

Nucleolus Precursor Body (NPB): A Distinct Structure in Mammalian Oocytes and Zygotes

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Nucleoli in mammalian oocytes and zygotes, sometimes referred to as nucleolus precursor bodies (NPBs), are compact and morphologically different from nucleoli in somatic cells. We applied a unique NPB analyzing method “enucleolation” technique to zygotes to remove the NPBs. It has been reported that oocyte NPBs are essential for embryonic development; in their absence, the oocytes complete maturation and can be fertilized, but no nucleoli are formed in the zygotes and embryos, leading to developmental failure. However, we found that when NPBs were removed from zygotes, the zygotes developed successfully to live-born pups. These results indicated that oocyte NPBs are essential for embryonic development, but zygote NPBs are not. In addition, the enucleolated zygotes formed somatic-type nucleoli during early embryonic development, demonstrating that somatic-type nucleoli do not originate from zygote NPBs. We summarize our recent investigation on NPBs, and provide additional comments and findings.

Keywords: embryo, mouse, NPB, nucleolus, oocyte

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Introduction

The oocyte nucleolus was first reported in 1835 in the jellyfish,¹ and in mammals it was first documented in the rabbit in 1842.² In 1964, Brown and Gurdon showed that anucleolate (no nucleoli) mutant *Xenopus* embryos exhibited arrested development due to their inability to synthesize new ribosomes, suggesting that the nucleolus is the site of rRNA (rRNA) synthesis and nascent ribosome assembly.³ Since then, the

nucleoli in somatic cells have been considered as the site of rRNA synthesis and ribosomal subunit assembly.⁴ Apart from those nucleolus studies, extensive studies on in vitro maturation and fertilization of mammalian oocytes have greatly advanced our fundamental knowledge of mammalian reproduction since the mid-20th century.⁵ Thus it is well established that mammalian oocytes grow in the ovary, mature after periodic gonadotropic stimulation from the pituitary, and are ovulated into the oviduct and fertilized by the spermatozoon. During the growth phase, the oocyte nucleolus is engaged in rRNA synthesis and ribosome assembly much like the nucleolus in somatic cells; however, in oocytes the nucleolus changes its morphology and decreases its rRNA synthetic activity as the cells approach full-size.⁶ Finally, in the fully-grown oocytes, a single large nucleolus, which does not contain DNA, is formed in the nucleus (germinal vesicle: GV).⁷ This nucleolus is transcriptionally inactive and structurally distinct from the nucleoli in somatic cells, and has been termed a nucleolus precursor body (NPB).⁸ A single large and morphologically distinguishable nucleolus is seen in oocytes from various species, such as starfish^{9,10} and sea urchins,¹¹ although it is not clear whether the nucleoli in invertebrate oocytes is similar in character to mammalian NPBs. In contrast, the *Xenopus* oocyte contains as many as 1,500 nucleoli.¹² And in spite of these advances in understanding, the actual reason that the NPB evolved in mammals is unknown and remains a topic of great interest.

