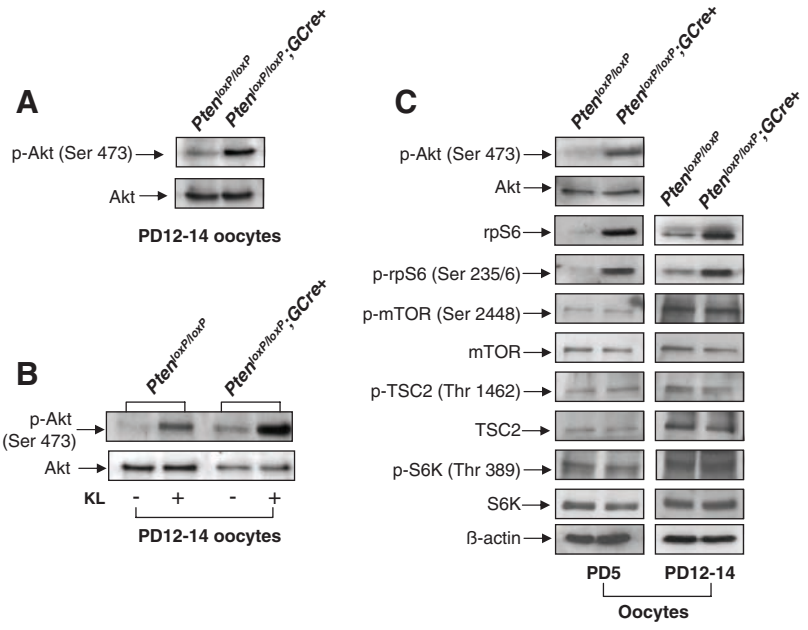

Fig. 4. Enhanced Akt signaling in *Pten*$^{loxP/loxP}$;*GCre*$^{+}$ oocytes leads to elevated expression and activation of rpS6. Oocytes were isolated from ovaries of *Pten*$^{loxP/loxP}$;*GCre*$^{+}$ and *Pten*$^{loxP/loxP}$ mice at PD5 and PD12 to 14, and Western blots were performed (*10*). (**A**) Levels of p-Akt (Ser^{473}) and total Akt in PD12 to 14 *Pten*$^{loxP/loxP}$;*GCre*$^{+}$ and *Pten*$^{loxP/loxP}$ oocytes. (**B**) Activation of Akt (p-Akt, Ser^{473}) by KL treatment (100 ng/ml, 2 min) in PD12 to 14 *Pten*$^{loxP/loxP}$;*GCre*$^{+}$ and *Pten*$^{loxP/loxP}$ oocytes. Levels of Akt were used as internal controls. (**C**) Signaling studies in *Pten*$^{loxP/loxP}$;*GCre*$^{+}$ and *Pten*$^{loxP/loxP}$ oocytes at PD5 and PD12 to 14, showing levels of p-Akt (Ser^{473}), rpS6, p-rpS6 (Ser$^{235/6}$), p-mTOR (Ser^{2448}), p-TSC2 (Thr^{1462}), and p-S6K (Thr^{389}). Levels of total Akt, mTOR, TSC2, S6K, and β-actin were used as internal controls. All experiments were repeated at least three times. For isolation of PD5 oocytes for Western blot, 10 to 15 *Pten*$^{loxP/loxP}$;*GCre*$^{+}$ or *Pten*$^{loxP/loxP}$ mice were used for each lane. For isolation of PD12 to 14 oocytes, 3 to 5 *Pten*$^{loxP/loxP}$;*GCre*$^{+}$ mice or 6 to 10 *Pten*$^{loxP/loxP}$ mice were used per lane. In each lane, 30 to 40 μg of protein sample was loaded.

ablation of *Pten*, which is caused by excessive activation and depletion of primordial follicles. Thus, our findings may have broad physiological and clinical implications, contributing to in-depth understanding of both normal ovarian physiology and the development of ovarian diseases. In humans, POF is defined as a primary ovarian defect characterized by absent menarche (primary amenorrhea) or by premature depletion of ovarian follicles or arrested folliculogenesis before the age of 40 years (secondary amenorrhea), with an estimated incidence of 1% (*11*). We hypothesize that genetic variations leading to overactivation and depletion of follicles may be among the possible causes of POF in humans. On the other hand, the retardation of follicle activation and/or excessive primordial follicle atresia, both of which may be caused by underactivation of the PI3K pathway in oocytes, can also lead to POF, albeit from opposite directions. Recognition of the importance of the PTEN-PI3K signaling network in oocytes opens up new prospects for our understanding of the physiological and pathological processes of the mammalian ovary.

