Subcellular Distribution of Mitochondrial Ribosomal RNA in the Mouse Oocyte and Zygote

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Mitochondrial ribosomal RNAs (mtrRNAs) have been reported to translocate extra-mitochondrially and localize to the germ cell determinant of oocytes and zygotes in some metazoa except mammals. To address whether the mtrRNAs also localize in the mammals, expression and distribution of mitochondrion-encoded RNAs in the mouse oocytes and zygotes was examined by whole-mount in situ hybridization (ISH). Both 12S and 16S rRNAs were predominantly distributed in the animal hemisphere of the mature oocyte. This distribution pattern was rearranged toward the second polar body in zygotes after fertilization. The amount of mtrRNAs decreased around first cleavage, remained low during second cleavage and increased after third cleavage. Staining intensity of the 12S rRNA was weaker than that of the 16S rRNA throughout the examined stages. Similar distribution dynamics of the 16S rRNA was observed in strontium-activated haploid parthenotes, suggesting the distribution rearrangement does not require a component from sperm. The distribution of 16S rRNAs did not coincide with that of mitochondrial-specific heat shock protein 70, suggesting that the mtrRNA is translocated from mitochondria. The ISH-rearrangement does not require a component from sperm. The distribution of 16S rRNAs did not coincide with that of mitochondrial-specific heat shock protein 70, suggesting that the mtrRNA is translocated from mitochondria. The ISH-scanning electron microscopy confirms the extra-mitochondrial mtrRNA in the mouse oocyte. Chloramphenicol (CP) treatment of late pronuclear stage zygotes perturbed first cleavage as judged by the greater than normal disparity in size of blastomeres of 2-cell conceptuses. Two-third of the CP-treated zygotes arrested at either 2-cell or 3-cell stage even after the CP was washed out. These findings indicate that the extra-mitochondrial mtrRNAs are localized in the mouse oocyte and implicated in correct cytoplasmic segregation into blastomeres through cleavages of the zygote.

INTRODUCTION

Producing different cell types through segregation of specific cellular components is a key aspect of embryogenesis, which is implemented at the very beginning of the embryogenesis in some species. The anterior-posterior axis is laid down within Nematoda zygote immediately after sperm entry following which the zygote goes through a series of asymmetric cleavage to produce certain progenitor cells including germ cells [1]. In Drosophila, several gene products are regionally localized in the oocyte cytoplasm to define the body plans of the future embryo [2,3] and specify the complement of future germ cells [4]. These are regarded as pre-patterned oocytes and zygotes since different domains of cytoplasm harbour distinct developmental properties. Even in vertebrates such as Xenopus, several RNAs are reported to subcellularly localize in the oocyte [5–10].

Localization of germ plasm and its subsequent segregation to germ cells in early embryogenesis has been investigated particularly thoroughly. In both Drosophila and Xenopus, germ plasm is localized in the oocyte, where it is clearly defined as a region rich in mitochondria and possessing “nuage” or germinal bodies, polar granules in Drosophila and germinal granules in Xenopus, which characterize prospective germ cells in developing embryos [4,9]. The germinal body contains nucleic acids and that germ cell determinant capability is lost when the germ plasm is irradiated with short-wavelength UV, but can be restored if intact germ plasm is transplanted into the irradiated zygotes [11]. Surprisingly the nucleic acid in question is mitochondrial ribosomal RNA (mtrRNA) that lies outside this organelle [12,13]. MtrRNA injected into the UV-irradiated Drosophila zygote restored the formation of pole cells [14] indicating that this class of RNA may be essential for pre-patterning of the germ plasm in Drosophila, sea urchin [15] and Xenopus [16] zygote.