During the maturation of mammalian oocytes, the NPBs disappear, and upon

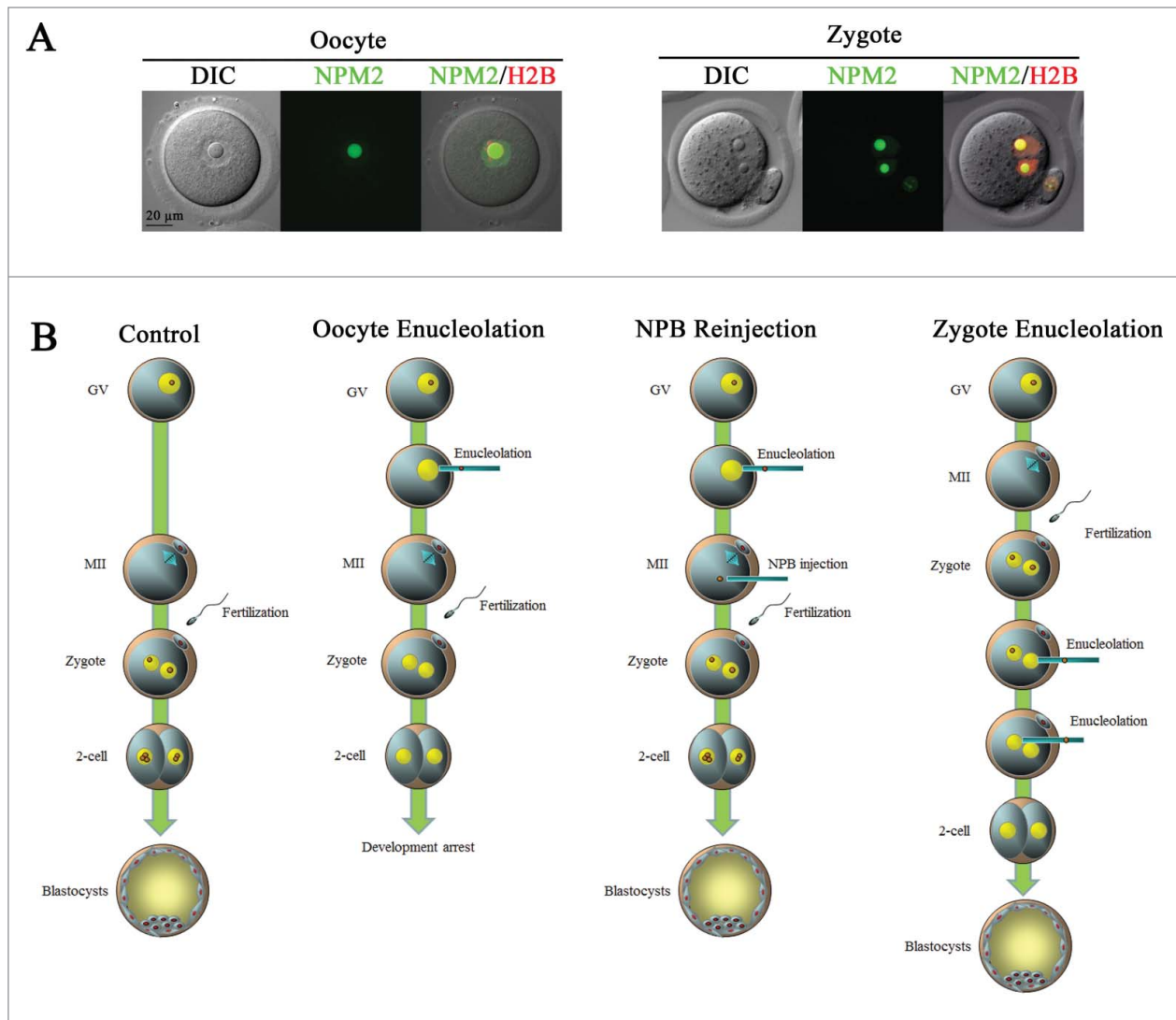


Figure 1. (A). The morphology of oocyte nucleolus precursor bodies (NPBs) is similar to that of zygote NPBs. Confocal imaging was performed after an injection with *NPM2-EGFP* (green) and *H2B-mCherry* (red) mRNAs. **(B).** Experimental summary of oocyte and zygote enucleation. NPBs were microsurgically aspirated from germinal vesicles (GVs) to produce enucleated oocytes (Oocyte Enucleation). Oocyte NPBs were re-injected into previously enucleated oocytes at MII (NPB Re-injection). The NPBs of zygotes were aspirated from both pronuclei (Zygote Enucleation).

fertilization the NPBs are formed again in male and female pronuclei of zygotes (Fig. 1A). The zygote NPBs are transcriptionally inactive and morphologically similar to the oocyte NPBs.⁸ Our recent studies showed that enucleated mammalian oocytes, whose NPBs had been removed micro-surgically at the GV-stage, were able to mature to metaphase II (MII) and to be fertilized.¹³⁻¹⁵ However, the enucleated oocytes neither formed NPBs in zygotes nor developed to blastocysts after fertilization (Fig. 1B). When oocyte NPBs were re-injected into previously enucleated oocytes at MII

and these oocytes were fertilized, they formed pronuclei with NPBs and developed to full term.¹³ Thus, zygotes inherit their NPBs from oocytes, and oocyte NPBs are essential for embryonic development. We have also shown that zygote NPBs are not required for embryonic development. When zygote NPBs were removed, the enucleated zygotes formed new nucleoli after several divisions and developed to live pups. Here, we summarize these studies on oocyte and zygote NPBs, and briefly discuss the role of NPBs in early embryonic development.

