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RESEARCH REPORT

De novo formation of nucleoli in developing mouse embryos originating from enucleolated zygotes

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ABSTRACT

The large, compact oocyte nucleoli, sometimes referred to as nucleolus precursor bodies (NPBs), are essential for embryonic development in mammals; in their absence, the oocytes complete maturation and can be fertilized, but no nucleoli are formed in the zygote or embryo, leading to developmental failure. It has been convincingly documented that zygotes inherit the oocyte nucleolar material and form NPBs again in pronuclei. It is commonly accepted that during early embryonic development, the original compact zygote NPBs gradually transform into reticulated nucleoli of somatic cells. Here, we show that zygote NPBs are not required for embryonic and full-term development in the mouse. When NPBs were removed from late-stage zygotes by micromanipulation, the enucleolated zygotes developed to the blastocyst stage and, after transfer to recipients, live pups were obtained. We also describe *de novo* formation of nucleoli in developing embryos. After removal of NPBs from zygotes, they formed new nucleoli after several divisions. These results indicate that the zygote NPBs are not used in embryonic development and that the nucleoli in developing embryos originate from *de novo* synthesized materials.

KEY WORDS: Nucleolus, Oocyte, Embryo, Mouse

INTRODUCTION

Fully grown mammalian oocytes contain specific nucleoli, termed the nucleolus precursor bodies (NPBs), which exhibit distinct structural and functional differences compared with nucleoli in somatic cells (supplementary material Fig. S1). In the process of oocyte maturation, the NPBs disappear with the onset of germinal vesicle breakdown (GVBD), but after fertilization they are formed again in female and male pronuclei of zygotes.

A previous study showed that oocytes from which NPBs were microsurgically removed (enucleolation) matured to metaphase II (MII) (Ogushi et al., 2008), but after fertilization or parthenogenetic activation no NPBs in pronuclei were formed and the embryos typically stopped development at the two-cell stage. However, when the oocyte NPBs were re-injected into previously enucleolated oocytes that were subsequently matured to MII and then fertilized, NPBs were formed in pronuclei and these embryos developed to full term. These results suggested that zygotes inherit NPBs from the oocyte NPBs, and that oocyte NPBs are thus essential for embryonic development or at least for some of its stages.

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It is commonly accepted that, as the mammalian embryo develops, NPBs are gradually transformed into nucleoli that are typical for differentiated cells (Fléchon and Kopecny, 1998). Morphologically, the zygote NPBs are indistinguishable from oocyte NPBs. NPBs in oocytes and zygotes show a highly compacted dense fibrillar morphology, whereas somatic cells have fibrillo-granular reticulated nucleoli (Fléchon and Kopency, 1998; Derenzini et al., 1990; Olson et al., 2000). In mice, the fully grown oocytes do not synthesize ribosomal RNA (rRNA), but synthesis of the heterogeneous nuclear RNA continues until resumption of meiosis (Rodman and Bachvarova, 1976). At the end of the two-cell stage, the embryos become transcriptionally active and begin to synthesize rRNA (Schultz, 2002; Zatsepina et al., 2003). In parallel with this, NPBs are transformed into reticulated nucleoli. Here, we challenge this commonly accepted dogma and demonstrate that zygote NPBs are not necessary for embryonic development and that nucleoli in more advanced embryos do not originate from zygote NPBs.

RESULTS AND DISCUSSION

Enucleolation of zygotes

The scheme of our experiments is shown in supplementary material Fig. S2. Mouse oocytes contain a single NPB in the nucleus, called the germinal vesicle (GV), and zygotes usually contain multiple NPBs in each pronucleus at the early phase of pronuclear formation. During pronuclear development, these NPBs fuse and form a single NPB (supplementary material Fig. S3A and Movie 1). Most of the zygotes (85%; 233/275) thus contained a single NPB in each pronucleus at the late phase of pronuclear formation (supplementary material Fig. S3B). We microsurgically aspirated NPBs from both female and male pronuclei 10 h after intra-cytoplasmic sperm injection (ICSI) (Zygote-ENL group) (Fig. 1A; supplementary material Fig. S4A and Movie 2). This enucleolation is similar to the method reported for oocytes (supplementary material Fig. S4B and Movie 3) (Ogushi et al., 2008). Zygotes from which a small amount of nucleoplasm was aspirated and removed served as sham-operated controls (Zygote-Sham group).