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The Maternal Nucleolus Is Essential for Early Embryonic Development in Mammals

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With fertilization, the paternal and maternal contributions to the zygote are not equal. The oocyte and spermatozoon are equipped with complementary arsenals of cellular structures and molecules necessary for the creation of a developmentally competent embryo. We show that the nucleolus is exclusively of maternal origin. The maternal nucleolus is not necessary for oocyte maturation; however, it is necessary for the formation of pronuclear nucleoli after fertilization or parthenogenetic activation and is essential for further embryonic development. In addition, the nucleolus in the embryo produced by somatic cell nuclear transfer originates from the oocyte, demonstrating that the maternal nucleolus supports successful embryonic development.

To create a totipotent zygote, the oocyte and spermatozoon combine not only their nuclear DNA but also certain RNAs, proteins, and organelles. Some zygotic material is strictly of maternal or paternal origin. For example, mitochondria originate exclusively from the oocyte (*1*), whereas the centriole in most mammals (excluding rodents) comes from the spermatozoon (*2*, *3*). Another zygotic organelle that seems to be supplied by the oocyte is the nucleolus. The nucleoli in fully grown oocytes are compact and transcriptionally inactive (*4*–*7*). It is not known how or to what extent this nucleolar material contributes to the construction of zygotes and early embryos. Our results demonstrate that the nucleolus in the zygote and early embryo is exclusively maternally inherited and originates from the material that is present in the oocyte germinal vesicle. Moreover, using nuclear transfer experiments, we demonstrate that nucleoli originating from a somatic cell or even from an embryonic stem (ES) cell cannot substitute for the original oocyte nucleolar material.

The scheme for our analysis is shown in fig. S1. Nucleoli were microsurgically removed from fully grown oocytes before gonadotropic stimulations in pigs and mice (Fig. 1, A and B, fig. S2, and movies S1 and S2) (*8*). The oocytes from which we aspirated a small amount of nucleoplasm served as controls [fig. S1, sham-operated (Sham)]. The success of enucleolation was checked immunocytochemically (Fig. 1C and fig.

S3A). In sham-operated oocytes, the immunofluorescence against H3K9triMet (trimethylated histone H3 at Lys[9]), C23 (nucleolin), and NPM2 (nucleoplasmin 2), as well as the staining against chromatin by DAPI (4′,6′-diamidino-2-phenylindole) and Hoechst (bisbenzimide H33342), demonstrated a single nucleolus surrounded by a heterochromatin rim (Fig. 1C, Chromatin, and fig. S3A, Hoechst). With this approach, no nucleoli were detected in enucleolated oocytes. After the enucleolation, the germinal vesicle envelope became sealed, as confirmed by labeling against lamin A/C (nuclear lamin A and lamin C) (Fig. 1C and fig. S3B, Lamin A/C), and the chromatin remained inside the germinal vesicle and was not detectable around isolated nucleoli (Fig. 1C, Chromatin, and fig. S3A, Hoechst). Maturation of enucleolated oocytes appeared normal, with regular spindle formation and subsequent polar body extrusion, and progressed in a similar time course as in controls to metaphase II at 52% (93/180) in the pig (fig. S3, D and F) and 90% (533/592) in the mouse (fig. S3B). Karyotyping of mouse oocytes at metaphase II revealed a normal haploid set of chromosomes in both sham-operated and enucleolated oocytes (n = 20 chromosomes, fig. S3C). Analysis of the kinetics of activities of meiotic cell cycle molecules cyclin-dependent kinase 1 (CDK1) and mitogen-activated protein (MAP) kinase demonstrates that these kinase activities fluctuated similarly in the enucleolated and control pig oocytes (fig. S3E). These results suggest that oocyte nucleoli do not contain the essential factor(s) for oocyte maturation.

