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Supporting Online Material for

The Maternal Nucleolus Is Essential for Early Embryonic Development in Mammals

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Materials and Methods

Pig Oocyte Manipulations

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. Fully grown germinal vesicle (GV) stage oocytes were collected from ovarian follicles 4-6 mm in diameter. Oocytes were cultured in M199 (Nissui Pharmaceutical) supplemented with 10% fetal calf serum (FCS, Dainippon Sumitomo Pharma), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate, and 25 mM sodium bicarbonate at 38.5°C under an atmosphere of 5% CO_2 in air.

Enucleolation was carried out as described previously (8). Briefly, the immature GV-stage oocytes were freed from cumulus cells and cultured for at least 1 hr in the culture medium described above. They were then centrifuged and manipulated in Hepes-M199 (M199 containing 25 mM Hepes, 0.1% polyvinyl alcohol (PVA), 10 mM sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate) with 7.5 μg/ml cytochalasin B (Sigma). Oocytes from which a small volume of nucleoplasm was aspirated were used as control (sham-operated) oocytes.

EA, IVF, and ICSI (electro-activation, *in vitro* fertilization, and intracytoplasmic sperm injection) were carried out as reported previously (S1). The manipulated oocytes exhibiting a first polar body at 30 hr after the beginning of culture were selected and subjected to EA, then cultured for 4 hr in PZM3 (S2) supplemented with 5 μg/ml of cytochalasin B, and thereafter after several washings with Hepes-M199, transferred into PZM3 without cytochalasin B and covered with a liquid paraffin oil. For IVF, the manipulated oocytes were incubated with capacitated spermatozoa from a fertile boar in BO solution (S3) with 3 mg/ml bovine serum albumin (BSA, Wako Pure Chemical) for 8 hr, then washed and further cultured in PZM3. For ICSI, the oocytes were injected with capacitated spermatozoa and cultured in PZM3. The formation of pronuclei was checked after 10 hr or 18 hr post EA, insemination or ICSI.

Somatic cell nuclear transfer was carried out as follows. The enucleolated and sham-operated oocytes with first polar bodies were selected after 30 hr of culture. These oocytes were incubated for 20 min in Hepes-M199 containing 12 μg/ml Hoechst 33342 (Sigma), 3 μg/ml nocodazole (Sigma), and 7.5 μg/ml cytochalasin B. The spindle with chromosomes was removed in Hepes-M199 with 3 μg/ml nocodazole and 7.5 μg/ml cytochalasin B by a micromanipulator under a fluorescence microscope (BX51, Olympus Optical) (S4). After enucleation, these oocytes were

further cultured for 1 hr in culture medium. Pig cumulus cells detached from freshly collected oocytes were used as the donors of nuclei for transfer. After cumulus cells were sucked in and out several times to damage the cell membrane, the nucleus was then aspirated into a pipette (inner diameter, 8-10 μm) and introduced deeply into the cytoplasm either of enucleolated or sham-operated oocytes. Oocytes were then washed 5 times in Hepes-M199 and cultured for 4 hr in 0.5 ml of culture medium in a 4-well dish before being subjected to EA and then cultured for 4 hr in PZM3 supplemented with 5 μg/ml cytochalasin B. Finally, they were transferred into PZM3 without cytochalasin B, covered with a liquid paraffin oil, and further cultured for 6 days.

The empty zonae pellucidae were prepared by sucking out all the contents of the ooplasm by a micromanipulator. The nucleoli isolated in the enucleolation procedure were transferred into the empty zona pellucida by using an injection pipette and were cultured for 26 hr under the same conditions in parallel with enucleolated oocytes. Enucleolated oocytes having a first body were selected, and the above nucleoli were injected into them. The nucleolus-reinjected (previously enucleolated and nucleolus-reinjected) oocytes were cultured for an additional 4 hr and subjected to EA, IVF, or ICSI. The pronucleus formation was then assessed.

Mouse Oocyte Manipulations

Three- to four-week-old ICR female mice were injected with 5 IU of equine chorionic gonadotropin (eCG, ASKA Pharmaceutical). Fully grown GV oocytes were collected 44 hr after injection. They were released directly into HTF-Hepes medium and their cumulus cells were removed by pipetting. Oocytes were then cultured for at least 30 min in αMEM (Sigma) with 4 mg/ml BSA, 25 μg/ml sodium pyruvate, 50 μg/ml gentamicin, and 0.1 mg/ml dibutyryl cyclic AMP (Sigma) at 37°C under an atmosphere of 5% CO_2 in air until used for enucleolation.

The mouse oocytes at the GV stage were manipulated with a micromanipulator equipped with a PIEZO drive (Prime Tech) in modified HTF-Hepes medium (Nippon Medical & Chemical Instruments) supplemented with 0.1 mg/ml dibutyryl cyclic AMP and 7.5 μg/ml cytochalasin D (Sigma). Oocytes from which a small volume of nucleoplasm was sucked by micromanipulation were used as controls (sham-operated oocytes). After enucleolation, oocytes were cultured in αMEM with 4 mg/ml BSA, 25 μg/ml sodium pyruvate and 50 μg/ml gentamicin.

The oocytes matured up to metaphase II over 16 hr in culture, and then they were selected and

activated parthenogenetically by incubating them in Ca-free CZB (S5) supplemented with 7.5 mM $SrCl_2$ and 5μg/ml cytochalasin B for 3 hr and then in KSOM (Chemicon International). For IVF, the manipulated oocytes were inseminated with capacitated spermatozoa in mTBM (S6) for 5-6 hr, and then cultured in KSOM. In the case of ICSI, oocytes were injected with sperm heads essentially as described by Yoshida and Perry (S7) and cultured in KSOM. The formation of pronuclei was examined between 8 to 12 hr after $SrCl_2$ activation, insemination or injection of spermatozoa.