We checked the success of enucleolation indirectly using immunocytochemistry (Fig. 1B). In the sham-operated zygotes, immunofluorescence labeling of B23 (nucleophosmin 1), as well as staining of the chromatin by DAPI (4′,6-diamidino-2-phenylindole) or Hoechst (bisbenzimide H33342), demonstrated a single NPB in both pronuclei (Fig. 1B, Zygote-Sham; supplementary material Fig. S4C, upper pronucleus). No NPBs were detected in enucleolated zygotes (Fig. 1B, Zygote-ENL; supplementary material Fig. S4C, lower pronucleus). After enucleolation, the pronuclear envelopes were sealed and the chromatin remained inside the pronuclei, as confirmed by labeling of lamin A/C (nuclear lamin A and C) and staining with DAPI (Fig. 1B, Zygote-ENL; supplementary material Fig. S4C).

Zygotes without NPBs develop to live-born pups

When NPBs were removed from oocytes, the enucleolated oocytes matured to MII (99%; 516/520) at a rate comparable to that of the

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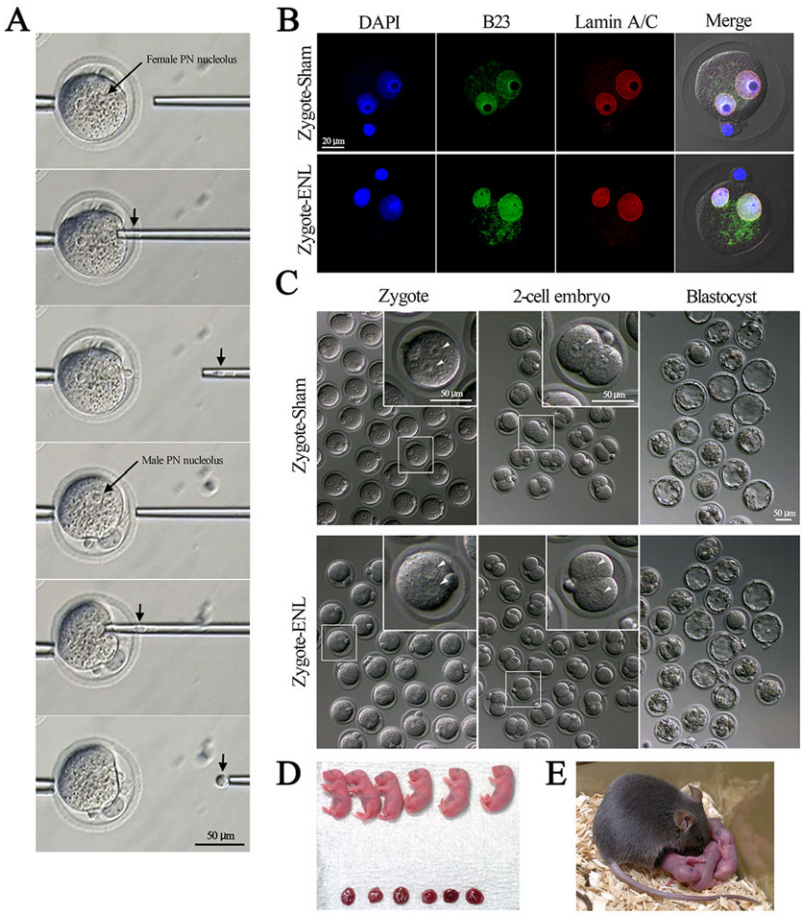