In both species, enucleolated and mature oocytes were efficiently activated parthenogenetically (pig: 100%, 212/212; mouse: 75%, 157/208) or by fertilization (pig: 57%, 93/164; mouse: 69%, 129/186). Whereas round prominent nucleoli were detectable in germinal vesicles and in both the male and female pronuclei of control zygotes, they were not visible in germinal vesicles or pronuclei originating from enucleolated oocytes (Fig. 1, D to F). After activation, CDK1 and MAP kinase in enucleolated oocytes were inactivated in a similar time course as in controls (fig. S3E). At the pronucleus or four- to eight-cell stage, the proteins and DNA were synthesized at similar levels in both the experimental and control groups (fig. S4).

Next, we tested the recovery of nucleolus formation in the pronuclei by reinjecting the oocyte nucleoli into the enucleolated oocytes (fig. S1, Nucleolus reinjected). Isolated pig oocyte nucleoli were kept for 26 hours in the empty zonae pellucidae prepared by sucking out all the contents of the ooplasm by a micromanipulator (Fig. 1B), and mouse oocyte nucleoli were kept in the perivitelline space under the zona pellucida for 14 hours (fig. S3A). The nucleoli were then reinjected into enucleolated mature oocytes. Reinjected nucleoli disappeared in the oocyte cytoplasm (fig. S5). After activation, the nucleolus-reinjected oocytes formed pronuclei that contained prominent nucleoli (pig: 53%, 61/115; mouse: 69%, 77/112), whereas no nucleoli were visible in embryos from enucleolated oocytes (Fig. 2 and fig. S6). In addition, after in vitro fertilization, zygotes from the nucleolus-reinjected enucleolated oocytes contained nucleoli in their pronuclei (fig. S7). Next, we tested whether somatic cell nucleoli can substitute for the original oocyte material by injecting a somatic cell nucleus via the somatic cell nuclear transfer (SCNT) method. After enucleolation and maturation of pig and mouse oocytes, nuclei of cumulus cells were injected into cytoplasts of enucleated oocytes at metaphase II (fig. S1, Enucleolated-SCNT). Cytoplasts from non-enucleolated mature oocytes served as controls (fig. S1, Sham-SCNT). The exposure of somatic nuclei to the oocyte cytoplasm resulted in nuclear envelope breakdown and premature chromosome condensation (pig: 59%, 73/123; mouse: 73%, 38/52). These reconstructed oocytes were then activated parthenogenetically. No nucleoli were seen in newly formed pseudo-pronuclei in the enucleolated group, whereas controls always contained a single nucleolus or multiple nucleoli (Fig. 2). Similarly, no nucleoli were visible in pseudo-pronuclei after injection of nuclei of pluripotent ES cells into enucleolated oocytes (Fig. 2B, Enucleolated-ESCNT). This indicates that somatic and ES