Somatic cell nuclear transfer was carried out essentially as described by Kishigami *et al.* (S8). Enucleolated and sham-operated oocytes at metaphase II were selected after 14 hr of culture and incubated in modified HTF-Hepes medium supplemented with 5 μg/ml of cytochalasin B, and then the spindle with chromosomes was aspirated from the oocyte cytoplasm for enucleation. Mouse cumulus cells detached from freshly collected oocytes, and ES cells (E14tg2A, 129 Ola background) detached from the culture dish by trypsinization were used as the donors of nuclei for transfer. They were aspirated and expelled several times in and out of an injection pipette (diameter, 6-8 μm) to damage the cell membrane and then injected into corresponding cytoplasts. The injected oocytes were then washed 5 times in modified HTF-Hepes medium and cultured for 1 hr in αMEM with 4 mg/ml BSA, 25 μg/ml sodium pyruvate, and 50 μg/ml gentamicin. These reconstructed oocytes were then subjected to $SrCl_2$ activation as described above and cultured in KSOM for the corresponding time intervals.

The isolated mouse nucleoli were injected into previously enucleolated oocytes that were matured to metaphase II as described above. The nucleoli used for reinjection were kept in the perivitelline space of corresponding oocytes. The nucleolus-reinjected oocytes were cultured for an additional 2 hr and then subjected to $SrCl_2$ activation, IVF, or ICSI.

For embryo transfer, 4-6 week B6D2F1 females and 8-10 week B6D2F1 males were used to identify the transferred embryos by hair color. Two-cell embryos after IVF from sham-operated, enucleolated, and nucleolus-reinjected enucleolated oocytes were transferred into oviducts of d0.5 pseudo-pregnant ICR female mice. Caesarian section was performed on day E19.5. Surviving pups were fostered to an ICR foster mother that had given birth on either the same day or 1 day earlier.

Mouse Zygote Manipulations

Six to eight-week-old ICR female mice were superovulated by injection of 5 IU eCG followed

by 5 IU human chorionic gonadotropin (hCG, ASKA Pharmaceutical), and mated with 8-10 week ICR male mice. Zygotes were recovered from the oviducts of plugged female mice at about 20 hr post hCG. They were released directly into HTF-Hepes medium, and cultured in KSOM.

Somatic cell nuclear transfer to mitotic zygotes was carried out as described by Egli *et al*. (10). Zygotes were incubated in KSOM for 5-6 hr after collection, transferred into the medium containing 0.1 μg/ml nocodazole, and left in it for 8 hr. Following 1 μM MG132 (Calbiochem) treatment for 15 min, the mitotic spindle was aspirated from zygotes under modified HTF-Hepes medium containing 1 μM MG132 and 5 μg/ml cytochalasin B. After several washings with HTF-Hepes medium, they were cultured in KSOM for 1 hr in order to allow them to recover. The cumulus cells were synchronized in M phase by 0.1 μg/ml nocodazole for 8 hr, and their metaphase spindles were injected into mitotic enucleated zygotes. Injected zygotes were cultured in KSOM after several washings with HTF-Hepes medium.

For somatic cell nuclear transfer to zygotes at the pronucleus stage, enucleation was carried out as previously described by McGrath and Solter (S9). Zygotes were incubated in HTF-Hepes medium supplemented with 5 μg/ml cytochalasin B, and then their pronuclei were aspirated from them. Mouse cumulus cells detached from freshly collected oocytes were injected into the corresponding cytoplasts. Some zygotes were co-injected with cumulus cell nuclei along with the nucleolus isolated from freshly collected oocytes. The injected zygotes were then washed 5 times in modified HTF-Hepes medium and cultured in KSOM for the corresponding time intervals.

Fluorescence Microscopy

To elucidate the chromatin localization after enucleolation, sham-operated and enucleolated mouse oocytes were stained with 12 μg/ml Hoechst 33342 and observed under a fluorescence microscope.

For immunofluorescence staining, sham-operated and enucleolated pig and mouse oocytes or embryos were fixed and permeabilized in 4% (pig) or 2% (mouse) paraformaldehyde in phosphate buffered saline (PBS)-PVA (pH 7.4) containing 0.2% Triton X-100 for 40 min. Only for mouse nuclear envelope staining, the oocytes at the GV stage were fixed in methanol at 4°C, and subsequently permeabilized in PBS-PVA containing 0.2% Triton X-100 for 40 min. After blocking the samples in PBS containing 1 mg/ml BSA (PBS-BSA), the oocytes (embryos) were incubated

with the appropriate primary antibodies at 4°C overnight, washed several times in PBS-BSA and incubated with secondary antibodies for 40 min at room temperature. DNA was counterstained with 14.3 μM 4',6-diamidino-2-phenylindole (DAPI) for 20 min at 4°C. The oocytes (embryos) were mounted on slides in Vectashield Mounting Medium (Vector Laboratories) and evaluated under a fluorescence microscope.

