, because enucleated cytoplasts generated from ES cells have failed to reprogram somatic cells (Do and Scholer, 2004).

Here, we reinvestigate whether genomic reprogramming factors are located in the nucleus or cytoplasm of GV oocytes and whether they could improve the reprogramming of somatic cell nuclei. We have attempted to produce cloned mice from differentiated adult somatic cells treated with lysates of GV oocyte cytoplasm, because the production of live offspring is clear evidence of nuclear reprogramming.

MATERIALS AND METHODS

Collection of fully grown GV oocytes

Ovaries were collected from BDF1 strain mice, 45 hours after an injection of 5 IU equine chorionic gonadotropin. Fully grown oocytes were collected in mFHM medium (Lee et al., 2006) containing 100 μg/ml dibutyryl cyclic AMP (dbcAMP) (Sigma-Aldrich, St Louis, MO, USA). The oocytes were stored in mKSOM medium (Specialty Media, Phillipsburg, NJ, USA) containing 100 μg/ml dbcAMP at 37°C under 5% CO_2 in air until use.

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HDAC activity assay

HDAC activity was measured using the CycLex HDAC Deacetylase Fluorometric Assay Kit (#CY-1150; Cyclex, Nagano, Japan). Each sample containing 150 nuclei or 150 enucleated or 150 intact oocytes was transferred into 3 μl of assay buffer and frozen at –80°C. After thawing, each sample was put into 2 μl of lysis buffer, vortexed for 10 seconds and kept on ice for 20 minutes. Samples were moved to each well of 96-well microplates and incubated by mixing fluorescence-labeled acetylated peptide (substrate), HDAC and lysyl endopeptidase at 37°C. Fluorescence intensity was measured for 30 to 60 minutes at 1- to 2-minute intervals using a microtiter plate fluorometer. The rate of reaction was measured at constant reaction velocity. Diluted crude HDAC from MCF4 cells was used as a positive control, and MCF4 cells treated with trichostatin A (TSA) were used as negative controls according to the manufacturer's instructions. The positive control activity was set arbitrarily to 100% and all other bands were expressed relative to that as a mean percentage±s.e.m.

Nuclear transfer to GV oocytes and maturation

Isolated GV oocytes were transferred to mFHM medium containing cytochalasin D (1 μg/ml) for 30 minutes. This produced a concavity in the oocyte surface to insert an enucleation pipette (inner diameter 25 μm) to remove the GV nucleus using a piezo-actuated micromanipulator system (Prime Tech, Aburaki, Tokyo, Japan). Enucleated oocytes were washed and cultured in mKSOM medium containing 100 μg/ml dbcAMP until injection. Cumulus cells were injected into enucleated oocytes using injection pipettes (inner diameter 6-8 μm). Oocytes were then cultured in mKSOM for various times to obtain the following meiotic stages: germinal vesicle (GV, 0-3 hours); metaphase I (MI, 7 hours); anaphase-telophase I (AI-TI, 9-11 hours) and MII (17 hours).

Nuclear transfer to MII oocytes and activation

Cumulus cell donor and the recipient oocyte were collected from BDF1 females. Nuclear transfer to MII oocytes, and oocyte activation methods were as described (Kishigami and Wakayama, 2007; Wakayama, 2007).

Somatic cell permeabilization and preparation of oocyte cytoplasmic lysates

Cumulus cells were washed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (Gibco-BRL, Gaithersburg, MD, USA) and incubated in 200 ng/ml streptolysin O in Hanks' solution for 40 minutes at 37°C. In preliminary experiments, 90% of cells were permeabilized, as judged by vital staining with 0.4% Trypan Blue (Gibco-BRL). Oocyte cytoplasmic lysates were prepared as follows: 500 GV oocytes were collected and zonae pellucidae were removed using acidic Tyrode's solution. Zona-free oocytes were broken down in 5 μl droplets of HEPES-buffered medium (HEPES-CZB) (Chatot et al., 1990) containing an ATP-generating system (1 mM ATP, 10 mM creatine phosphate, 25 μg/ml creatine kinase, 100 μM GTP) and protease inhibitors (Sigma-Aldrich). Entire GVs were removed (Fig. 1B). Removal of all GVs was confirmed using nuclear staining with 12 μg/ml Hoechst 33342 (Polysciences, Warrington, PA, USA) for 15 minutes. Permeabilized cumulus cells were incubated in 5 μl droplets of oocyte cytoplasmic lysates for 45 minutes at 37°C with 1000 cells per lysate. Another group of treated cumulus cells was cultured for 2 hours with RPMI-1640 medium (Gibco-BRL) containing 10% fetal calf serum (FCS), antibiotics and 2 mM $CaCl_2$ to reseal the plasma membrane (Fig. 1C). Control permeabilized cumulus cells were incubated in the same medium without oocyte cytoplasmic lysates. Treated cumulus cells were then used for nuclear transfer.

Reprogramming of fibroblasts by GV cytoplasmic lysate treatment

Mouse fibroblasts collected from the tails of adult mice were cultured in DMEM medium until used. Cells were collected and separated by trypsinization and the cell suspensions were permeabilized and treated with GV lysate, and then resealed membrane as above. Treated cells were cultured in DMEM or ES medium for 1, 2, 3 or 4 weeks, corresponding to 2, 4, 6 or 8 passages. ES cells were used as controls. After culture, cells were collected to examine Oct4 expression and nuclear lamin A (LMNA, a marker of differentiated cells) repression using RT-PCR and real-time quantitative RT-PCR.

Polymerase chain reaction

mRNA was extracted using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway), according to the manufacturer's instructions and eluted in 15 μl DEPC-treated water. Reverse transcription (RT) was performed with 8 μl mRNA using the Superscript First-Strand synthesis System (Invitrogen, Carlsbad, CA, USA) and oligo(dT)15 primers. Quantitative RT-PCR reactions were performed in triplicate on a DNA Engine OPTICON4 (MJ Research, San Francisco, CA, USA) with IQ SYBR Green (Bio-Rad, Hercules, CA, USA). Sequences of the primers used were as follows: OCT4 primer set (GenBank Accession Number, NM_013633; sense, 5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′; antisense, 5′-CTCGAACCACATCCTTCTCT-3′ for a 312 bp product); LMNA primer set (GenBank Accession Number, NM_001002011; sense, 5′-GCAGGCATGGAGTTCTCTTC-3′; antisense, 5′-CCGTCATTC CCATCTCTGT-3′ for a 253 bp product) and GAPDH primer set (GenBank Accession Number, NM_008084; sense, 5′-AGGTCGGTGTGAACGGATTTG-3′; antisense, 5′-TGTAGACCATGTAGTTGAGGTCA-3′ for a 123 bp product). RT-PCR conditions were 95°C for 3 minutes and 35~40 cycles of 95°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute. Quantitative RT-PCR conditions were 95°C for 10 minutes and 44 cycles of 95°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute. Data were analyzed using the 2-ddCt method (Livak and Schmittgen, 2001) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control.

Immunofluorescence

Oocytes and embryos were fixed and then treated as described (Bui et al., 2004). Primary antibodies used were: rabbit anti-phospho-histone H3 at serine 10 (P-H3-S10; Cell Signaling Technology, Danvers, MA, USA); rabbit anti-phospho-histone H3 at serine 28 (P-H3-S28; Upstate Cell Signaling Solutions, Charlottesville, VA, USA); rabbit anti-trimethyl-histone H3 at lysine 9 (Me-H3-K9; Abcam, Cambridge, UK); mouse anti-α-tubulin or anti-β-tubulin (Sigma-Aldrich) for staining microtubules; mouse anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for nuclear membranes; mouse anti-Cdx2 (BioGenex, San Ramon, CA, USA) for the trophectoderm (TE) cells; and rabbit anti-Oct4 (H-134, Santa Cruz Biotechnology) for the blastocyst inner cell mass (ICM). The secondary antibodies were Alexa-Fluor-568-labeled goat anti-mouse and Alexa-Fluor-488-labeled chicken anti-rabbit antibodies (Molecular Probes, Eugene, OR, USA). Control fertilized embryos were produced using intracytoplasmic sperm injection (ICSI) at the same time.