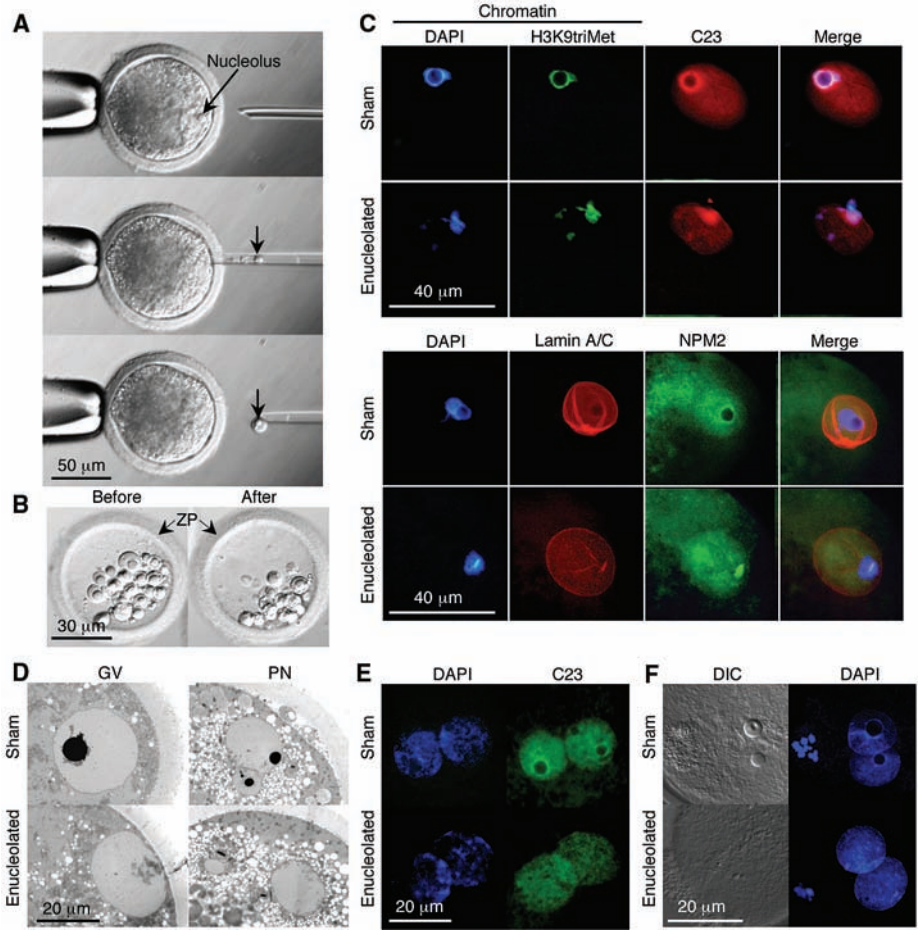

Fig. 1. Enucleolation of immature oocytes (A to C) and the absence of nucleoli in pronuclei of zygotes originating from enucleolated oocytes (D to F). (**A**) The nucleolus in centrifuged pig oocytes at the germinal vesicle (GV) stage was aspirated and isolated. (**B**) Isolated nucleoli before (left) and 26-hours after (right) culture without disassembling. Arrows indicate the empty zonae pellucidae (ZP). (**C**) Immunofluorescence labeling was performed with the indicated antibodies in green (H3K9triMet and NPM2) and in red (C23 and Lamin A/C). The absence of a nucleolus is evident in enucleolated oocytes. (**D** to **F**) No nucleolus formation in pronuclei (PN) is observed in light-microscopic observations (D) and immunofluorescence labeling against C23 (green) (E) of pig zygotes, as well as in differential interference contrast (DIC) images of mouse zygotes after in vitro fertilization (F). DAPI staining marks chromatin in blue.

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cell nuclei (including nucleolus) cannot substitute for the original oocyte nucleolar material.

The development of pig embryos originating from enucleolated oocytes arrested after a few cleavages (Table 1 and Fig. 3A). In contrast, embryos from sham-operated oocytes developed to blastocysts with detectable nucleoli (fig. S8, Pig). The cleavage rate was essentially the same at 48 hours after activation. However, no nucleoli were detected in the nuclei of two-cell embryos originating from enucleolated oocytes (Fig. 3B, Pig). At this stage, these embryos without nucleoli were still viable, having the ability to synthesize proteins at similar levels when compared to control embryos originating from sham-operated oocytes (fig. S4, A and B). We observed the same developmental defects in the embryos from enucleolated oocytes after SCNT (Table 1 and Fig. 3B, Pig), and intracytoplasmic sperm injection (table S1). Unexpectedly, pig embryos that were enucleolated and then reinjected with oocyte nucleoli cleaved regularly with visible nucleoli in blastomere nuclei (Fig. 3B, Pig) and developed to blastocysts (Table 1, table S1, Fig. 3A, and fig. S8, Nucleolus reinjected). The developmental ability of embryos, including SCNT embryos, thus depends on the presence of the oocyte nucleolar material that was either released into the oocyte cytoplasm at germinal vesicle breakdown or reinjected into the cytoplasm of previously enucleolated oocytes. In the mouse (table S2), embryos originating from sham-operated oocytes developed to the blastocyst stage, and the blastomere nuclei contained visible nucleoli (fig. S8, Mouse). No nucleoli were detected in embryos originating from enucleolated oocytes (Fig. 3B, Mouse), and their development was arrested between the two- and four-cell stages. After nucleolus reinjection into enucleolated and mature mouse oocytes, the nucleoli were detected in embryo nuclei, and their developmental potential was restored (table S2). Furthermore, when we transferred two-cell embryos that were derived from enucleolated oocytes and from nucleolus-reinjected oocytes to recipients' uteri, we obtained live-born pups at a ratio comparable to that from control oocytes (table S3). Thus, the inability of enucleolated oocytes to develop does not reflect the mechanical damage caused by micromanipulation but is a direct consequence of the absence of nucleolar material.