The Dynamics of NPBs

During oogenesis in mammals, oocytes become arrested at the diplotene stage of the first meiotic prophase and begin growing. Dynamic changes in nucleolar morphology during oogenesis and embryogenesis in mammals have been observed by electron microscopy from the 1960s.¹⁶ The reticulated nucleoli of non-growing and growing oocytes are composed of fibrillar centers, dense fibrillar components, and granular components.¹⁷⁻¹⁹ The oocyte nucleolus is the site of active rRNA synthesis and ribosome

production.²⁰ The nucleoli contain chromatin, although the chromatin is moved out of the nucleoli during oocyte growth, and finally in fully grown oocytes the nucleoli are transformed into a single compacted NPB, which no longer contains DNA, and has no rRNA synthetic activity (Fig. 2).^{7,18} After the GV breakdown of oocytes, NPB materials diffuse into the oocyte cytoplasm, and upon fertilization, NPBs are formed again in male and female pronuclei of zygotes.⁸ The compact NPBs remain as the core in the process of nucleolus formation during the development of embryos,⁸ and reticulated nucleoli are gradually formed around the NPBs. Then, the nucleoli become transcriptionally active (at the end of the 2-cell stage in mouse embryos) to synthesize rRNA, and at the morula stage the somatic-type nucleoli are formed and the original NPBs disappear.²² Although the dynamics of NPBs during oocyte maturation and early embryonic development has been well characterized, their function has not been elucidated.

The Enucleation Technique

About 10 years ago, a new method for analyzing the NPB was introduced by Fulka and his colleagues.²³ They showed that oocyte NPBs can be microsurgically removed (enucleation) in a process akin to enucleation in animal cloning.²⁴ In our recent experiments, we applied this enucleation method to zygotes, from which we were thereby able to remove the NPBs.¹⁴

The enucleation method is technically impressive, but its effectiveness is sometimes questioned by other researchers. That is because the perinucleolar chromatin rings are tightly associated with NPBs in oocytes and zygotes, as shown by the staining of DNA and epigenetic markers such as HP1 β and H3K9me.^{15,25,26} Thus a question arises as to how NPBs could be selectively removed without any damage to the DNA. A previous report established that there is no DNA damage during enucleation.¹³ This report showed that when oocyte NPBs were re-injected into previously enucleated oocytes at MII, the oocytes were

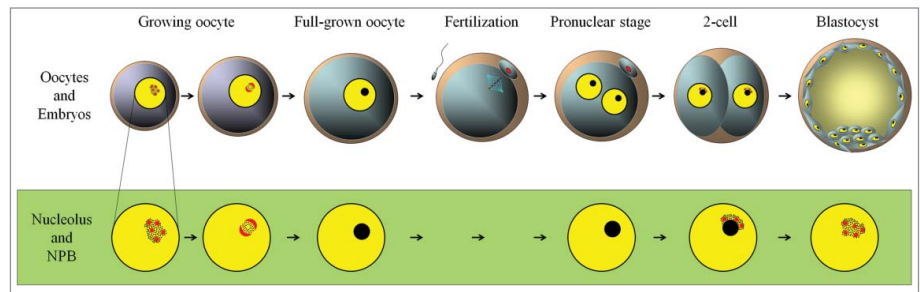


Figure 2. The nucleoli of growing oocytes in mammals are composed of fibrillar centers (red), dense fibrillar components, and granular components (black dots). At the end of the growth phase, nucleoli are inactivated to become NPBs (black). During oocyte maturation, the nucleoli (NPBs) disappear, and upon fertilization, NPBs (black) are formed in pronuclei. At the end of the 2-cell stage, fibrillar centers and fibrillar components (red), and granular components (dots) develop on the surface of NPBs. This figure is based on **Figure 2** from the study by Hyttel (2011; reference (21)) with some modifications.