Fig. 1. Embryonic development and normal growth of offspring from enucleolated zygotes. (A) NPBs were aspirated from female and male pronuclei (PN) of zygotes. Arrows indicate NPBs. (B) Immunofluorescence labeling was performed with the indicated antibodies (green, B23; red, lamin A/C). DAPI staining marks chromatin in blue. The absence of NPBs is evident in the enucleolated zygotes. In total, ~21 zygotes were analyzed in each group. (C) Embryonic development of enucleolated zygotes (Zygote-ENL). These zygotes developed to blastocysts similarly to sham-operated zygotes (Zygote-Sham). No NPBs were observed in the nuclei of zygotes or two-cell embryos originating from enucleolated zygotes. Arrowheads indicate pronuclei and nuclei. Insets show higher magnifications of boxed areas in main panels. (D) Offspring (top) with normal placentae (bottom) originating from enucleolated zygotes. (E) An adult offspring from an enucleolated zygote with full fertility.

controls (sham-operated: 96%; 690/716). The enucleolated and subsequently matured oocytes did not develop to blastocysts after ICSI (supplementary material Table S1). Moreover, after the transfer of two-cell-stage embryos into recipients' oviducts, they never developed to live-born pups (Table 1). These results confirm those of a previous study showing that the oocyte NPB is essential for early embryonic development after fertilization (Ogushi et al., 2008). Here, when NPBs were removed from late zygotes, no NPBs were detected in the nuclei of enucleolated zygotes or two-cell embryos (Fig. 1C, Zygote-ENL); however, surprisingly, these zygotes cleaved and developed to blastocysts (Fig. 1C; supplementary material Table S1).

We used immunostaining to characterize the blastocysts that originated from enucleolated zygotes. In mouse blastocysts, the transcription factor OCT4 (POU5F1 – Mouse Genome Informatics) is restricted to the inner cell mass (ICM), and the trophectoderm (TE) is marked by CDX2 (Niwa et al., 2005). The numbers of ICM and TE cells in the blastocysts originating from enucleolated zygotes were similar to those in the blastocysts originating from sham-operated zygotes (supplementary material Fig. S5). After the transfer of two-cell-stage embryos originating from enucleolated zygotes, live-born pups were obtained (32%) (Fig. 1D; Table 1). All these offspring grew into adults with full fertility (Fig. 1E). These observations clearly suggest that advanced zygote NPBs are not essential for embryonic development.

It was shown that zygote NPBs are exclusively of maternal origin and are essential for normal embryonic development (Ogushi et al., 2008). However, contrary to that report, our present findings suggest that zygote NPBs are not essential for embryonic development. This paradox is difficult to explain. A previous study (Ogushi and Saitou, 2010) examined the effect of timing of NPB re-injection into enucleolated mouse oocytes and into early- and late-stage zygotes upon further development. When the NPBs were injected into MII oocytes (originating from GV-enucleolated oocytes) and these oocytes were then fertilized, good embryonic development up to the

Table 1. Full-term development of enucleolated oocytes and zygotes

Group	Total number of oocytes and zygotes examined	Number (%) of oocytes and zygotes forming two-cell embryos	Number of transferred embryos (number of recipients)	Number (%) of live offspring	Average body weight of offspring (g) ±s.e.m.	Average weight of placenta (g) ±s.e.m.
Oocyte-sham	109	93 (85)[a]	91 (6)	23 (25)	1.50±0.03	0.14±0.006
Oocyte-enucleolation	142	104 (74)[b]	104 (8)	0 (0)	–	–
Zygote-sham	103	91 (88)	91 (7)	45 (49)[A]	1.35±0.02	0.12±0.0003
Zygote-enucleolation	103	91 (88)	74 (7)	24 (32)[B]	1.44±0.03	0.13±0.0004

The frequencies of embryos and offspring were analyzed using the chi-square test. (a,b and A,B) Values with different superscripts in the same column differ significantly (P<0.05).

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blastocyst stage was reported. By contrast, after the injection of NPBs into zygotes (originating from GV-enucleolated oocytes) at the early (8 h after insemination) and late (15 h after insemination) pronuclear formation stages, blastocyst development and full-term development were severely compromised. Combined with the present results, these findings indicate that the oocyte NPB material is only essential at the very early stage of pronuclear formation.