The following primary antibodies were used: mouse monoclonal anti-C23 antibody (1:100, sc-8031, Santa Cruz Biotechnology); mouse monoclonal anti-lamin A/C antibody (for pig nuclear envelope, 1:100, sc-7292, Santa Cruz Biotechnology); rabbit polyclonal anti-lamin A/C antibody (for mouse nuclear envelope, 1:100, sc-20681, Santa Cruz Biotechnology); mouse monoclonal anti-α tubulin antibody (1:400, T9026, Sigma); mouse monoclonal anti-B23 antibody (1:100, B0556, Sigma); goat anti-NPM2 antiserum (1:100, kindly provided by Dr. M. M. Matzuk); human nuclear ANA-centromere autoantibody (CREST) antiserum (1:100, CS1058, Cortex Biochem); and rabbit monoclonal anti-trimethylated histone H3 at lysine 9 antibody (1:250, ab8898, Abcam). Secondary antibodies were Alexa Fluor 594-labeled rabbit anti-mouse IgG, Alexa Fluor 568-labeled goat anti-mouse IgG, Alexa Fluor 488-labeled goat anti-rabbit IgG, Alexa Fluor 488-labeled donkey anti-goat IgG, Alexa Fluor 488-labeled goat anti-human IgG, and Alexa Fluor 488-labeled goat anti-mouse IgG (1:400, Molecular Probes).

Processing of Semi-Thin Sections

Freshly enucleolated or sham-operated pig oocytes, and zygotes originating from nucleolus-reinjected, enucleolated, and sham-operated pig oocytes (20 hr after sperm insemination) were fixed for 3 hr in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at 4°C. After repeated washings in 0.1 M cacodylate buffer (pH 7.2) and 1 hr post-fixation in 1% osmium tetroxide, the samples were dehydrated through an ascending ethanol series, embedded in epoxy resin (Durcupan ACM, Fluka Chemie) and sectioned by an ultramicrotome (Leica Ultracut R, Leica Microsystems). Serial semi-thin sections stained with toluidine blue were used for gross morphological evaluation under the light-microscope. Male and female pronucleus formation and the localization of nucleoli in both pronuclei were assessed.

Determination of Meiotic Stage

Some sham-operated and enucleolated pig oocytes before and after culture were mounted on slides, fixed in an acetic-ethanol (1:3, v/v) solution, stained with 1% aceto-orcein, and evaluated under a Nomarski interference microscope. The meiotic oocyte maturation stages were classified as: germinal vesicle stage (GV), metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII). After activation, the oocytes were further cultured, and their nuclear stage was examined after staining. Oocytes forming pronuclei were classified as being in the pronucleus stage (PN).

Karyotyping

Air-dried C-banded chromosomes were prepared according to the previously described methods (S10,S11).

CDK1 and MAP Kinase Assay

The kinase assay was carried out as previously described by Ogushi *et al*. (S1). In brief, sham-operated and enucleolated oocytes were collected before or at 6, 12, 18, 24, or 30 hr of culture, and also at 1, 4, and 8 hr after EA. The sample containing one oocyte was washed three times in PBS-PVA and transferred into an Eppendorf tube with 1 μl of PBS-PVA. Then, 4 μl of ice-cold extraction buffer was added, and samples were kept at –80°C until the appropriate kinase assay. After thawing, the samples were centrifuged at 13,000 *g* for 2 min, and then supplemented with 5 μl of kinase buffer containing 0.3 μCi/μl [γ-^{32}P] ATP (250 μCi/25 μl, 5,000 Ci/mmol, Amersham Biosciences) and 5 μl of substrate solution. The resulting mixtures were then incubated for 20 min at 37°C. The substrate solution was composed of 4.25 μl of histone H1 (5 mg/ml, from calf thymus; Roche Diagnostics) and 0.75 μl of myelin basic protein (5 mg/ml, from bovine brain; Sigma). The reaction was terminated by the addition of 5 μl of 4-times-concentrated SDS sample buffer (S12). The samples were boiled for 5 min and loaded onto a 15% SDS-polyacrylamide gel for the separation of labeled histone H1 and myelin basic protein. After running, the gels were fixed, dried, and autoradiographed.

Detection of DNA Replication

DNA replication in zygotes derived from enucleolated and sham-operated pig oocytes was evaluated after incubating the oocytes with 5-bromo 2'-deoxyuridine (BrdU). The cells were labeled

with 100 μM BrdU (Roche Diagnostics) for 1 hr beginning at 10 hr after EA, when the pig oocytes start to replicate DNA actively (S13). The oocytes were then washed in PBS-PVA and fixed for 40 min in 4% paraformaldehyde containing 0.2 % TritonX-100. After being washed 3 times with BSA-PBS, DNA was denatured by incubating the oocytes in 4 N HCl for 1 hr. The acid was neutralized in 0.1 M Tris-HCl buffer (pH 8.5) for 15 min. After being washed 3 times with BSA-PBS, the oocytes were incubated with the anti-BrdU monoclonal antibody conjugated with Alexa Fluor 488 (1:100, Molecular Probes) for 1 hr at room temperature. The DNA was counterstained with 200 μg/ml propidium iodide (Sigma) for 20 min at 4°C. The oocytes were washed 3 times with BSA-PBS, mounted on glass slides with Vectashield Mounting Medium, and observed under a fluorescence microscope.

Analysis of Protein Synthesis

To assess the protein synthesis of enucleolated oocytes, enucleolated and sham-operated pig oocytes at the PN stage 10 hr after EA and 4-8 cell stage embryos 44 hr after EA were labeled for 4 hr with 500 μCi/ml [^{35}S]-methionine (1,000 Ci/mmol: Amersham Biosciences) in 25 mM Hepes-buffered PZM3 medium without amino acids. In some groups, 35 μM cycloheximide (CHX) (S14) was added to the medium (control group). Following several washes in PBS-PVA, each group with 10 labeled oocytes or embryos was transferred to an Eppendorf tube with 2 μl of PBS-PVA, and 8 μl of SDS sample buffer was added. The mixture was then boiled for 5 min. Some samples were loaded onto a 10.5% SDS-polyacrylamide gel for separation of labeled proteins. After running, the gels were fixed, dried, and autoradiographed. The remaining groups of labeled oocytes with or without CHX were transferred to 5 ml of the scintillation fluid (ACS II Amersham Aqueous Counting Scintillant, Amersham Biosciences) and [^{35}S]-methionine was measured using a liquid scintillation counter (Biometra).