Quantitative analysis of methylation H3-K9 level in nuclei

Embryos were observed using an Olympus Fluoview FV1000 confocal scanning laser microscope. Fluorescence intensities of nuclei in pronuclear and cleavage stage embryos were measured by manually outlining all nuclei in the display. For blastocysts, at least 15 nuclei were selected from the ICM and TE cell populations based on morphology (small and compact in the ICM; elongated in the TE). Nuclei were selected randomly except for metaphase or anaphase-telophase stages, as shown in strong green in Fig. 6B, part h, indicating a typical high level of Me-H3-K9. The total intensity in each nucleus was measured from five different regions and the background value for the cytoplasm was subtracted. This was multiplied by the nuclear volume to represent the total amount of fluorescence for the nucleus.

Statistical analysis

Immunostaining experiments were repeated three times and at least 50 immunostained oocytes were examined in each group. Each HDAC assay was performed on at least three replicates. The data were normalized by arcsine transformation for each replication. The transformed values were analyzed using one-way ANOVA and $P<0.05$ was considered to be statistically significant.

RESULTS

The GV oocyte cytoplasm contains factors involved in histone deacetylation

Because $HDAC_1$ in the GV stage nucleus is inactive and only becomes active after GVBD (Kim et al., 2003), first we injected somatic nuclei into either GV nuclei or the cytoplasm of GV oocytes

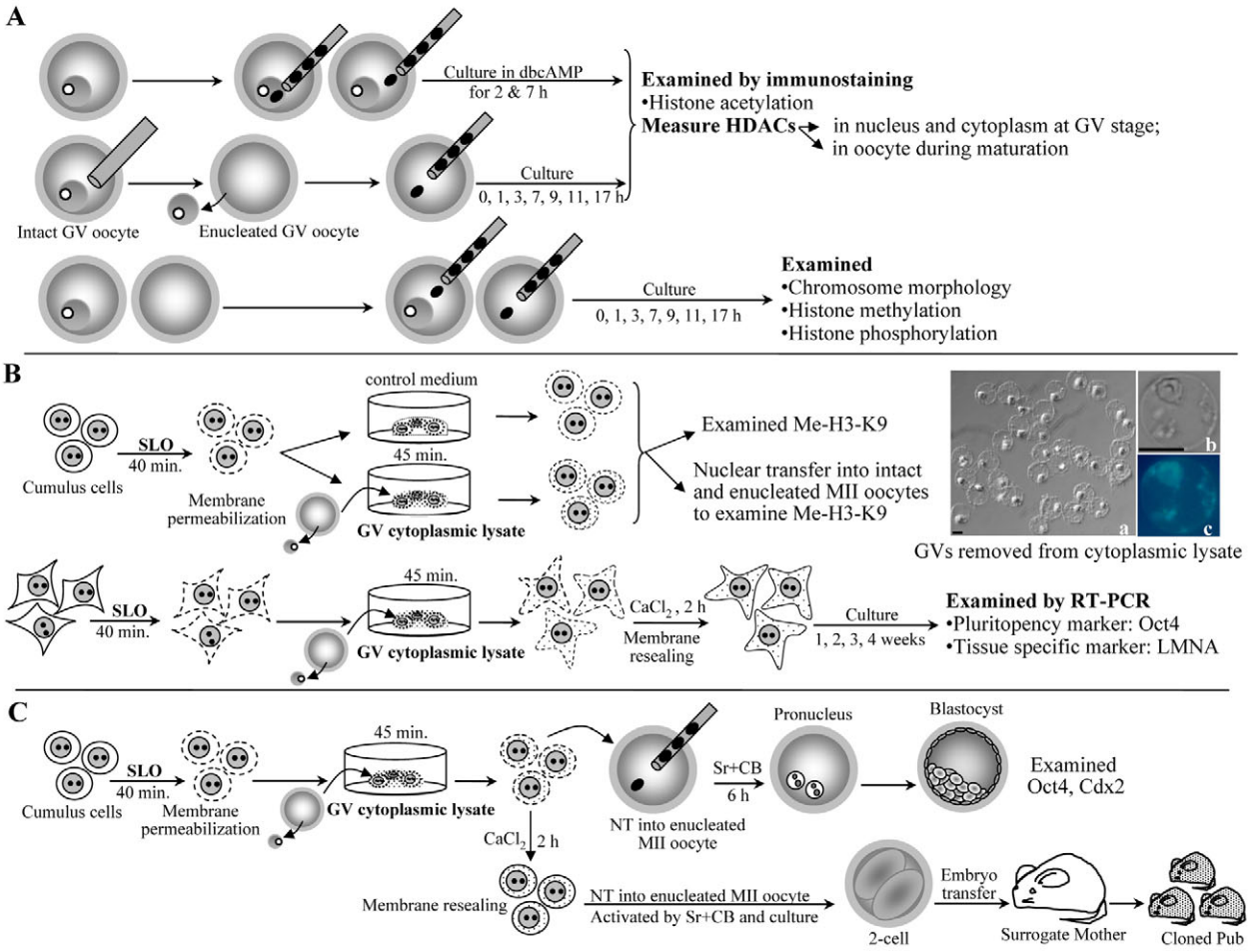

Fig. 1. Schema of the experiments. (**A**) Injection of mouse cumulus cell nuclei (black) into GV nucleus, GV cytoplasm, GV intact or GV enucleated oocytes. (**B**) Cumulus cells were permeabilized with streptolysin O (SLO, 40 minutes), washed and incubated for 45 minutes in HEPES-CZB medium containing an ATP-generating system with or without (control) GV oocyte cytoplasmic lysate. Intact and treated cumulus cells were examined for the intensity of histone H3 methylation at lysine 9 (Me-H3-K9). GV nuclei were removed from the GV oocyte lysate before use (a). Scale bar: 35-40 μm. (b,c) Hoechst-stained GV nucleus at high magnification. Scale bar: 20 μm. The lower figure indicated fibroblasts treated as above, their membrane resealed and cultured for 1, 2, 3 or 4 weeks. These cells were then collected to examine Oct4 and nuclear lamin A (LMNA) using RT-PCR. (**C**) Cumulus cells were treated with GV oocyte cytoplasmic lysate and transferred into enucleated MII oocytes. The oocytes were activated and cultured until the blastocyst stage to examine Oct4 and Cdx2 immunoreactivities. Some embryos were transferred to pseudopregnant surrogate mothers to obtain cloned pups.

(Fig. 1A). Oocytes were then cultured for 2 hours in medium containing dbcAMP to prevent GVBD and we examined histone H3 acetylation at lysines 9 and 14 (Ac-H3-K9 and Ac-H3-K14). Interestingly, we found that there was a difference in the level of histone acetylation of somatic chromosomes when injected into the nucleus versus the oocyte cytoplasm. Ac-H3-K9 and Ac-H3-K14 levels were maintained in somatic chromosomes injected into the oocyte nucleus, but they decreased in the oocyte cytoplasm after 2 hours (100% of 40 oocytes examined for each case of Ac-H3-K9 and Ac-H3-K14, see Fig. 2B, parts a-f for Ac-H3-K14). Thus, the cytoplasm of the GV oocyte contained factors that reduced histone acetylation in somatic chromosomes. However, these could not induce histone deacetylation completely, even 7 hours after injection (100% of 45 oocytes examined for each case of Ac-H3-K9 and Ac-H3-K14, see Fig. 2B, parts g-l for Ac-H3-K14). In the control nontreated dbcAMP group, there were similar results for nontreated and treated oocytes after 2 hours; however, both H3-K9 and H3-K14 become deacetylated in oocyte and somatic chromosomes after GVBD (see Fig. 2C for Ac-H3-K14).