Although some genes products are localized in mammalian oocytes [17,18], it is widely accepted that early development is essentially regulative rather than pre-patterned in mammals [19–21]. While mechanical perturbation to the cytoplasm, for instance, does not perturb embryogenesis [22–24], there are a growing number of findings that challenge the absence of pre-patterning in mammals [25]. Moreover, the existence of a regulative capacity does not necessarily rule out pre-patterning of the oocytes [26–29]. Several mammalian homologues to the posterior group genes of Drosophila have been identified, and many of them are expressed in the mouse primordial germ cells [30]. Little is, however, known about the molecular organization of the mouse oocyte and zygote in which no distinctive germ plasm has been identified and the occurrence of extra-mitochondrial mtrRNAs has not been reported. The purpose of the present study was therefore to investigate if such mtrRNA is localized in the mouse oocyte or zygote and, if so, whether has any role in patterning the embryo.

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**MATERIALS AND METHODS**

**Collecting and culturing mouse oocytes and zygotes**

All oocytes and zygotes were obtained from PO (Pathology, Oxford) albino closed-bred mice. Media and conditions for recovery and culture of oocytes and zygotes, and for strontium-activation for haploid parthenogenetic development, were as reported previously [27,31]. All experiments regarding animals were carried out under the UK legislation for animal welfare (Animals (Scientific Procedures) Act 1986, Home Office, UK, scienceandresearch.homeoffice.gov.uk/animal-research/).

Chloramphenicol (CP, SIGMA, www.sigmaaldrich.com), cycloheximide (CH, Merck, www.merckbiosciences.com) and a-amanitin (AM, Merck) were dissolved into dimethysulfoxide (DMSO, SIGMA) to make stock solutions. Stock solutions were diluted 1:100 in KSOM-AA medium to obtain the final concentrations in the medium; CP 200 μg/mL [32], CH 10 μg/mL [33] and AM 100 μg/mL [34]. These final concentrations were determined by referring to previous reports. For vehicle control, 1% DMSO in KSOM-AA was used.

MitoTracker Red H2-CMXRos (Molecular Probes, probes.invitrogen.com), which is chemically reduced and non-fluorescent unless oxidized [35], was prepared according to the manufacturer’s instruction and applied final concentration of 400 nM to the treated conceptuses for 30 minutes at 37°C before fixation. The conceptuses were fixed in 4% paraformaldehyde (PFA) in calcium- and magnesium-free Dulbecco’s phosphate buffered saline (PBS(-)) for 5 minutes at room temperature, then washed with PBS(-) and mounted on glass slides with 60% glycerol in PBS(-) for 24 hours and quickly frozen in liquid nitrogen.

**Whole-mount in situ hybridization**

Mitochondrial DNA was isolated from E6.5-7.5 (plug day noon = E0.5) mouse conceptuses by alkaline hydrolysis protocol designed for purification of plasmids from *Escherichia coli* (QIAprep Spin, QIAGEN, www1.qiagen.com). Using the purified DNA as a template, the following PCR primers containing partial T3 and T7 promoter (below, small caps) were used to generate templates for antisense and sense RNA probes.

1. 837 bp fragment of 12S rRNA fwd-pt-T3 cactaaaggGACGGTTTGTGTCGTCGACTTCATT, rev-pt-T7 cactaatgggCGGTGTTGTCGACCTTCATT
2. 883 bp fragment of 16S rRNA fwd-pt-T3 cactaaaggGTACCGAAGGGAAGATGA, rev-pt-T7 cactaatgggCAGTTGGAACCCCTGGTTAGC
3. 656 bp fragment of ATPase 6 (ATP6) fwd-pt-T7 cactaggGGAACAAATCTTATTGCTCA, rev-pt-T3 cactaaGGGCTTATTTGAGGGAAGATTG
4. 869 bp fragment of Cytochrome b oxidase subunit 1 (Cox1) fwd-pt-T7 cactaatgggGGTCAMGGGACGGTTACCT, rev-pt-T3 cactaatgggTGATTGTTGTCGACCTTCATT