NPBs in fully grown maturation-competent mouse oocytes as well as in zygotes are enclosed with a ring of heterochromatin. Oocytes without this close association can be fertilized, and they form pronuclei but typically reach only the two-cell stage (Inoue et al., 2008). This indicates the importance of an NPB-chromatin association, but it is not clear why this close contact is so important. After fertilization, extensive chromatin reprogramming occurs in both pronuclei. Pericentric heterochromatin reorganizes and forms a ring(s) around NPB(s) (Probst and Almouzni, 2011; Fulka and Langerova, 2014). Pericentric satellites are methylated but there are distinct differences between both pronuclei (maternal versus paternal). NPBs seem to be thus involved in regulation of correct satellite transcription and pericentric chromatin organization (Probst and Almouzni, 2011). We checked the localization of the chromosome centric regions (kinetochores) in zygotes without NPBs during pronuclear formation using live-cell imaging after injection of *EGFP-CENP-C* mRNA (supplementary material Fig. S6 and Movie 4). In the embryos originating from sham-operated oocytes (GV-Sham), EGFP-CENP-C signals were localized around the NPBs. However, in the embryos originating from enucleolated oocytes (GV-ENL), EGFP-CENP-C signals were spread throughout the nucleoplasm. These results indicate that NPBs seem to be scaffolding for the chromosome centric region, although the function was not elucidated.

De novo nucleolus formation in embryos originating from enucleolated zygotes

We next used live-cell imaging to examine NPB formation in developing embryos originating from enucleolated zygotes after an injection with *EGFP-NPM2* (nucleoplasmin 2) mRNA (Fig. 2; supplementary material Fig. S7 and Movie 5). NPM2 is an oocyte-specific nuclear protein and a component of oocyte and zygote NPBs (Burns et al., 2003; Inoue and Aoki, 2010). In the embryos originating from sham-operated zygotes, we detected robust EGFP-NPM2 signals in NPBs throughout early embryonic development, and the signal intensity was greater in the NPBs than in the nucleoplasm until the morula stage (supplementary material Fig. S7 and Movie 5). After the four-cell stage, the sham-operated zygotes formed nucleoli around the NPB material. The nucleoli showed weak EGFP-NPM2 signals (Fig. 2). The embryos originating from enucleolated zygotes did not show such a robust EGFP-NPM2 signal, although a weak signal was observed throughout the nucleoplasm (supplementary material Fig. S7 and Movie 5). Interestingly, at the four-cell stage, these embryos also formed nucleoli, although they showed weak and homogeneous EGFP-NPM2 signals throughout the nucleoli (Fig. 2B).

We then checked nucleolus formation by immunostaining. Embryos at each stage (two-, four- and eight-cell embryos, morulae, and blastocysts) and somatic cells (NIH3T3) were immunostained against typical nucleolus markers [B23 and upstream binding factor (UBF; UBTF – Mouse Genome Informatics)] (Fig. 3; supplementary material Fig. S8). The nucleoli in the somatic cells were consistently stained with these antibodies, whereas the compact zygote NPBs were not stained. In the sham-operated zygotes, B23 was localized in the nucleoplasm (Fig. 3), and UBF was essentially undetectable (supplementary material Fig. S8). After the two-cell stage, B23 and UBF gradually accumulated around the compact NPBs, and then in the blastocysts the compact NPBs material disappeared completely. The distributions of B23 and UBF became essentially the same as those in the reticulated nucleoli (Fig. 3; supplementary material Fig. S8, Zygote-Sham). The embryos originating from enucleolated zygotes had no visible NPBs at the two-cell stage. They formed nucleoli, similar to the reticulated nucleoli of somatic cells, directly at the four-cell stage (Fig. 3; supplementary material Fig. S8, Zygote-ENL). The nucleoli were B23 and UBF positive, although they did not include compact NPB material. These findings suggest that zygotes without NPBs are able to form nucleoli *de novo*. Our data thus clearly indicate that NPBs of zygotic origin are not necessary for embryonic development, but their material rather passively persists in early embryo blastomeres.