Statistical Analysis

The frequencies of oocytes and embryos at each stage were analyzed using the chi-square test. *P* values less than 0.05 were considered to indicate statistical significance.

Supporting References and Notes

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Table S1. *In vitro* development of pig zygotes after intracytoplasmic sperm injection (ICSI).

The values in parentheses represent the percentages of the oocytes cleaved at 48 hr and forming blastocysts at 6 days after ICSI.

type of pig oocytes	Sham	Enucleolated	Nucleolus reinjected
total no. of oocytes examined	83	154	160
total no. (%) of oocytes cleaved	41 (49)	56 (36)	68 (43)
total no. (%) of oocytes forming blastocysts	29 (35)	0 (0)	50 (31)

Table S2. *In vitro* development of mouse parthenotes derived from enucleolated oocytes.

The values in parentheses represent the percentages of the oocytes cleaved at 24 hr and forming blastocysts at 3.5 days after activation.

type of mouse oocytes	Sham	Enucleolated	Nucleolus reinjected
total no. of oocytes examined	120	132	80
total no. (%) of oocytes cleaved	37 (31)	26 (20)	41 (51)
total no. (%) of oocytes forming blastocysts	26 (22)	0 (0)	26 (33)

Table S3. Developmental potency of mouse embryos derived from nucleolus-reinjected oocytes.

type of mouse oocytes	Sham	Enucleolated	Nucleolus reinjected
total no. of embryos transferred (recipients)	72 (4)	45 (3)	133 (7)
total no. of pregnant recipients	2	0	5
pups	6	0	17

Table S4. *In vitro* development of mouse zygotes after somatic cell nuclear transfer (SCNT).

The values in parentheses represent the percentages of the zygotes cleaved at 36 hr and forming blastocysts at 4 days post hCG injection.

type of mouse zygotes	Zygote SCNT	Zygote SCNT+Nucleolus	M-phase Zygote SCNT
total no. of zygotes examined	70	92	37
total no. (%) of zygotes cleaved	32 (46)	28 (30)	22 (59)
total no. (%) of zygotes forming blastocysts	0 (0)	0 (0)	18 (49)

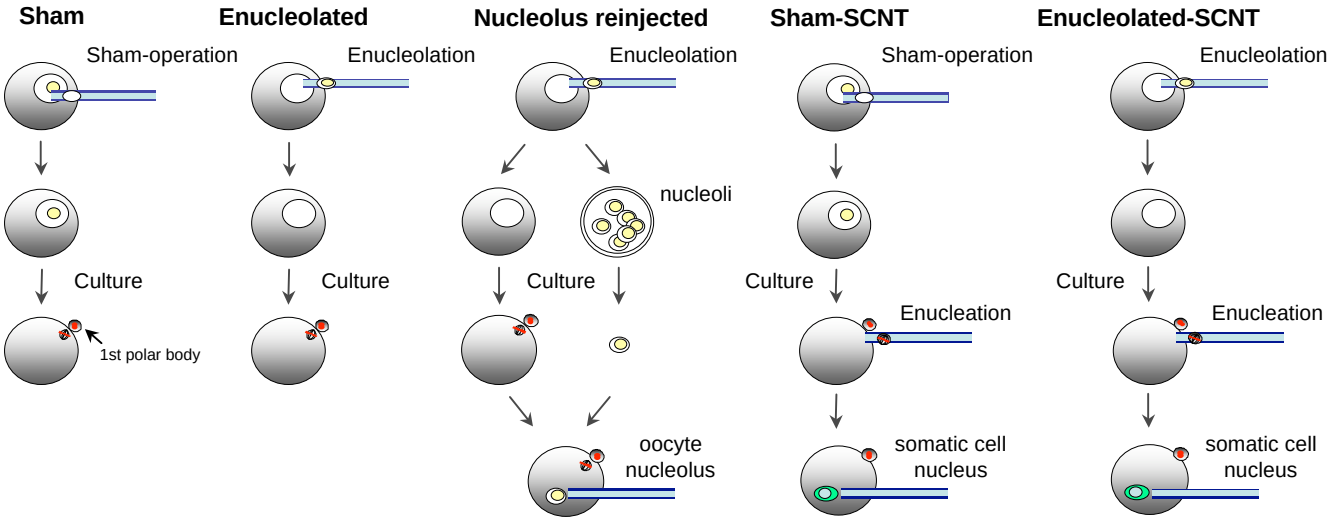

Figure S1. Experimental scheme of oocyte enucleolation and somatic cell nuclear transfer (SCNT) experiments. Nucleoli were microsurgically aspirated from germinal vesicles (GVs) to produce "enucleolated oocytes" (Enucleolated). Oocytes from which we aspirated small amount of nucleoplasm served as controls (sham-operated oocytes; Sham). The isolated nucleoli were kept in the empty zonae pellucidae which were prepared by sucking all the contents of ooplasm by micromanipulator and reinjected into enucleolated mature oocytes having a first polar body (Nucleolus reinjected). Enucleation followed by somatic cell nuclear transfer was performed with sham-operated (Sham-SCNT) and enucleolated (Enucleolated-SCNT) mature oocytes.