These DNA fragments were further amplified using the following adaptor primers to follow up the PCR-generated templates were subjected to sequencing (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, www.appliedbiosystems.com) and homology search (Nucleotide-nucleotide Basic Local Alignment Search Tool, National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) to confirm the relevant gene identities and specificities. The 2.4 kb PCR-generated cDNA of mouse EMK (ELKL Motif Kinase) cloned into pBluescript KS+ (mEMK c24, a gift from Prof. Ohno) was also used. The expression of 12S and 16S rRNAs, ATP6, Cox1 and mEMK mRNAs and Cytochrome b mRNA in the mouse MII oocytes had been confirmed by either blot hybridization [39] or EST analysis [40]. For digoxigenin (DIG) labeled in vitro transcription, T3 and T7 RNA polymerases (Roche, www.roche-applied-science.com) were used according to the manufacturer’s instruction. Whole-mount in situ hybridization (ISH) for oocytes and zygotes was done as described in previous report [41].

For scanning electron microscopic (SEM) visualization of the ISH, frozen sections were prepared in accordance with previous report [42] with some modifications. The whole-mount hybridized and washed oocytes were embedded in 2% low-melting point temperature agarose (NuSieve GTG, FMC, www.fmc.com) in PBS(-) before fixation. The conceptuses were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer at room temperature [42] with some modifications. The whole-mount hybridized and washed oocytes were embedded in 2% low-melting point agarose (NuSieve GTG, FMC, www.fmc.com) in PBS(-) before fixation. The conceptuses were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer [32] and mounted on glass slides with 60% glycerol in PBS(-). Fluorescent images were photographed using a CCD camera (MagnaFire, Optronics, www.optronics.com) on a conventional epifluorescent microscope (Axioplan, Zeiss, www.zeiss.com).

**Morphometric analysis of 2-cell conceptuses**

Cultured conceptuses were treated with acidified-Tyrode’s saline to release membrane tension and leave blastomere adherence [36]. After treatment, conceptuses were incubated in KSOM-AA medium for 30 minutes at 37°C before being fixed and processed as described in previous section. The specimens were photographed using a digital camera (Coolpix 995, Nikon, www.nikon.com) on a differential interference contrast microscope (DMLB, Leica, www.leica-microsystems.com), and the photomicrographs were analyzed with the ImageJ software [37,38] runs on a Macintosh personal computer (PowerMac G4, Apple, www.apple.com).

**Whole-mount immunofluorescence**

Oocytes and zygotes were fixed in 4% PFA on ice for at least overnight, then washed in PBS(-) containing 0.1% Tween 20 (PBTr). Washed specimens were transferred to 0.2 ml PCR tubes containing 100 μl of 10 mM citrate buffer (8.2 mM tri-sodium citrate and 1.8 mM citrate), and antigen-retrieved at 97°C for 30 minutes using a PCR machine. Prior to applying primary antibodies, specimens were blocked for at least 60 minutes on ice with 10% heat-inactivated FCS and 2 mg/mL of bovine serum albumin (BSA fraction V, SIGMA) supplemented PBTr. Anti-mitochondrion
Distribution of mitochondrial 16S rRNA

From second meiotic metaphase arrested (MII) oocyte through to 8-cell morula stage conceptuses, both 12S and 16S rRNAs were localized identically but the ISH staining intensity of 12S rRNA (Figure S1A) was slightly weaker than that of 16S rRNA (Figure S1B). Considering single pre-processed mitochondrial transcripts are split into both mtrRNAs, this might be due to a difference of hybridization efficiencies between two probes rather than a difference of the actual amount of mtrRNAs. Therefore only ISH results of 16S rRNA are represented further. In the MII oocyte, the 16S rRNA was distributed at the presumptive animal hemisphere region—the half of the oocyte toward the MII spindle (Figure 1A, asterisk and S2B), the vegetal hemisphere being almost devoid of such RNA (Figure 1A and S2B). After fertilization, the distribution of the 16S rRNA changed to being localized around the pronuclei of the zygote (Figure 1B). Closer examination revealed that it lay preferentially between the pronuclei (Figure 1B, chevrons) and second polar body (Figure 1B, asterisk). The intensity of staining decreased after the first cleavage (Figure 1C) and remained low until the 4-cell stage (Figure 1D and 1E), before gradually increasing after the third cleavage (Figure 1F). Expression of ATP6 (Figure S1C) and Cox1 (Figure S1D) were undetectable during these stages.