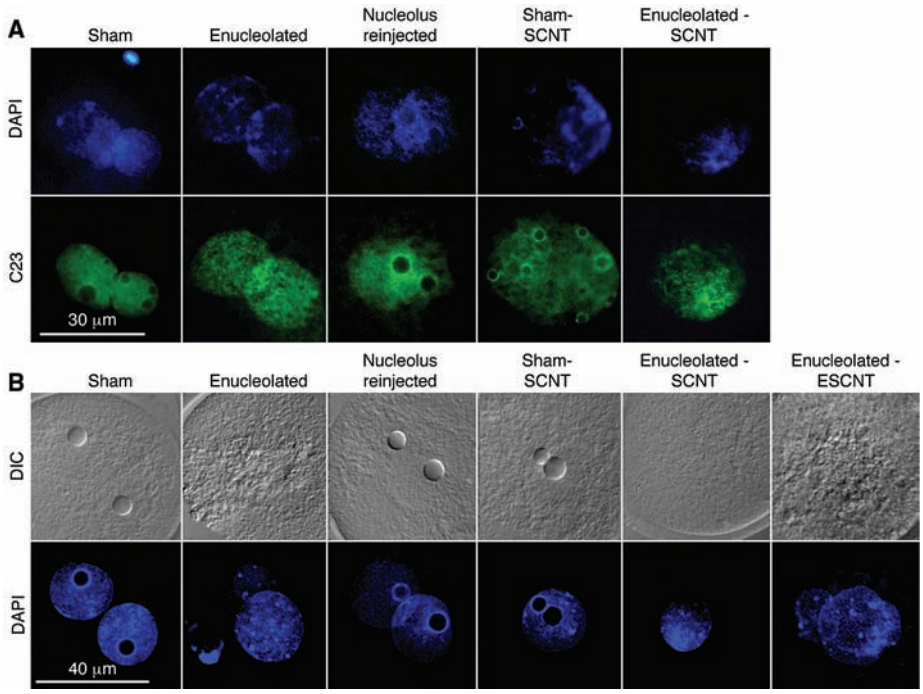

Fig. 2. The presence of nucleoli in pronuclei of activated enucleolated oocytes after reinjection of isolated oocyte nucleoli or after SCNT. No nucleolus was formed in the embryos originating from enucleolated (Enucleolated) oocytes, from somatic cell (cumulus cell) nucleus–transferred enucleolated (Enucleolated-SCNT) oocytes, and from ES cell nucleus–transferred enucleolated (Enucleolated-ESCNT) oocytes. Nucleoli in pronuclei were detected by immunofluorescence labeling against C23 (green) in the pig (**A**) and by DIC microscopy in the mouse (**B**). DAPI staining marks chromatin in blue.

Table 1. In vitro development of pig parthenotes and SCNT embryos derived from enucleolated oocytes. The values in parentheses represent the percentages of oocytes cleaved at 48 hours and forming blastocysts at 6 days after activation.