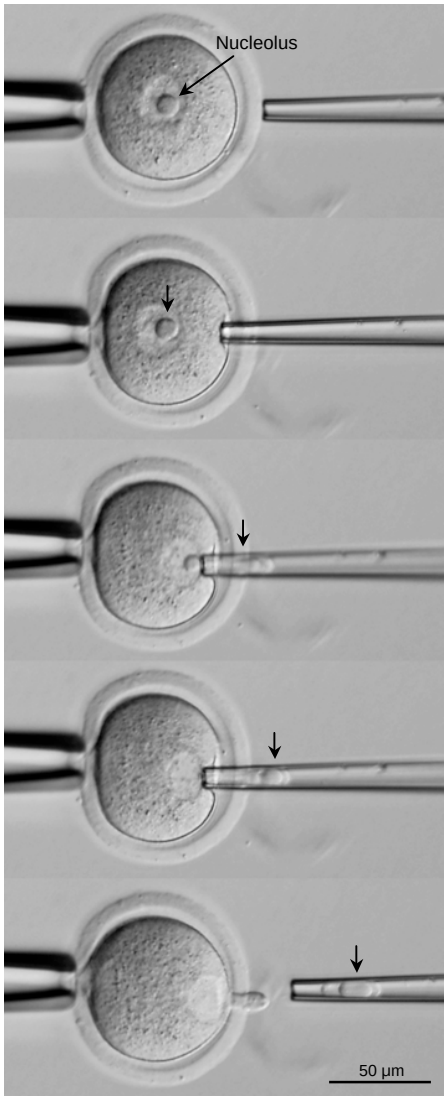

Figure S2. Enucleolation of mouse oocytes at the germinal vesicle stage. Arrows indicate the nucleoli. An injection pipette penetrates through the zona pellucida and its tip is then pushed against the germinal vesicle membrane. With mild suction, the nucleolus is preferentially aspirated into the injection pipette. The nucleolus can be then detached from the oocyte.