fertilized and developed to full term. Moreover, the chromosome spreads of enucleolated oocytes showed undamaged chromosomes.²⁷

The procedure used to enucleated oocytes without sucking out the DNA is outlined in **Figure 3**. First, the NPBs of oocytes or zygotes were aspirated from outside of the nuclear envelopes. Oocytes and zygotes were treated with an inhibitor of actin polymerization, cytochalasin B, to soften the cell membrane. Using a square-ended injection pipette, the zona pellucida was punctured by a PIEZO pulse, and then the tip of the pipette was pushed into the cytoplasm to reach the nuclear envelope near the NPB, while leaving the cell membrane intact. Thereafter, gentle suction was applied through the cell membrane and the nuclear envelope. As a result of this suction, the NPB penetrated the nuclear envelope and moved into the mouth of the pipette with the surrounding cell membrane. In this process, the nuclear envelope seemed to work as a filter, such that all the chromatin remained inside the nuclear envelope. In other words, the NPB was able to pass through the nuclear envelope without breaking it, perhaps due to the NPB behaving as a liquid-like droplet.¹² Moreover, when NPBs were injected into the cytoplasm of enucleated GV oocytes, the injected NPBs were disassembled in the oocyte cytoplasm and gradually reassembled in the GV.²⁸ The NPB materials dispersed in the cytoplasm by the NPB disassembly may pass through the nuclear envelope into the

nucleoplasm. However, the details of how NPBs pass through the nuclear membrane remain to be determined.

De novo Formation of Nucleoli in Developing Embryos

In our recent report, we applied this enucleation method to zygotes and yielded interesting results. After removal of NPBs from zygotes, the embryos originating from enucleated zygotes formed new somatic-type nucleoli after several divisions. This result contradicts a classical dogma in developmental biology (embryology), since it is commonly accepted that nucleoli in developing embryos originate from zygote NPBs that are then, as the embryos develop, gradually transformed into fully differentiated nucleoli.²⁹

In mouse embryos, proteins required for nucleolar function, such as upstream binding factor (UBF), fibrillarin and B23, begin to assemble at the periphery of NPBs before rDNA transcription resumes.³⁰ During embryonic development, the differentiation of reticulated nucleoli is strictly limited to the periphery of NPBs, while the core of NPBs remains compact and detectable in the following cleavage stages up to the morula.³¹ At the blastocyst stage, reticulated somatic-type nucleoli are formed in every nucleus (Fig. 2). Thus, the nucleolar material originating from zygote NPBs has been thought to be required for the assembly of fully functional somatic-type nucleoli at a