Next, HDAC activity levels in the nucleus and cytoplasm of GV oocytes were assayed. HDACs were expressed in both the nucleus and cytoplasm of GV oocytes and the intensity between nucleus and cytoplasm was similar (Fig. 3A). However, HDAC activities in the GV oocyte could not induce histone deacetylation completely in injected somatic chromosomes, especially in the GV nucleus (Fig. 2B). One possibility is that GV nuclei might contain inhibitors of HDACs, so even if the amounts of HDACs are similar between the GV nucleus and cytoplasm, nuclei injected into GV nuclei still retain high levels of histone acetylation.

We then tested whether HDAC activities increased after GVBD. To do this, the activity levels of HDACs were measured during oocyte maturation. Oocytes were collected at various times of culture to obtain specific stages of meiosis: 0 hours (GV), 3 hours (GVBD), 7 hours (MI) and 17 hours (MII). Oocytes were then assayed for HDAC activity. HDAC activities did not change significantly during oocyte maturation (Fig. 3B). Finally, to examine whether the cytoplasm alone could induce histone deacetylation, enucleated GV oocytes were injected with cumulus cell nuclei and

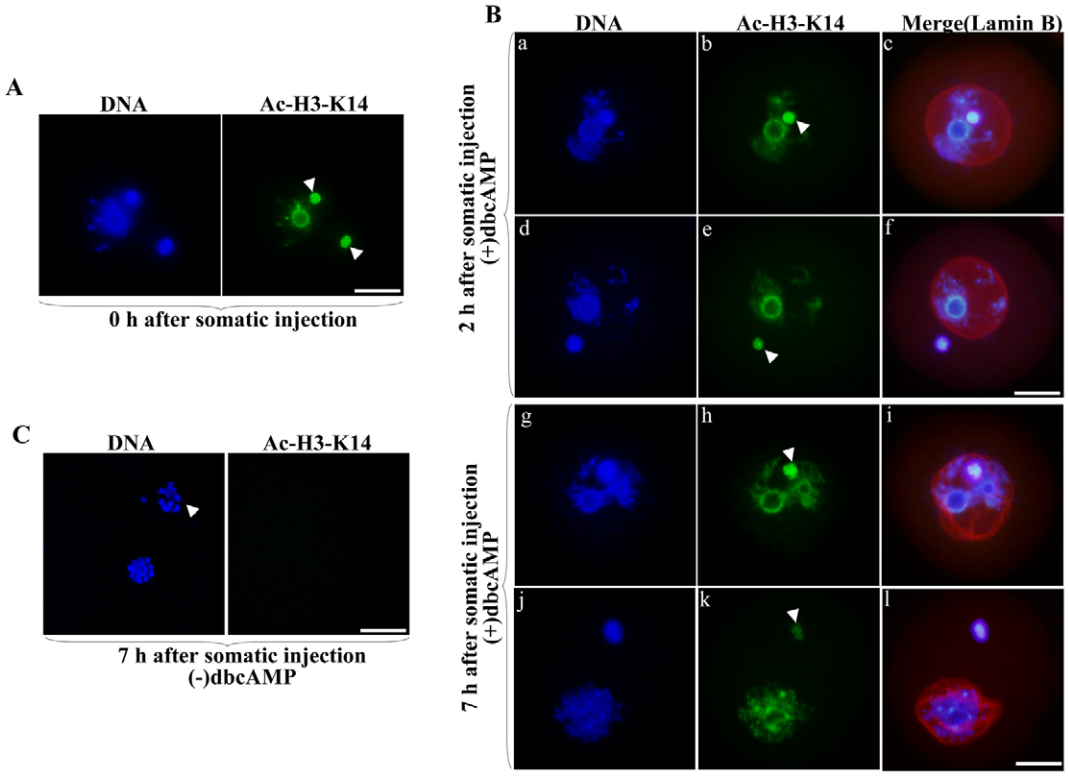

Fig. 2. Expression of histone H3 acetylation at lysine 14 (Ac-H3-K14) in somatic nuclei injected into the nucleus or cytoplasm of mouse GV oocytes. (**A**) Immediately after somatic injection into both the nucleus and cytoplasm. (**B**) (a-f) 2 hours after somatic injection into the nucleus and the cytoplasm. (g-l) 7 hours after somatic injection into the nucleus and the cytoplasm. (**C**) Control oocyte non-treated dbcAMP at 7 hours. Scale bars: 20 μm. Arrows indicate injected cumulus cell nuclei.

histone acetylation was examined during oocyte maturation. The histone acetylation level was high in somatic chromosomes immediately after injection (100% at 0 hours for both Ac-H3-K9 and Ac-H3-K14) (see Fig. 3C for Ac-H3-K9) and decreased 3 hours after injection. Histone H3 became deacetylated 7 hours after injection when the somatic chromosomes reached the MI-like stage and by 17 hours for the MII-like stage (100% for both Ac-H3-K9 and Ac-H3-K14) (see Fig. 3C for Ac-H3-K9). Thus, histone H3 can be deacetylated under the effect of the oocyte cytoplasm without the need for oocyte-specific nuclear factors.

Although more than 17 isoforms of HDACs have been identified in mammalian cells, which of them induce histone deacetylation during oocyte maturation is unknown. If GVBD was blocked, the acetylation levels of both H3-K9 and K14 after 7 hours were decreased in the cytoplasm (Fig. 2), if maturation progressed through the GVBD stage without GV nuclei being present, deacetylation was complete after 7 hours (Fig. 3C). However, the HDAC activity levels in the GV nuclei were slightly lower than in the cytoplasm (Fig. 3A). Thus, the enhancement in HDAC activity upon GVBD bears no relation to the amounts of HDACs present.