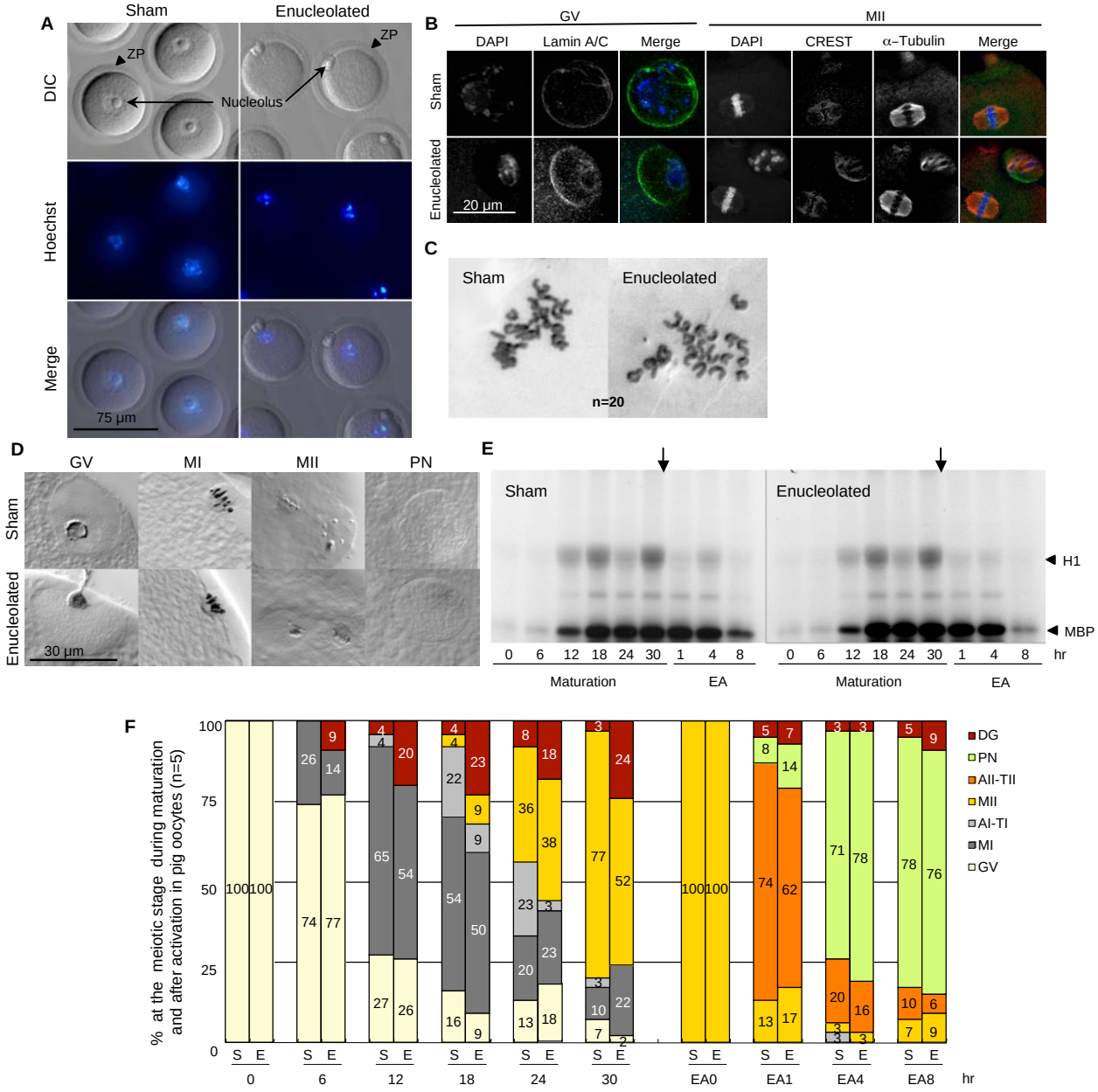

Figure S3. Meiosis progression of enucleolated oocytes. (A) Enucleolated mouse oocytes at the germinal vesicle stage. The merged differential interference contrast (DIC) and fluorescence (Hoechst) images demonstrate the absence of chromatin around nucleoli. Left panels show the sham-operated (Sham) oocytes with nucleoli surrounded by a positive fluorescence (chromatin) inside the germinal vesicles. Right panels demonstrate the absence of Hoechst fluorescence in isolated nucleoli that are located under the zonae pellucidae (arrows indicate the nucleoli, and arrowheads indicated zonae pellucidae, ZP). (B) Nuclear morphology in enucleolated and sham-operated mouse oocytes during maturation. The nuclear envelope was immunostained with anti-lamin A/C antibody in green, the spindle was stained with anti-α-tubulin antibody in red, and centromeres were stained with CREST antibody in green. DNA was counterstained with DAPI in blue. (C) Karyotyping of mouse enucleolated oocytes at metaphase II. The enucleolated oocytes have normal chromosome numbers (n=20). (D) Nuclear morphology of enucleolated and sham-operated (Sham) pig oocytes during maturation and after activation. Meiosis progressed normally in the enucleolated oocytes. (E) The kinetics of CDK1 and MAP kinase activities during maturation and after electro-activation (EA, arrows) of enucleolated and sham-operated pig oocytes. The activities of CDK1 and MAP kinase were measured by the phosphorylation of histone H1 (H1) and myelin basic protein (MBP) as the corresponding substrates. The patterns of activation and inactivation of CDK1 and MAP kinase in enucleolated oocytes were similar to those in sham-operated oocytes. (F) Meiotic stages during maturation and after electro-activation (EA) of sham-operated (S) and enucleolated (E) pig oocytes. Meiosis in enucleolated oocytes progressed at a time course similar to that of sham-operated oocytes. GV: the germinal vesicle stage; MI: metaphase I; AI-TI: anaphase I-telophase I; MII: metaphase II; AII-TII: anaphase II-telophase II; PN: the pronucleus stage; and DG: degenerated.