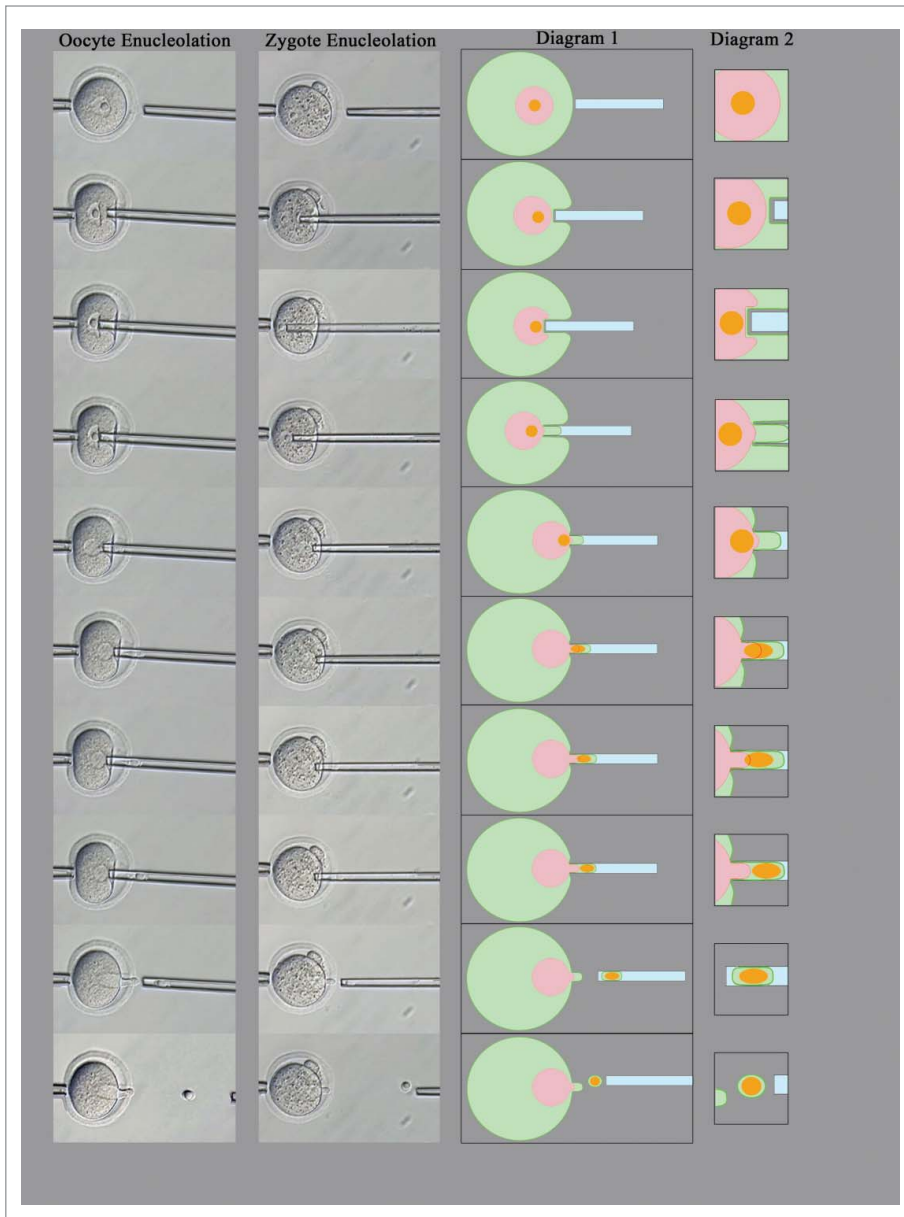


Figure 3. Enucleation of oocytes and zygotes. An injection pipette is used to penetrate the zona pellucida, and its tip is pushed against the nuclear envelope. Due to the mild suction from the outside cell membrane and nuclear envelope, the NPB is preferentially aspirated into the injection pipette through the nuclear envelope. The NPB can then be detached from the zygote. Diagrams show the cytoplasm (green), nucleoplasm (red), and NPBs (orange).

later stage of embryonic development.²² However, our study showed that reticulated nucleoli develop in enucleolated zygotes; we observed nucleolus formation in enucleolated zygotes during early embryonic development by immunostaining against nucleolus markers for somatic cells (B23 and UBF). The embryos derived from enucleolated zygotes had no visible nucleoli at the 2-cell stage, but

directly formed somatic-type nucleoli at the 4-cell stage. The nucleoli were B23- and UBF-positive and did not have compact cores like those observed in embryos from intact zygotes. It has not been determined whether nucleoli in the embryos derived from enucleolated zygotes are fully functional in rRNA synthesis; however, at least the embryos developed to live-born pups at a rate similar to sham-operated

zygotes. These results indicate that reticulated somatic-type nucleoli develop even without NPBs in early embryos.