	Type of pig oocyte				
	Sham	Enucleolated	Nucleolus reinjected	Sham-SCNT	Enucleolated-SCNT
Total number of oocytes examined	148	228	194	156	200
Total number and % of oocytes cleaved	128 (86)	168 (74)	174 (90)	120 (77)	175 (88)
Total number and % of oocytes forming blastocysts	92 (62)	0 (0)	72 (37)	48 (31)	0 (0)

Cytoplasts of oocytes at metaphase II and mitotic zygotes support development of SCNT embryos (*9*). In contrast, cytoplasts of interphase zygotes, after the removal of pronuclei, have been incapable to support such development (*10*). When the pronuclear membrane and chromatin of interphase zygotes are selectively removed but other pronuclear components, including the nucleolus, are left in the cytoplasm, these cytoplasts support development of SCNT embryos (*11*). These results suggested that an undefined activity or material(s) in pronuclei of zygotes facilitates the reprogramming of transferred somatic cell nuclei (*9*, *11*). To determine whether the maternal nucleolus alone facilitates the somatic cell reprogramming in zygotic cytoplasts, we cotransferred the cumulus cell nuclei and the isolated oocyte nucleoli into cytoplasts from interphase enucleated mouse zygotes (Zygote-SCNT). When the somatic cell nuclei were transferred into the cytoplast of M-phase enucleated zygotes, the resulting embryos assembled visible nucleoli (fig. S9, M-phase Zygote-SCNT) and developed to the blastocyst stage (table S4). In contrast, the embryos from Zygote-SCNT cleaved at 36 hours after human chorionic gonadotropin injection, but they arrested at the two-cell stage or fragmented, and they never developed to blastocysts. These two-cell embryos derived from zygote-SCNT never assembled nucleoli in their blastomere nuclei (fig. S9, Zygote-SCNT). Coinjection of oocyte nucleoli along with somatic cell nuclei resulted in two-cell–stage embryos with prominent nucleoli in their nuclei (fig. S9, Zygote-SCNT+Nucleolus). This nucleolar coinjection, however, did not rescue embryonic development, and embryos were typically arrested between the two- and four-cell stages (table S4). This indicates that, in addition to the nucleolar material, some other