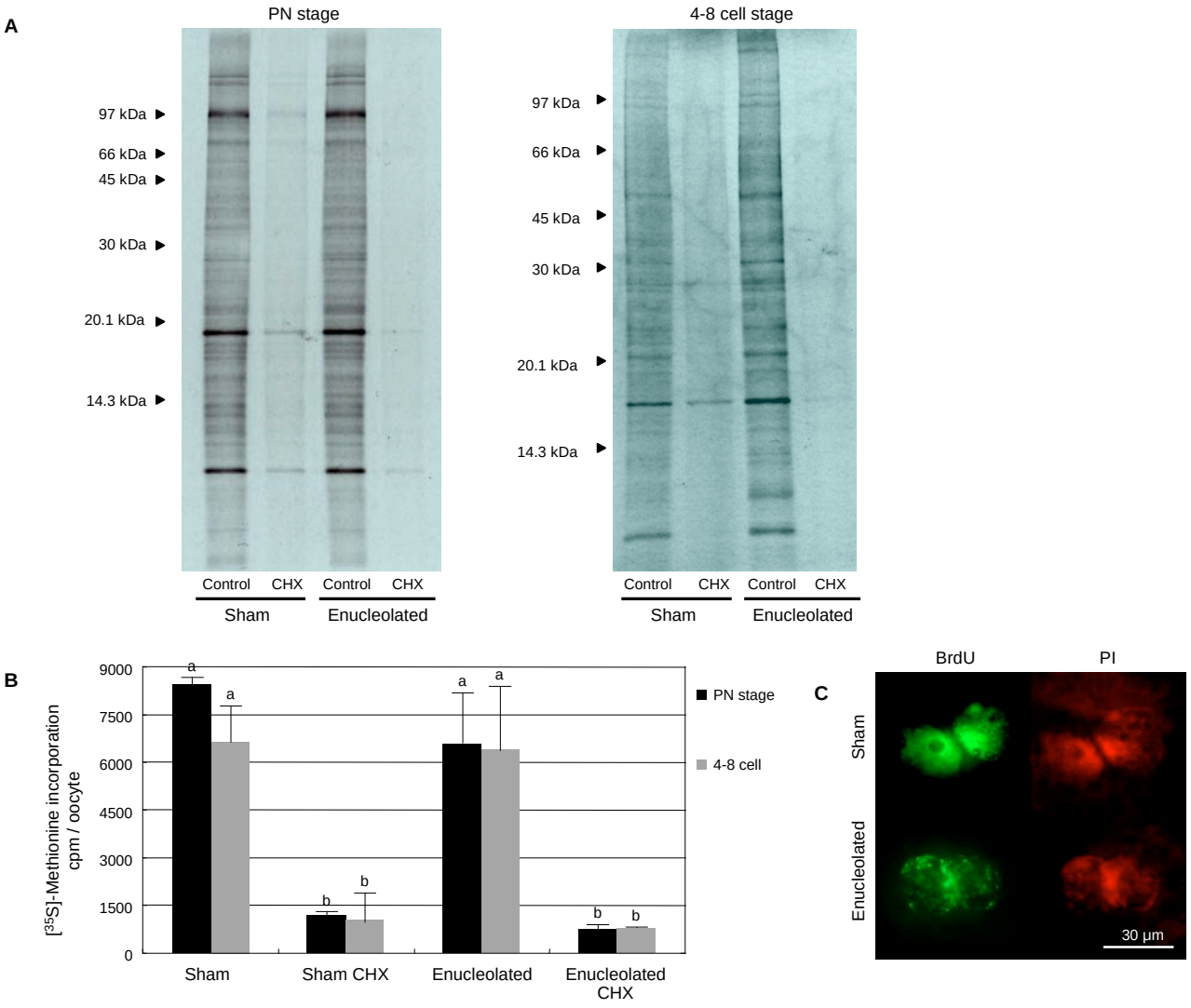

Figure S4. Protein and DNA syntheses in pig embryos originating either from enucleolated or sham-operated (Sham) oocytes. (A) The autoradiographs indicate that there were no differences in [^{35}S] methionine-labelled proteins between enucleolated and sham-operated embryos at the pronucleus stage (PN stage) 14 hr after electro-activation (EA) as well as in 4-8 cell stage 48 hr after EA. Each lane represents the pattern of 10 activated oocytes or embryos with or without a protein synthesis inhibitor, 35 μM cycloheximide (CHX). (B) Uptakes of [^{35}S]-methionine by enucleolated and sham-operated oocytes at the PN stage and 4-8 cell stage were measured by scintillation counting. The uptake of labeled methionine by enucleolated oocytes was similar to that by sham-operated oocytes. Assays were performed in triplicate with bars indicating standard error. $^{a,b}P<0.05$. (C) The replication of DNA in enucleolated and sham-operated oocytes at the PN stage was assessed after Brd-UTP (BrdU, green) incorporation. Both enucleolated and sham-operated oocytes showed positive fluorescence in pronuclei. DNA was counterstained with propidium iodide (PI, red).

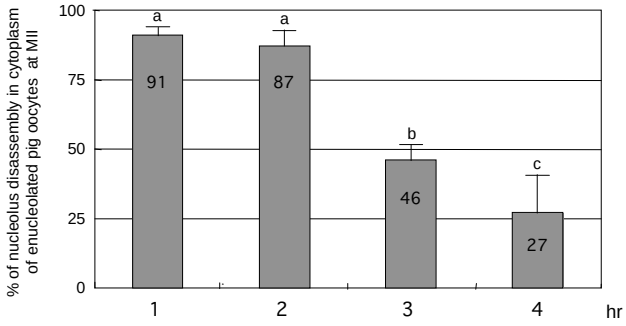

Figure S5. Disassembly of injected nucleoli in enucleolated pig oocytes at metaphase II. Isolated oocyte nucleoli were transferred to the cytoplasm of enucleolated oocytes that were cultured for 26 hr. Assays were performed in triplicate with bars indicating standard error. $^{a\text{-}c}P<0.05$.

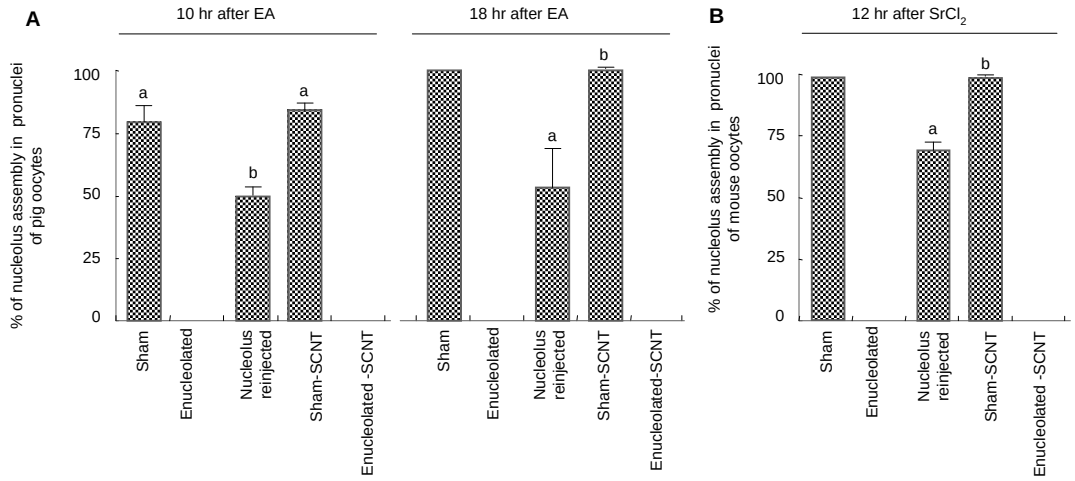

Figure S6. Percentage of nucleolus assembly in pronuclei of (A) pig oocytes after electro-activation (EA) and (B) mouse oocytes after $SrCl_2$ treatment. Assays were performed in triplicate with bars indicating standard error. ^{a,b} $P<0.05$.

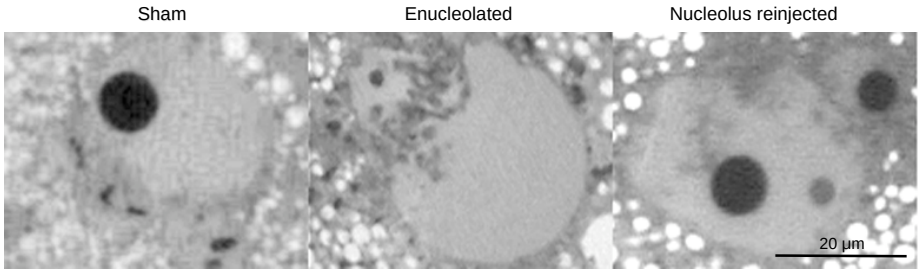

Figure S7. Semi-thin sections of pig oocytes after in vitro fertilization. Nucleolar formation was observed in both pronuclei in sham-operated (Sham) and nucleolus-reinjected (Nucleolus reinjected) oocytes.