In nonmanipulated mouse embryos, proteins required for nucleolar function, such as RPA116 (POLR1B – Mouse Genome Informatics), UBF, fibrillarin and B23, begin to assemble at the periphery of NPBs before rDNA transcription resumes (Zatsepina et al., 2003). Reticulated nucleoli are gradually formed on the periphery of NPBs and this is accompanied by active rDNA transcription, whereas the NPB cores themselves remain intact as solid spherical bodies (Fléchon and Kopecny, 1998; Maddox-Hyttel et al., 2005). Finally, at the late morula or blastocyst stage, the mature nucleoli can be detected (Geuskens and Alexandre, 1984). Based on these observations, it is commonly accepted that the nucleolar material originating from zygote NPBs is required for the gradual

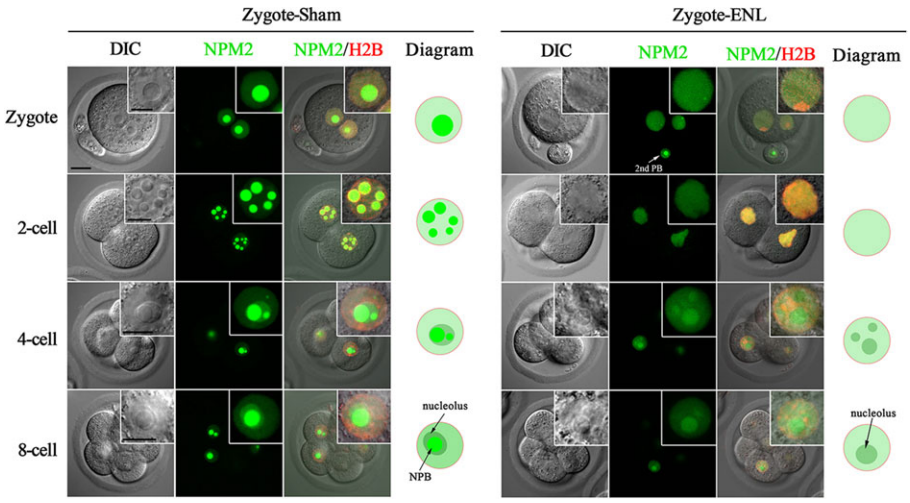

Fig. 2. NPB and nucleolus formation in embryos originating from enucleolated zygotes. Confocal imaging was performed after an injection with *EGFP-NPM2* (green) and *H2B-mCherry* (red) mRNA into the oocytes at MII. In sham-operated zygotes (Zygote-Sham), NPBs showing a robust EGFP signal were observed throughout early embryonic development. In four-cell and eight-cell embryos, nucleoli showing a weak EGFP signal were formed around the NPBs. In the enucleolated zygotes (Zygote-ENL), no robust signal was observed except for the second polar body (2nd PB, indicated by arrow); however, weak signals (reticulated nucleoli) were observed in the embryo after the four-cell stage. Diagrams show each stage of nuclei. Insets show higher magnifications of main panels. In total, at least ten zygotes and embryos were analyzed in each group. Scale bars: 20 µm (main panels); 10 µm (insets).