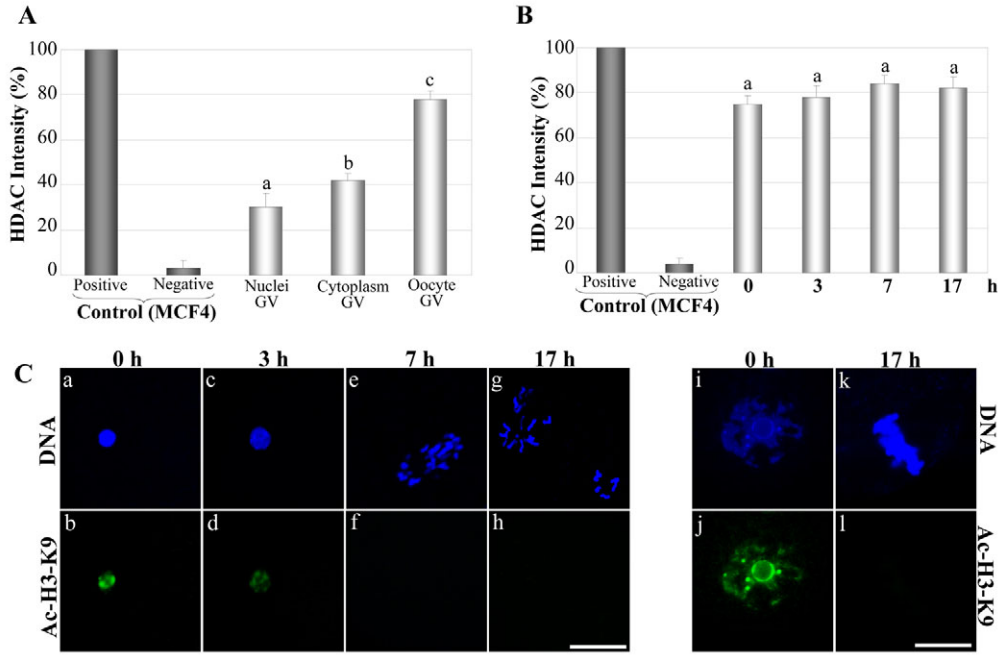

Fig. 3. Expression of HDAC and histone acetylation in mouse oocytes. (**A**) Expression of HDAC activity in nuclei, cytoplasm and oocytes at the GV stage. The positive and negative controls were crude nuclear extract from MCF4 cells and were used according to the procedure outlined in the Cyclex HDAC assay kit. (**B**) Expression of HDAC activity in oocytes during maturation at various meiotic stages: GV (0 hours), GVBD (3 hours), MI (7 hours) and MII (17 hours). (**C**) Deacetylation of histone H3-K9 in somatic nuclei transferred into enucleated GV oocytes (a-h) or in control intact GV oocytes (i-l). Oocytes were cultured at various times to obtain the equivalent meiotic stages: GV-like stage (0 hours); GVBD (3 hours); MI-like stage (7 hours) and MII-like stage (17 hours). Scale bars: 20 μm.

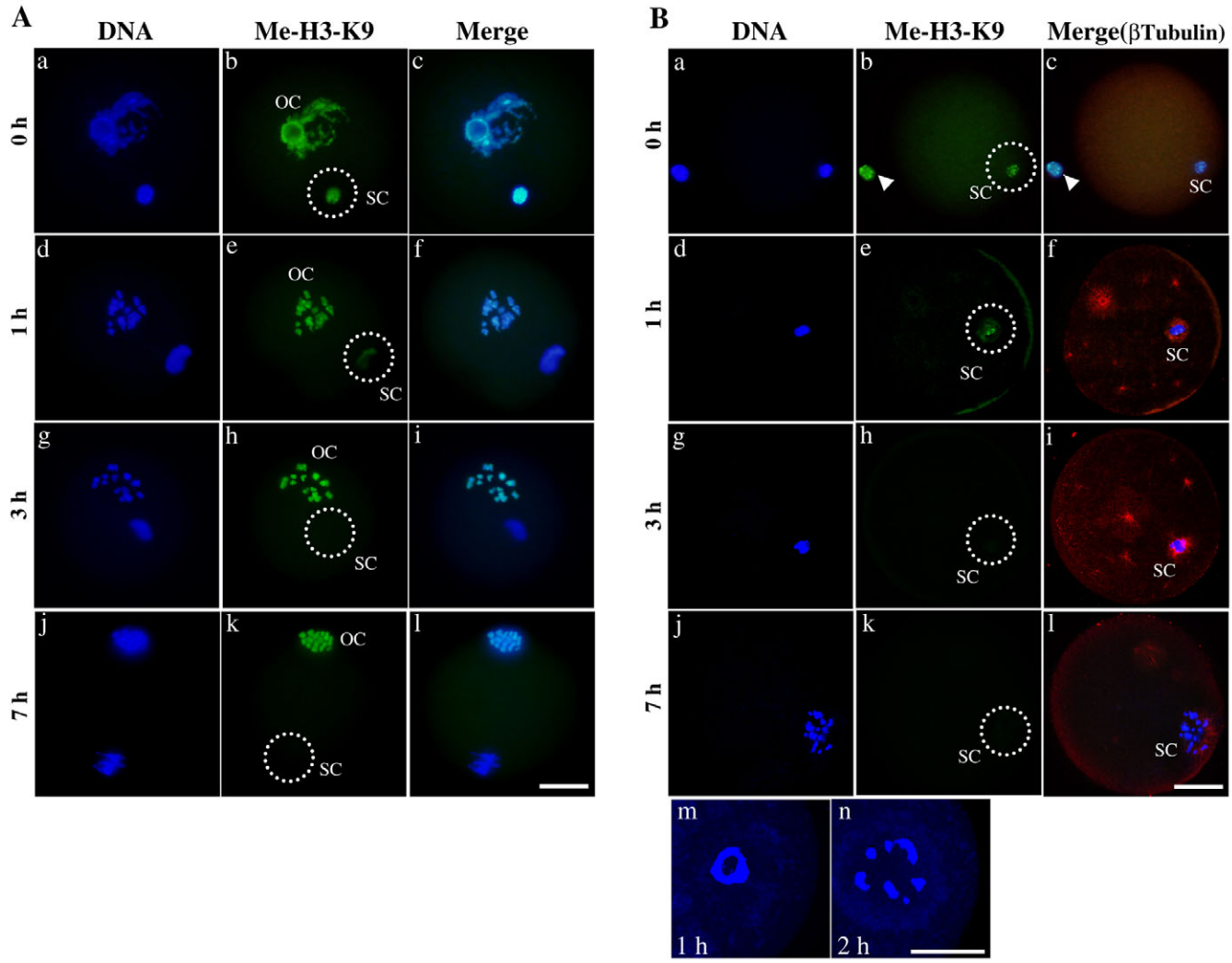

Fig. 4. Demethylation of histone H3-K9 in somatic nuclei after being injected into intact mouse GV oocytes and enucleated GV oocytes. (**A**,**B**) Oocytes were examined after culture for 0, 1, 3 and 7 hours. Methylation of H3-K9 is shown in green and microtubules are stained for β-tubulin in red. (B) After nuclear transfer in enucleated GV oocytes, somatic chromosome formed structures similar to fully grown GV chromosome after 1 hour (m) and similar to late GV chromosome after 2 hours (n). OC, oocyte chromosome; SC, somatic chromosome. Arrow indicates an intact cumulus cell before injection. Scale bars: 20 μm.

Because the total HDAC activity levels do not change during oocyte maturation, there might be some HDAC isoforms that induce increased histone deacetylation activity after GVBD.

Cytoplasm of the GV oocyte induces histone demethylation in somatic chromosomes and remodels them

We compared chromosome morphology along with histone H3 methylation and phosphorylation of somatic cell nuclei injected into intact or enucleated GV oocytes (Fig. 1A). We know that histone H3 methylation at lysine 9 (Me-H3-K9) is epigenetically stable, as it is maintained in the oocyte chromosomes during maturation (Bui et al., 2007) and even in somatic nuclei injected into MII oocytes (Bui et al., 2006). Surprisingly, histone H3 in somatic chromosomes (SC), which was originally methylated at lysine 9, started to decrease in 90% of injected oocytes after 1 hour (Fig. 4A, part e and Fig. 4B, part e in SC). This became demethylated completely 7 hours after injection (Fig. 4A, part k and 4B, part k in SC). This was maintained during oocyte maturation (17 hours after nuclear transfer) (Fig. 5A; see Fig. S1A in the supplementary material). This histone H3 demethylation pattern was similar in somatic chromosomes injected into both intact and enucleated oocytes (Fig. 5A).

We tested whether there was any difference in the effect of the GV cytoplasm versus the GV nucleus on the pattern of histone H3 methylation in microinjected somatic nuclei. Somatic nuclei were injected into either the nucleus or the cytoplasm of GV oocytes, and the constructs were cultured in medium containing dbcAMP for 3 hours to examine Me-H3-K9. Histone H3 in the somatic nuclei became demethylated 3 hours after injection into the GV cytoplasm but remained unchanged after injection into the GV nucleus at the same time (Fig. 5B). Thus, GV stage cytoplasm can induce histone demethylation but the GV nucleus cannot.