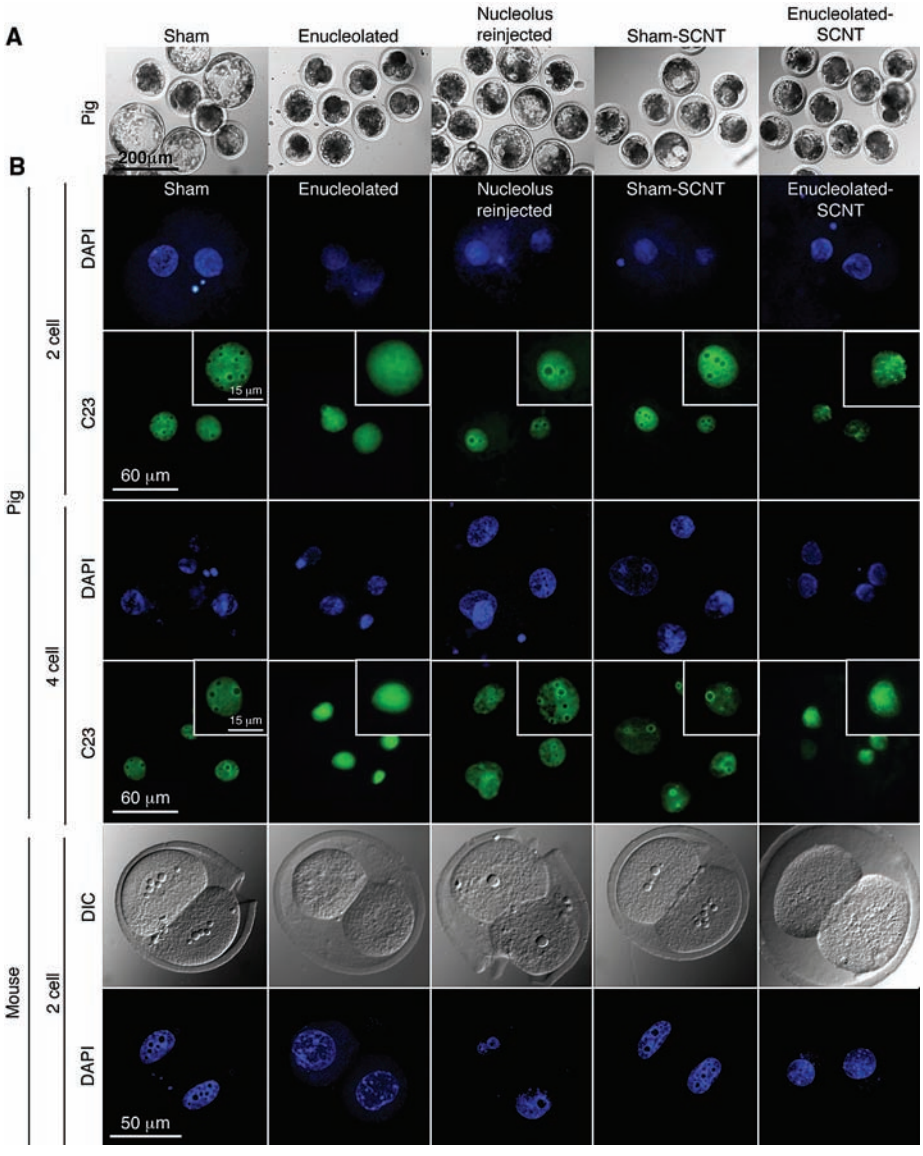

Fig. 3. Requirement of the oocyte nucleolus for early embryonic development. (**A**) Embryos originating from enucleolated and from enucleolated-SCNT oocytes, which were lacking the oocyte nucleolar material, were fragmented or arrested at the four- to eight-cell stage 6 days after electroactivation, whereas the embryos with nucleoli developed to blastocysts. (**B**) The absence of nucleoli in the embryos originating from enucleolated and enucleolated-SCNT oocytes. Nucleoli were visualized by immunofluorescence labeling against C23 (green) in the pig and by DIC microscopy in the mouse. Insets show nuclei from pig embryos at higher magnification. DAPI staining marks chromatin in blue.

nucleoplasmic components are essential for the successful development of zygote-SCNT embryos.

Our experiments demonstrate that nucleoli in mammalian zygotes are exclusively maternally inherited via the oocyte. We have also showed the necessity of the entire nucleolar complement for successful embryonic development. Because the nucleolus in the spermatozoon is eliminated during spermiogenesis (*12*–*14*), the oocyte nucleolar material is essential for the reassembly of newly formed nucleoli in both female and male pronuclei. We have found that after SCNT or ES cell nuclear transfer, the nucleolus apparently originates from the maternal source (oocyte), but we cannot exclude a possible minor contribution from the somatic and ES cells. This may be one of the reasons why cytoplasts of oocytes after germinal vesicle breakdown, as well as cytoplasts of mitotic zygotes, support development after nuclear transfer, whereas cytoplasts of oocytes before germinal vesicle breakdown, as well as cytoplasts of interphase zygotes, do not (*9*, *15*). The substances that are stored either in germinal vesicles or in pronuclei and that are essential for the reprogramming of somatic cell nuclei are not yet defined, but our results document that one of them is the nucleolus.

After fertilization, embryos drive their development for one or several cell cycles using the stockpile of molecules originating from oocytes. Therefore, it is speculated that early protein synthesis uses maternally stored ribosomes and RNAs. Functional nucleoli are assembled later on, when the embryonic genome becomes activated (*5*, *6*, *16*, *17*). The nucleolar materials originating from oocytes may represent the precursor molecules required for the assembly of fully functional nucleoli at a later stage, as the embryo develops (*16*). Our results suggest that the oocyte nucleolus provides the materials to build nucleoli not only in pronuclei of zygotes but also in pseudo-pronuclei formed after SCNT. Thus, the oocyte nucleolar material is essential for successful early embryonic development in mammals.

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