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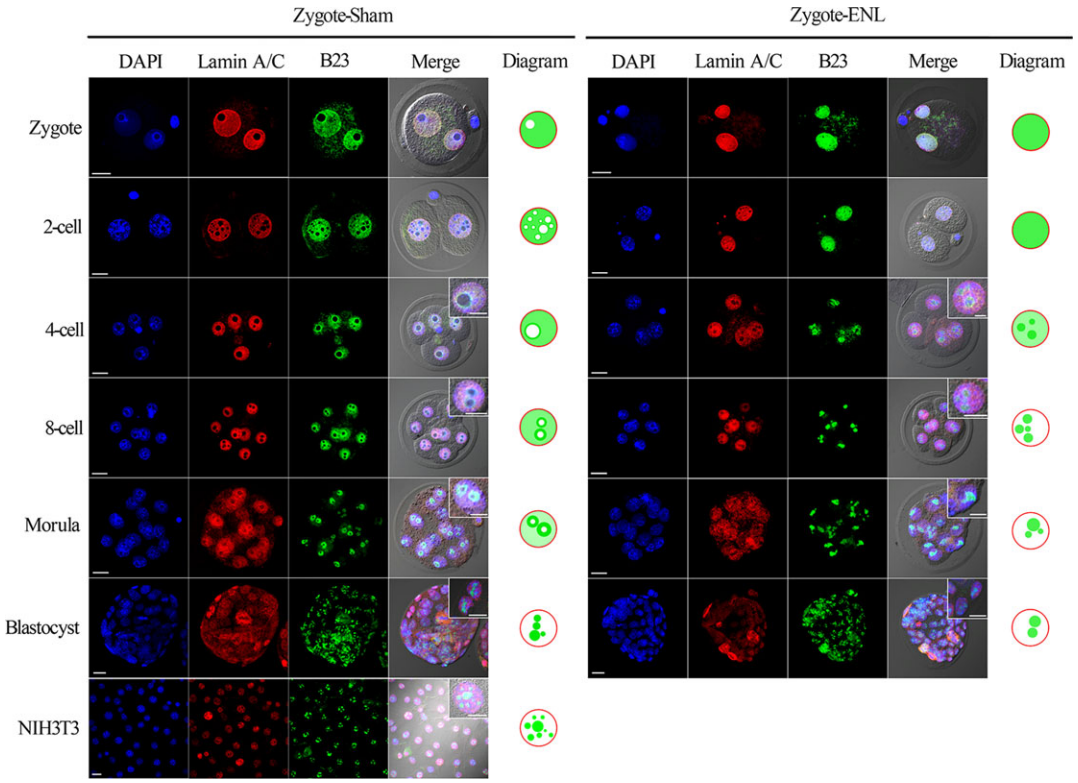

Fig. 3. Nucleolus formation in embryos originating from enucleolated zygotes. Immunofluorescence labeling was performed with the indicated antibodies (green, B23; red, lamin A/C). DAPI staining marks chromatin in blue. In zygotes and two-cell embryos, the absence of NPBs is evident after enucleolation (Zygote-ENL). Both types of embryos formed reticulated nucleoli during embryonic development. Somatic cells (NIH3T3 cells) were also stained in the same manner. Diagrams show each stage of nuclei. Insets show higher magnifications of main panels. In total, at least 20 zygotes and embryos were analyzed in each group. Scale bars: 20 µm (main panels); 10 µm (insets).

assembly of fully functional nucleoli at a later stage of embryonic development (Tesarík et al., 1987). However, we have shown here that reticulated nucleoli develop even without zygote NPBs in early embryos.

Conclusion

We have demonstrated that zygote NPBs are not essential for embryonic development and are not required for the formation of nucleoli in developing embryos. These results run counter to the common view that nucleoli in developing embryos originate from zygote NPBs, and indicate that embryos are able to form fully differentiated nucleoli *de novo*.

MATERIALS AND METHODS

Animals

B6D2F1 (C57BL/6×DBA/2) mice, aged 8-10 weeks, were used to produce oocytes. Surrogate pseudopregnant females used as embryo transfer recipients (see below) were ICR strain mice mated with vasectomized males of the same strain. B6D2F1 and ICR mice were purchased from Japan SLC.

Oocyte enucleolation

Enucleolation was carried out as described previously (Ogushi et al., 2008). Briefly, the oocytes at the GV stage were manipulated with a micromanipulator equipped with a PIEZO drive (Prime Tech) in HEPES-buffered Chatot, Ziomet and Bavister medium (H-CZB) (Chatot et al., 1990) supplemented with 7.5 µg/ml cytochalasin B (Sigma-Aldrich). Oocytes from which a small volume of nucleoplasm was aspirated and removed served as controls (sham-operated oocytes).

The oocytes matured up to MII over 16 h in culture, and then they were selected and subjected to ICSI with sperm heads, as described by Kimura and Yanagimachi (Kimura and Yanagimachi, 1995) and cultured in KSOM (Millipore).

Zygote enucleolation

Cumulus-oocyte complexes (COCs) were collected from superovulated oviducts. COCs were placed in H-CZB and treated with 0.1% bovine testicular hyaluronidase (Sigma-Aldrich). The cumulus-free oocytes were then transferred to KSOM. After ICSI, the oocytes were cultured for 8-10 h and then enucleolated.