Phosphorylation of histone H3 at serine 10 (P-H3-S10) and serine 28 (P-H3-S28) are involved in chromosome condensation (Bui et al., 2004; Bui et al., 2007). Somatic nuclei injected into intact or enucleated GV oocytes were examined for levels of P-H3-S10 and

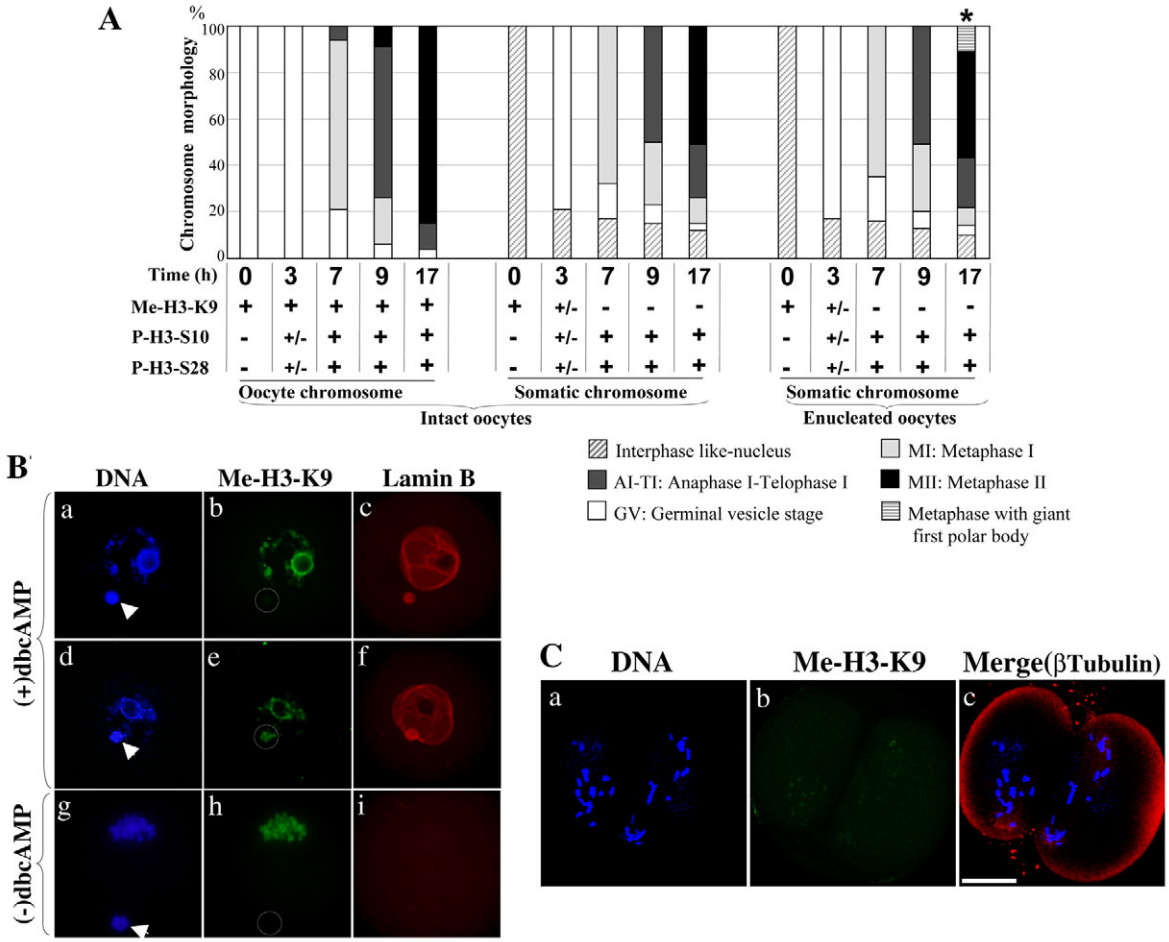

Fig. 5. Chromosome morphology and histone modifications. (**A**) The morphologies of mouse oocytes and injected donor nuclei are classified according to GV oocyte maturation status. Histone H3 methylation at lysine 9 (Me-H3-K9), phosphorylation at serine 10 (P-H3-S10) and serine 28 (P-H3-S28) of oocyte and somatic cell nuclei injected into intact and enucleated GV oocytes were examined simultaneously up to 17 hours after injection. (**B**) Expression of methylation H3-K9 in somatic nuclei injected into the cytoplasm (a-c) and nucleus (d-f), then cultured for 3 hours with dbcAMP. Control oocyte (g-i) was cultured same time without dbcAMP. Arrows indicate injected cumulus nuclei. (**C**) About 10% of enucleated GV oocytes extruded an extremely large first polar body at 17 hours after cumulus cell injection (c). Scale bar:20 μm.

P-H3-S28 to determine the cytoplasmic capacity for chromosome condensation. Histone H3 was not detected as being phosphorylated at either serine position in any somatic cell nucleus immediately after injection. It started to be phosphorylated at S28 and then at S10 1-3 h after injection in both intact and enucleated GV oocytes, and this was maintained during oocyte maturation (17 hours) (Fig. 5A; see Fig. S1B in the supplementary material). Histone H3 phosphorylation patterns in the SCs were similar to those of the oocyte chromosomes. Thus, the enucleated GV oocyte cytoplasm retained its capacity for chromosome condensation following the introduction of a somatic nucleus at interphase. There were also no differences in somatic chromosome morphology between enucleated and intact oocytes (Fig. 5A).

Interestingly, the interphase somatic nuclei formed heterochromatin ring-like structures similar to the nuclei of fully grown GV oocytes 1 hour after somatic nuclear injection into enucleated oocytes (Fig. 4B, part m). This structure appears to correspond to the oocyte-specific structure at the GVI stage as described in pig (Bui et al., 2007). After 2-3 hours, the somatic nuclei acquired morphology similar to the late GV stage in oocyte chromosomes, with individual filamentous bivalents (Fig. 4B, part n), as described for GVII-IV in pig oocytes (Bui et al., 2007). This morphology is specific for GV oocytes and has never been observed in somatic cell nuclei. Thus, these somatic chromosome-derived nuclei became remodeled from the somatic to the GV type (Fig. 5A), even without the GV nucleus. After 7 hours and 17 hours injection, somatic chromosome can reach to the MI- and MII-like stage, respectively (Fig. 4B, part j; see Fig. S1A in the supplementary material). Therefore, the somatic chromosome showed similar developmental stages of GV oocyte. However, about 10% of donor nuclei did not change, or remained at the GV stage even 17 hours after injection (Fig. 5A; see Fig. S1C in the supplementary material). Although there were no apparent differences in the nuclear morphology of donor cell nuclei injected into intact or enucleated GV oocytes (Fig. 5A), when they were observed 17 hours after injection, about 10% of the enucleated oocytes discharged a giant first polar body and resembled a two-cell embryo, as reported (Polanski et al., 2005). However, this was very rare in intact oocytes (Fig. 5A, asterisk; Fig. 5C). This was the only difference between intact and enucleated oocytes, which suggests that enucleation removed key cell cycle factors from the cytoplasm.