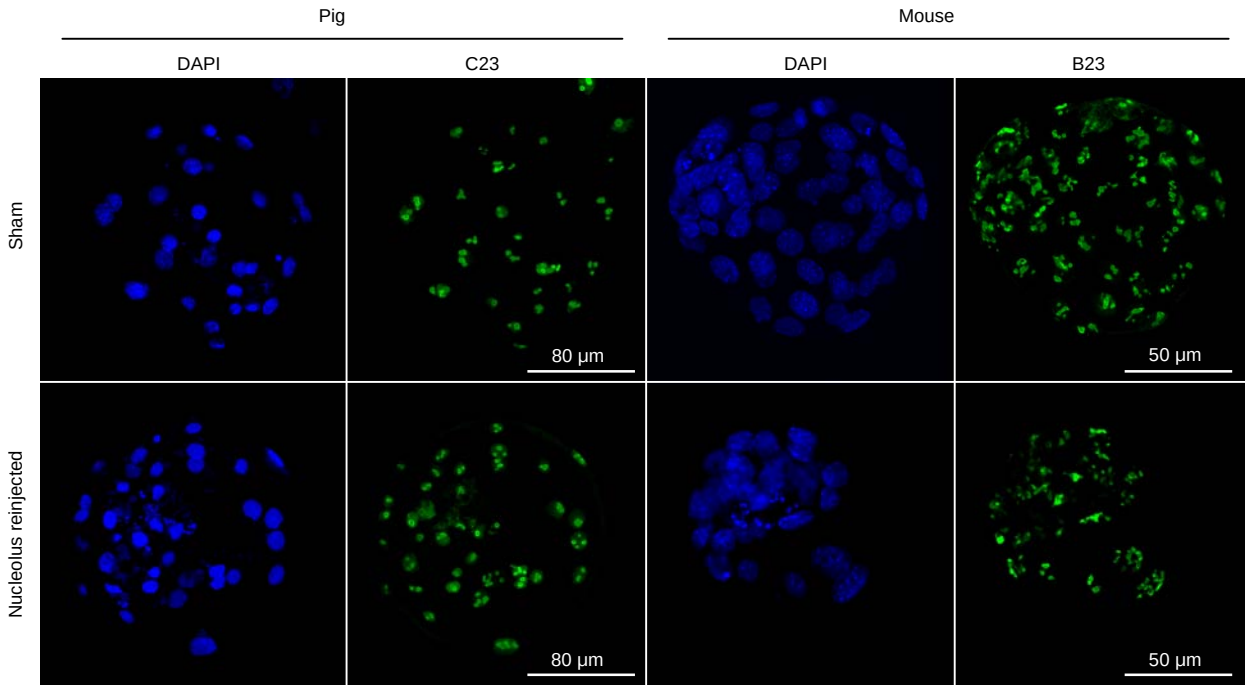

Figure S8. Nucleoli in blastomeres of pig and mouse parthenogenetic blastocysts originating from sham-operated and nucleolus-reinjected enucleolated oocytes. Nucleoli were immunostained with anti-C23 (nucleolin) antibody in the pig and anti-B23 (nucleophosmin/NPM) in the mouse in green, and DNA was counterstained with DAPI in blue.

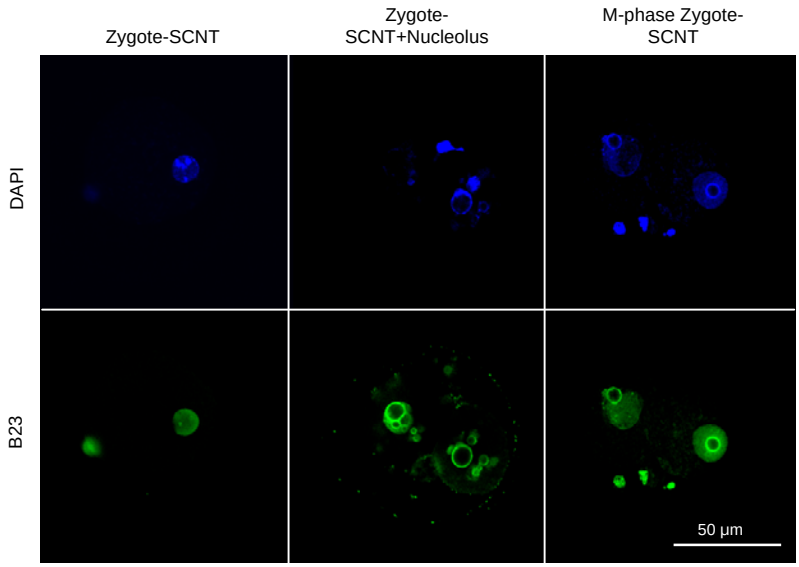

Figure S9. Nucleolus formation in nuclei of 2-cell mouse embryos originating from zygotes whose pronuclei were enucleated and subsequently injected with somatic cell nuclei (Zygote-SCNT). In contrast to the absence of nucleolus formation in the nuclei of blastomeres from Zygote-SCNT, nucleoli were observed in the Zygote-SCNT co-injected with the oocyte nucleolus (Zygote-SCNT+Nucleolus), and somatic nuclear transferred zygotes which were enucleated at M-phase (M-phase Zygote-SCNT). Nucleoli were detected by B23 (nucleophosmin/NPM) in green, and DNA was counterstained with DAPI in blue.