De novo nucleolus formation in embryos is also observed in NPM2 (nucleoplasm 2)-knockout mice (unpublished data). NPM2 is an oocyte-specific nuclear protein and a component of oocyte and zygote NPBs in mouse oocytes and zygotes.^{32,33} NPM2^{-/-} mouse oocytes and embryos never form NPBs in the nuclei. However, some embryos develop beyond the 2-cell stage, and a few develop to blastocysts after fertilization.³³ We examined nucleolus formation in developing NPM2^{-/-} mouse embryos after an injection with *B23-EGFP* mRNA. B23 is one of the proteins in reticulated somatic-type nucleoli in mouse embryos.²⁷ In NPM2^{+/+} embryos, at the end of the 2-cell stage, robust B23-EGFP signals were detected around the NPBs (Fig. 4), and then reticulated somatic-cell type nucleoli were gradually formed as the embryos developed (unpublished data). The NPM2^{-/-} embryos had no visible NPBs throughout embryonic development to blastocysts. However, robust EGFP signals were observed from the 2-cell stage and somatic-cell type nucleoli were formed directly at the end of the 2-cell stage (Fig. 4). These findings also indicate that zygotes without NPBs are able to form somatic-type nucleoli, and that zygote NPBs do not contribute to the nucleoli of somatic cells.

What is The Role of NPB?

When NPBs were removed from oocytes, the enucleolated oocytes matured to MII, but the enucleolated and matured oocytes did not develop to blastocysts after fertilization.^{13,14} Moreover, when 2-cell embryos derived from enucleolated oocytes were transferred into recipients' oviducts, they never developed to live-born pups. These results show that oocyte NPBs are essential for embryonic development after fertilization (Fig. 1B). However, when NPBs were removed from zygotes, the zygotes cleaved and developed to blastocysts.¹⁴ After the transfer of 2-cell embryos derived from enucleolated zygotes, live-born pups were obtained. All