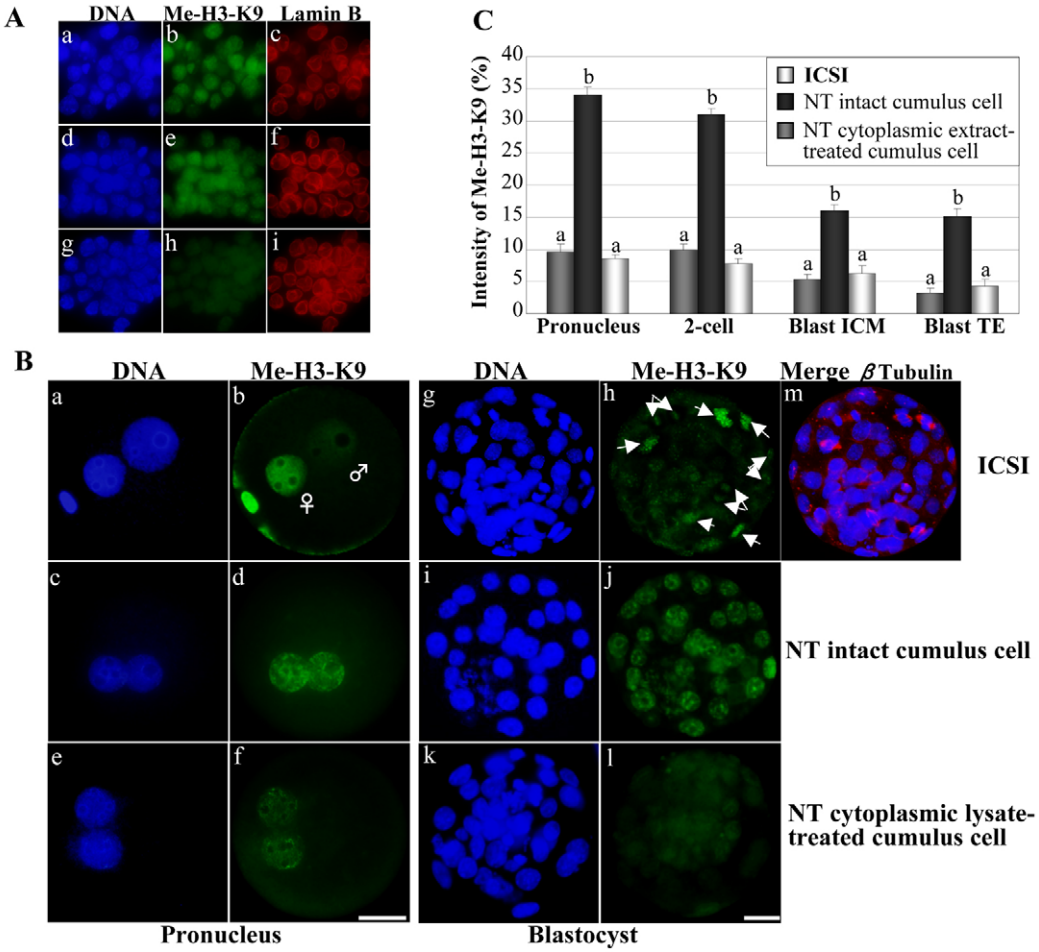

Fig. 6. Effects of mouse GV oocyte cytoplasmic lysates on somatic nuclei and cloned embryos. (**A**) Intensity of histone H3-K9 methylation in cumulus cell nuclei; Me-H3-K9 is shown in green and nuclear membranes are stained by lamin B in red. (b) Intact cumulus; (e) permeabilized cumulus without cytoplasmic lysates (control) and (h) permeabilized cumulus with cytoplasmic lysates. (**B**) Intact and cumulus cell nuclei treated with GV cytoplasmic lysates were transferred to enucleated MII oocytes, then the oocytes were activated and cultured to the blastocyst stage. ICSI-generated embryos were used as controls. The intensity of H3-K9 methylation of zygote and blastocyst stages is shown in fertilized embryos (b,h); in cloned embryos with intact cumulus cells (d,j); and in cloned embryos with cumulus cells treated with GV oocyte cytoplasmic lysate (f,l). Arrows indicate the nuclei at metaphase or anaphase/telophase stages with high levels of Me-H3-K9. Scale bars: 20 μm. (**C**) Quantification of methylated histone H3-K9 in pronuclear, two-cell and blastocyst stage embryos (ICM, inner cell mass; TE, trophectoderm). Each column represents the normalized mean value of these intensities per developmental stage, except for blastocysts, and distinguishes between ICM and TE. The data are presented as the mean±s.e.m.

Somatic nuclear histone H3-K9 is demethylated by GV oocyte cytoplasmic lysates

The low efficiency of cloning technology is thought to be caused by incomplete reprogramming of the donor cells after nuclear transfer. Therefore, the prevention of epigenetic errors such as DNA hypermethylation has been expected to lead to an improvement in the success rate in animal cloning through applying drugs to the donor cells before nuclear transfer (Enright et al., 2003; Shi et al., 2003). We found here that the GV cytoplasm has the potential to remodel somatic cell nuclei. Next, we tried to reprogram somatic cell nuclei by treatment with GV oocyte cytoplasmic lysates based on a method for the transdifferentiation of somatic cells (Hakelien et al., 2002). Permeabilized cumulus cells were exposed to pure lysates in which the oocyte nuclei had been removed and were then examined for histone methylation levels (Fig. 1B). The treated cumulus cells had low levels of histone H3 methylation (Fig. 6A, part h) compared with intact and with permeabilized control cumulus cells (Fig. 6A, parts b and e). Thus, the GV oocyte cytoplasmic lysate retains its demethylation activity, even without the nucleus. We then injected the treated cumulus cells into MII oocytes matured in vivo. The demethylation of H3-K9 in cumulus cells that had been treated with GV oocyte cytoplasmic lysates was maintained for 2 hours after injection into MII oocytes without activation (see SC in Fig. S2B′ in the supplementary material), whereas the cytoplasm of MII oocytes could not remove the methylation of H3-K9 in intact somatic nuclei (see SC in Fig. S2A′ in the supplementary material). Even after activation, the pronuclei from cytoplasmic lysate-treated cumulus cells showed low levels of histone H3-K9 methylation (see SP in Fig. S2D′ in the supplementary material).

GV cytoplasmic lysates elicit expression of Oct4 and repression of LMNA

It has been suggested that induction of Oct4 transcription and loss of gene expression for LMNA (lamin A, a marker of differentiated cells) in 293T cells can be achieved by treatment with extracts of undifferentiated human carcinoma cells and culture for 4 weeks

(Taranger et al., 2005). We examined the expression of Oct4 and repression of LMNA in GV cytoplasmic lysate-treated cells after culture for 1, 2, 3 and 4 weeks. Because treatment of fibroblasts with cytoplasmic lysates of mouse or pig GV oocytes promoted colony formation in medium used for embryonic stem cell culture (ES medium; H.-T.B., S.W., S.K., J.-H.K., N.V.T. and T.W., unpublished, the B6D2F1 fibroblasts were treated with mouse GV cytoplasmic lysates and cultured in DMEM with 10% FBS (DMEM medium) or in ES medium and then used for RT-PCR.

Expression of Oct4 was detected in treated fibroblast cells after 4 weeks of culture in both DMEM and ES medium. The dilute GV cytoplasmic lysate (500 GV oocyte cytoplasm in 5 μl medium) induced low expression of Oct4. Although the levels of Oct4 transcripts in treated fibroblast cells were lower than in the control ES cells, expression was significantly higher in ES medium than in DMEM. (Fig. 7A,B). Repression of LMNA was clearly shown in treated fibroblast cells at 4 weeks of culture in both media (Fig. 7A,C). Notably, LMNA repression paralleled the expression of Oct4 at the same time. Thus, the GV cytoplasmic lysate promoted the upregulation of a marker of pluripotency and the downregulation of a marker of differentiated cells. Strangely, expression of LMNA was increased in treated fibroblast cells for 1-3 weeks after culture in both media (Fig. 7C), whereas it remained unchanged in control non-treated cells cultured at the same time and condition (data not show). It has been suggested that many genes expressed early in the embryonic gonad formation and germline establishment are also expressed during follicular growth such as oocyte-expressed transcription factors Nr6a1, Pou5f1, Figla and Nobox (Pangas and Rajkovic, 2006). Beside that, fully grown GV oocytes regulate their own micro-environment by oocyte-secreted factors, such as bone morphogenetic protein 15 or growth differentiation factor 9 (Hussein et al., 2006). Therefore, the GV cytoplasmic lysate used in this study might have contained some differentiation factors that entered the treated fibroblasts to induce an early increase of tissue-specific markers for 1-3 weeks culture (Fig. 7C).