Briefly, zygotes were transferred into H-CZB containing 7.5 µg/ml cytochalasin B for 10 min. Then, NPBs were aspirated from both pronuclei in a micromanipulation chamber, which was put on a warmed stage (37°C) in an inverted microscope (Olympus). After enucleolation, the embryos were cultured in KSOM. The cleavage rate and morula/blastocyst formation rate were examined at 24 h and 3.5 days after ICSI, respectively.

Embryo transfer

Embryos at the two-cell stage were transferred at 0.5 days post coitum into oviducts of pseudopregnant ICR female mice that had been mated with vasectomized males. At 18.5 days post coitum, the offspring were delivered by Caesarean section. Surviving pups were fostered by an ICR foster mother.

Fluorescence microscopy

For immunofluorescence labeling, two-cell, four-/eight-cell, and morula/blastocyst embryos were collected at 24 h, 2.5 days and 3.5 days after ICSI, respectively. Embryos were fixed and permeabilized in 4% paraformaldehyde in PBS-polyvinyl alcohol (PVA) (pH 7.4) containing 0.2% Triton X-100 for 30 min. After blocking in PBS containing 1 mg/ml bovine serum albumin (PBS-BSA), the embryos were incubated with the appropriate primary antibodies at 4°C overnight, washed several times in PBS-BSA and incubated with secondary antibodies for 60 min

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at room temperature. The embryos were mounted on glass slides with ProLong Gold Antifade Reagent with DAPI (Molecular Probes) and evaluated using a confocal laser scanning microscope (FV1000-KDM; Olympus).

For somatic cell labeling, NIH3T3 cells were cultured in CultureSlides (BD Falcon). Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min. After being blocked in 3% BSA in PBS for 30 min, the cells were incubated with primary antibodies for 1 h. After several washes, the cells were incubated with secondary antibodies for 1 h. The cells were mounted with ProLong Gold Antifade Reagent with DAPI and observed under a confocal laser scanning microscope.

The following primary antibodies were used: goat polyclonal anti-B23 antibody (1:200, sc-6013, Santa Cruz Biotechnology); rabbit polyclonal anti-lamin A/C antibody (1:200, sc-20681, Santa Cruz Biotechnology); mouse monoclonal anti-UBF antibody (1:200, sc-13125, Santa Cruz Biotechnology); rabbit polyclonal anti-Oct3/4 antibody (1:200, sc-9081, Santa Cruz Biotechnology); and mouse monoclonal anti-Cdx2 antibody (1:200, AM392, BioGenex). The secondary antibodies were Alexa Fluor 488-labeled chicken anti-goat IgG, Alexa Fluor 568-labeled donkey anti-rabbit IgG, Alexa Fluor 568-labeled rabbit anti-mouse IgG, Alexa Fluor 488-labeled goat anti-mouse IgG and Alexa Fluor 568-labeled goat anti-rabbit IgG (1:400, Molecular Probes).

Live-cell imaging

mRNAs encoding enhanced green fluorescent protein (EGFP) coupled with NPM2 (EGFP-NPM2) and monomeric cherry (mCherry) fused with histone H2B (H2B-mCherry) plasmid were kindly provided by S. Ogushi (Kyoto University, Japan) and EGFP-CENP-C plasmid was kindly provided by T. S. Kitajima (RIKEN Center for Developmental Biology, Japan).

mRNA injection into oocytes was performed as described previously (Yamagata, 2010). Briefly, each mRNA was diluted to 50 ng/µl and a few picoliters of solution was injected into the MII oocyte cytoplasm using a micromanipulator equipped with a PIEZO drive with a glass micropipette (1-3 µm in diameter). After mRNA injection, the oocytes were manipulated (ICSI and enucleolation) as described above.

After enucleolation, the embryos were transferred to drops of CZB medium on a glass-bottomed dish, placed in an incubator on the microscope stage and incubated at 37°C under 5% CO_2 in air. For NPM2 and CENP-C imaging, the devices for imaging were as previously described by Yamagata et al. (Yamagata et al., 2009) and Kitajima et al. (Kitajima et al., 2011), respectively.

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Competing interests

The authors declare no competing financial interests.

Author contributions

H.K. designed and performed the experiments. All authors discussed the results and commented on the manuscript.

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Supplementary material

Supplementary material available online at
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