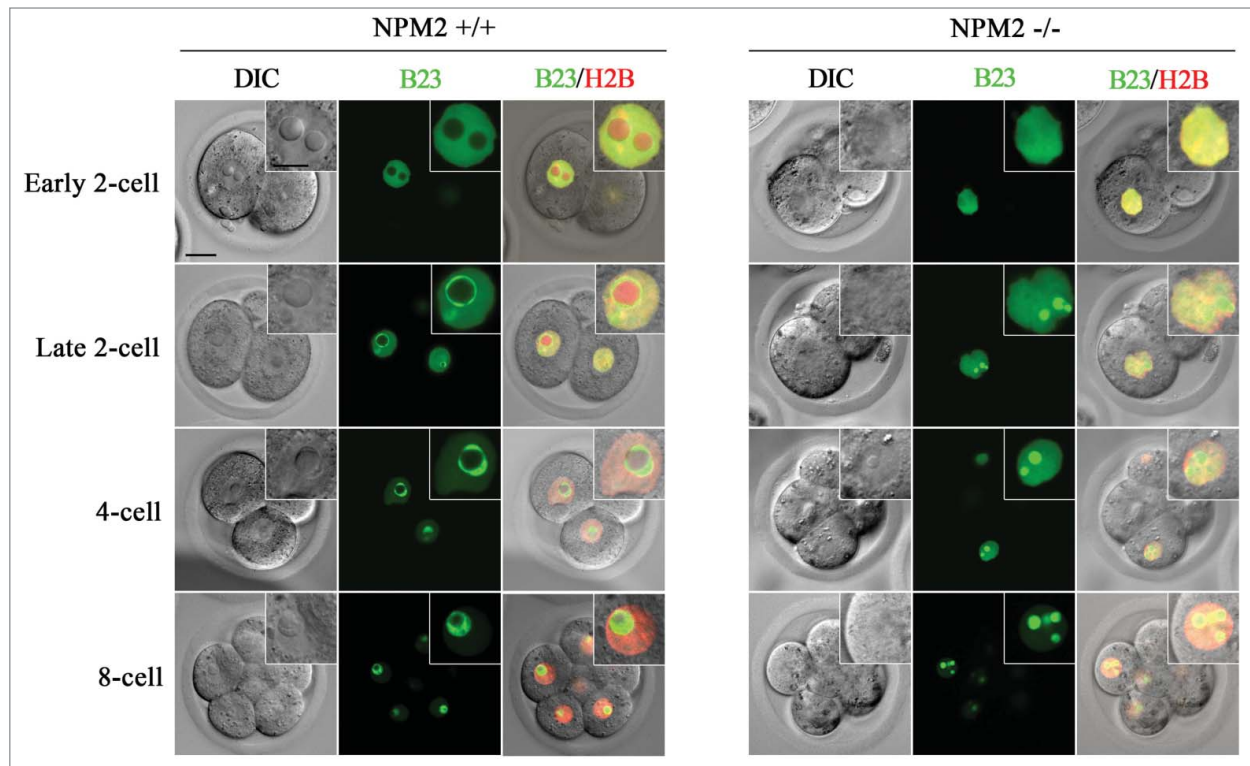


Figure 4. Nucleolus formation in the embryos originating from $NPM2^{-/-}$ zygotes. Confocal imaging was performed after an injection with *B23-EGFP* (green) and *H2B-mCherry* (red) mRNAs into the oocytes at MII. There is no NPB formation in $NPM2^{-/-}$ embryos after fertilization. At the late 2-cell stage, both types of embryos formed nucleoli that showed robust EGFP signals. Bars in the figures and insets indicate 20 μm and 10 μm , respectively.

of these offspring grew into adults with full fertility. These findings suggest that oocyte NPBs are essential for embryonic development,¹³ whereas zygote NPBs are not.¹⁴ This paradox could be explained by the difference of components between oocyte and zygote NPBs. However, when NPBs from 2-cell stage embryos were transferred into enucleolated oocytes at the MII stage, the resulting oocytes restored the developmental ability to blastocysts.³⁴ This result implies that NPBs from 2-cell stage embryos support the embryonic development instead of oocyte NPBs, and NPB materials function between fertilization and the first cleavage of embryos.

To determine the stage at which the NPB materials are required, a previous study investigated the timing of NPB re-injection into MII oocytes or zygotes originating from enucleolated oocytes.¹⁵ When the NPBs were re-injected into zygotes at the pronuclear stage, both blastocyst development and full-term development were severely retarded compared to those when the NPBs were re-injected

into oocytes at the MII stage. This finding again indicates that NPB materials are essential only between MII and the pronuclear stage of zygotes. During this step, male and female chromosomes are dynamically changed and NPBs are associated with heterochromatin. The NPBs (or NPB materials) may be important for decondensation and modification (reprogramming) of chromatin. In cloned embryos produced by somatic cell nuclear transfer, multiple NPB formation in pseudo-pronuclei is one of the apparent abnormalities.²⁴ This abnormality indicates that NPBs may have a functional role in chromosome decondensation or genome reprogramming.

NPM2 has been suggested to be the factor responsible for sperm chromatin decondensation (SCD).³⁵ However, male pronucleus formation following SCD has been shown to occur in enucleolated oocytes,^{13,35} as well as in $Npm2^{-/-}$ oocytes³⁵ after fertilization. These results suggest that other factors are also involved in SCD, and that SCD is not the main function of NPBs. A recent report showed

that NPBs were important for centromere satellite maintenance during pronucleus organization.²⁷ This study reported that the embryos derived from enucleolated oocytes showed abnormal chromatin remodeling, replication and expression of centric and pericentric satellite DNA accompanied by developmental arrest. The role of nucleoli in cell cycle regulation has been documented in yeasts³⁶⁻³⁸ and somatic cells.³⁹ In surf clam oocytes, the nucleolus, an RNA-rich structure inside the nucleolus, was associated with spindle formation and cell division.^{40,41} However, the critical role of NPBs in mammals remains to be determined.

Conclusion

Zygote NPBs are required neither for formation of somatic-type nucleoli during embryonic development nor for the full-term development of embryos, but oocyte NPB materials appear to be required at the early step of pronucleus organization in zygotes. The NPBs may have specific

functions which are completely different from those of somatic nucleoli. Elucidating the function of various molecules in NPBs and the role of NPBs in embryonic development remain future challenges.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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