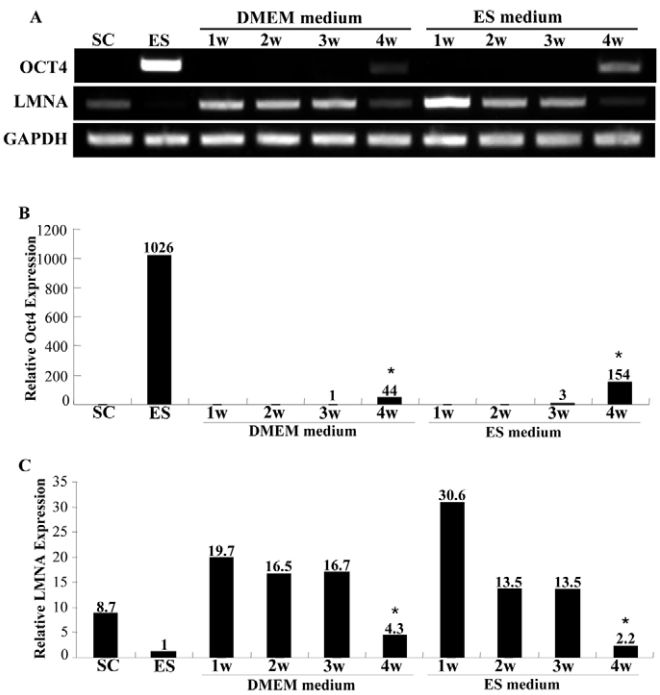

Fig. 7. Expression of Oct4, a pluripotency marker, and repression of nuclear lamin A (LMNA), a tissue-specific marker, in cells treated with GV cytoplasmic lysate and cultured in DMEM or ES medium. B6D2F1 strain mouse fibroblasts were treated with GV cytoplasmic lysate and cultured for 0, 1, 2, 3 or 4 weeks in DMEM with 10% FBS (DMEM medium) or in medium designed for embryonic stem cell culture (ES medium). (**A**) PCR analysis of Oct4 and LMNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control. Expression of Oct4 and repression of LMNA is clearly shown after 4 weeks of culture in both DMEM and ES medium. (**B**,**C**) Quantitative RT-PCR analysis of Oct4 (B) and LMNA (C). SC, somatic cell; ES, embryonic stem cell. Asterisks indicate the significant differences between expression of Oct4 and repression of LMNA at 4 weeks of culture.

Embryos reconstructed using somatic cells treated with cytoplasmic lysates from GV oocytes produce high-quality expanded blastocysts

To test whether the changes in histone methylation patterns described above were associated with nuclear reprogramming, reconstructed oocytes were activated, the resulting cloned embryos were cultured for 96 h and the quality of blastocysts was examined. At the pronuclear stage, cloned embryos derived from lysate-treated cumulus cells showed a lower intensity of methylation than those derived from intact cumulus cells, similar to control fertilized embryos in terms of the numbers of asymmetrically methylated female and male pronuclei (Fig. 6B, parts b,f; Fig. 6C). The low methylation level of H3-K9 in the embryos derived from the cumulus cells treated with cytoplasmic lysates was maintained until the blastocyst stage (96 hours), with no significant difference from normally fertilized blastocysts (Fig. 6B, parts h,l; Fig. 6C). By contrast, cloned embryos derived from intact cumulus cells showed high methylation levels at all embryonic stages (Fig. 6B, parts d,j; Fig. 6C).

Cdx2 and Oct4 are specific gene markers for the TE and ICM, respectively, at the expanded blastocyst stage (Nichols et al., 1998; Strumpf et al., 2005). To assess the quality of blastocysts, TE, ICM and total cell numbers were evaluated with markers for these genes as described (Kishigami et al., 2006; Van Thuan et al., 2006a). There was no significant difference between groups in the mean numbers of blastomeres expressing the Cdx2 protein at the blastocyst stage (see Fig. S3 in the supplementary material). However, blastocysts containing more than 15 ICM Oct4-positive blastomeres (designated Type I good embryos) (Fig. 8A) appeared significantly more frequently in the lysate-treated group than in the controls (20% and 7%) (Fig. 8B).

GV cytoplasm enhances somatic cell cloning efficiency

We aimed to produce live offspring by cloning from somatic cell nuclei using this system, because this endpoint is the strongest evidence for nuclear reprogramming. As it is difficult to prepare pure GV stage nuclear lysates, we performed controls in which donor cells were either left intact or permeabilized and exposed to the same medium lacking cytoplasmic lysates. As shown in Table 1, the rate of pronuclear formation and two-cell stage development were no different between groups. However, when cumulus cells were exposed to GV cytoplasmic lysates and then transferred into mature enucleated oocytes, we could obtain more live cloned offspring than in the intact or permeabilized cell group (3.1% from the cytoplasmic lysate-treated cells versus 0.4% to 0.9% from control groups; Table 1). These cloned mice have grown to fertile adulthood (Fig. 8C). Thus, the cytoplasm of the GV stage mouse oocyte can promote reprogramming of the differentiated somatic cell nucleus without the presence of the nucleus.

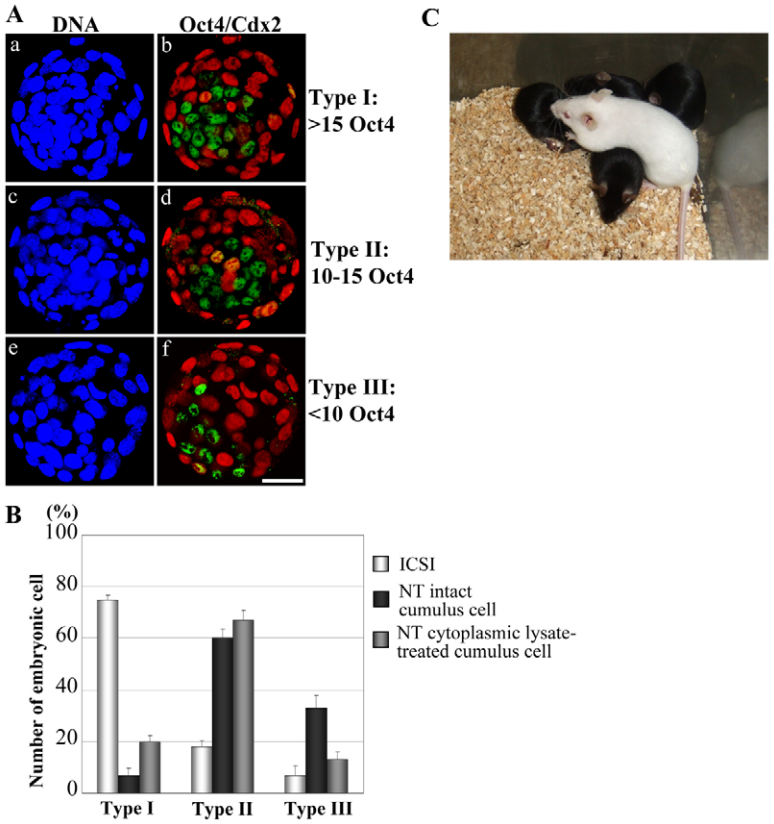

Fig. 8. Production of high-quality cloned embryos and offspring. (**A**) Expression of Oct4 and Cdx2 in expanded mouse blastocysts derived from normally fertilized embryos, from embryos produced by nuclear transfer with intact cumulus and from embryos produced by nuclear transfer with cumulus cells treated with GV oocyte cytoplasmic lysates. The classification is based on the numbers of ICM blastomeres expressing immunoreactivity for Oct4. Type I, more than 15 positive cells; type II, 10-15 positive cells; type III, fewer than 10 positive cells. Oct4 is stained green and Cdx2 is red. Scale bar: 20 μm. (**B**) Proportions of blastocysts classified into these three types. (**C**) Cloned mice were born from enucleated MII oocytes injected with cumulus nuclei treated with cytoplasmic lysates of GV oocytes.

DISCUSSION

For mammals, a number of studies show that epigenetic reprogramming is severely deficient in cloned embryos: most exhibit histone H3-K9 hypermethylation associated with DNA hypermethylation (Kang et al., 2002; Santos et al., 2003). Previously, we have shown that the methylation level of histone H3-K9 in somatic nuclei is stable after being injected into MII oocytes in a pig model (Bui et al., 2006). Here, we demonstrated for the first time that the GV oocyte cytoplasm, both intact and enucleated, could demethylate histone H3-K9 in somatic nuclei within 1 hour of transfer. However, the cytoplasm of MII oocytes appeared to lose this capacity (see Fig. S2 in the supplementary material). If demethylation of H3-K9 in somatic cells is one influential reprogramming factor, then our results show how clones produced from somatic cell nuclei treated with GV oocyte cytoplasmic lysates can develop to full term.

Until recently, histone methylation – unlike all other histone modifications – was considered irreversible (Bannister and Kouzarides, 2005). Methylation of histone H3-K9 remained high during maturation and activation in pig oocytes (Bui et al., 2007). The mechanisms responsible for protecting the maternal chromosomes from histone demethylation are not clear. Normally, histone methylation is stable epigenetically and is reversed only during gametogenesis and in early embryos. However, cloned embryos have higher levels of histone methylation than do natural embryos. This might result from an incomplete erasure of pre-existing methylation in the donor cells. It is possible that in somatic cell cloning with less epigenetic modifications, the lower levels of histone methylation might be reprogrammed more easily or completely. We found here that the GV stage cytoplasm induced demethylation of histone H3-K9 in transferred somatic cell nuclei. We conclude that the GV stage cytoplasm has the ability to remodel somatic cell nuclei in terms of altering chromatin structure and by epigenetic modifications.

Extracts of *Xenopus* oocytes or embryonic cells can remodel mammalian somatic cell genomes, as shown by the levels of DNA methylation or gene expression (Byrne et al., 2003; Simonsson and Gurdon, 2004; Freberg et al., 2007; Miyamoto et al., 2007). However, none of these studies managed to produce cloned offspring, so it is not clear whether these changes involve effective reprogramming. Our experiments document for the first time that donor nuclei can be manipulated epigenetically using mammalian GV stage oocyte cytoplasmic lysates as a step to enhance nuclear reprogramming. We also found that somatic nuclei could be reprogrammed partially to an embryonic state and express the

Table 1. Effect of GV oocyte cytoplasmic lysate for production of cloned mice by pre-treatment of cumulus cells

Cumulus cell treatment	Number of enucleated oocytes	Number (%) of pronuclear formation	Number (%) developed to two-cell embryos	Number (%) of full-term offspring
Intact control	311	282 (91)	231 (82)	2 (0.9)
Permeabilized control	356	295 (83)	248 (84)	1 (0.4)
GV cytoplasmic lysate treated	302	239 (79)	194 (81)	6 (3.1)

All two-cell embryos were transferred into oviduct of 0.5 dpc recipient females.

pluripotency marker Oct4, and could also dedifferentiate with the repression of LMNA. The somatic nuclei became demethylated in GV oocyte cytoplasmic lysates and the resulting embryos showed low levels of histone H3 methylation after nuclear transfer, similar to normally fertilized embryos. When they developed to blastocysts, the proportions of cells in the ICM increased significantly, as indicated by positive staining for Oct4 protein. Thus, embryos reconstructed from cumulus cell nuclei treated with GV oocyte cytoplasmic lysates resulted in high-quality blastocysts at a rate about three times greater than controls. Finally, we obtained live offspring after embryo transfer with a resulting 3.4-fold increase in the success rate of mouse cloning. Thus, factors present in the GV cytoplasm, not in the nucleus, can improve the reprogramming of somatic cell nuclei.

There has been much discussion about the likely effects of enucleation on cloning efficiency. For example, there is no difference seen in the success rate of cloning mice between two commonly used methods: enucleation followed by injection (Wakayama et al., 1998), versus injection followed by enucleation (Wakayama et al., 2003). This suggests that enucleation per se is not the main reason for the low efficiency of cloning. Here, we confirmed that the reconstructed oocyte retained a large amount of microtubule-organizing centers and could establish a bipolar spindle 3 hours after nuclear transfer (Fig. 4B, parts f,i), either with or without a GV nucleus, as for the MII spindle in the oocyte (Van Thuan et al., 2006b). Histones H3-S10 and H3-S28 of the transferred somatic cell genomes became phosphorylated and the chromosomes underwent condensation without the presence of the GV nucleus. This indicated that histone H3 kinase in the cytoplasm of GV oocytes regulated histone H3 phosphorylation and caused chromosome condensation (Bui et al., 2004). Some essential factors are present in the nucleus (Greda et al., 2006; Egli et al., 2007; Ogushi et al., 2008). However, there must be several reprogramming factors in the cytoplasm at the GV stage. One possibility is that the nucleus contains cell cycle-related factors rather than reprogramming factors. We reported that during the process of spindle-chromosome complex depletion, the main part of γ-tubulin, which is needed for correct mitotic spindle formation, is removed together with nuclear mitotic apparatus protein 1 (NuMA1), which is a matrix protein responsible for spindle pole assembly. These are essential for correct mitotic spindle formation (Van Thuan et al., 2006b). Therefore, even though the cytoplasm of enucleated oocytes might retain some genomic reprogramming factors, the subsequent reconstructed embryos fail to develop because of abnormal cell division or lack of essential vital factors. This hypothesis is supported by our observation that abnormal giant polar bodies were extruded only from enucleated oocytes (Fig. 5C).

Here, we demonstrate that genomic reprogramming factors in the GV cytoplasm could improve cloning efficiency. We investigated a possible strategy to enhance cloning efficiency through the pre-exposure of somatic cell nuclei to a GV cytoplasmic lysate before microinjection. However, although the mouse is the best model for studying the molecular mechanisms of cloning, using mouse GV cytoplasmic lysates has limitations in practice because we need to kill many animals and it takes a long time to prepare the lysates. However, this study has established the principle of using mammalian GV cytoplasmic lysates for reprogramming of donor somatic cell nuclei. It might be possible to prepare lysates suitable for practical applications using large domestic animals. For example, many porcine GV stage oocytes can be collected quickly by aspiration from ovaries freely available from commercially slaughtered animals. In this study, we aimed to concentrate on the molecular mechanisms of genetic reprogramming involved in cloning technology. Clearly, more studies need to be made on the factors in the GV oocyte cytoplasm that are involved in the remodeling of somatic cell nuclei during cloning by somatic cell nuclear transfer.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/23/3935/DC1

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