To test the efficiency of this ISH methodology, localization of transcripts of mEMK, a mouse homologue of *Nematoda par-1* [44,45], was also examined on the oocytes and zygotes (Figure S1E). As shown in the figure, the mEMK transcripts were distributed ubiquitously in the MII oocyte and zygote and dramatically decreased in abundance after the first cleavage. This pattern of expression of mEMK during these stages accords well with previous report in which RT-PCR was used to detect its transcripts [46]. This suggests that the negative results with ATP6 and Cox1 are due to difficulty of probe penetration rather than limited sensitivity of the ISH.

If the ISH for 16S rRNA is able to detect such RNAs within mitochondrion, their distribution should coincide with that of mitochondria. Immunofluorescent staining against mtHSP70 [47] clearly demonstrates that it is not the case. Consistent with previous reports where a mitochondrion-specific fluorescent dye was used [48,49], the immunofluorescence showed mtHSP70 to be distributed not only around MII spindle (Figure 2B and 2C) but evenly throughout the cytoplasm of MII oocytes (Figure 2A and 2D) and zygote (Figure 2E). At the 2-cell stage when the ISH staining intensity of the 16S rRNA had decreased, the immunofluorescence was firmly visible in the cytoplasm (Figure 2F). Hence it is likely that the DIG-labeled probes could have difficulties to penetrate into the mitochondria under the ISH conditions used in this study.

The combination of ISH and SEM further provided strong evidence showing few DIG-labeled probes could penetrate into the mitochondria. A small number of gold colloidal particles are observed on tubular cristae [50,51] of the mitochondrion (Figure 3A, chevrons), whereas the surrounding cytoplasm is almost deserted by the particles. The ISH-SEM also revealed that the 16S rRNAs are extra-mitochondrially distributing at the certain place of cytoplasm (Figure 3B and S2D). In these SEM fields, the particles are wide spread including inside the mitochondrion (Figure 3B, chervon), except the endoplasmic reticulum (Figure 3B, asterisk) in which the 16S rRNA is not expected to present.

**Distribution dynamics of the 16S rRNA after parthenogenetic oocyte activation**

*In vitro* activation of oocyte revealed that the rearrangement in distribution of 16S rRNA in zygotes occurs independently of the presence of sperm components. Thus, the strontium-activated

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**RESULTS**

**Distribution of mitochondrial 16S rRNA**

Figure 1. Subcellular distribution of 16S rRNA in the mouse oocyte and zygotes. Higher magnification of the MI oocyte (A) shows a predominant distribution of the 16S rRNA in animal hemisphere (asterisk: position of MI spindle). Distribution of the 16S rRNA is rearranged to peri-pronuclei (chevrons) accumulation toward the polar body (asterisk: position of MII spindle). Distribution of the 16S rRNA in animal hemisphere (Figure 1B). Closer examination revealed that it lay preferentially between the pronuclei (Figure 1B, chevrons) and second polar body (Figure 1B, asterisk). The intensity of staining decreased after the first cleavage (Figure 1C) and remained low until the 4-cell stage (Figure 1D and 1E), before gradually increasing after the third cleavage (Figure 1F). Expression of ATP6 (Figure S1C) and Cox1 (Figure S1D) were undetectable during these stages.

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**Distribution dynamics of the 16S rRNA after parthenogenetic oocyte activation**

*In vitro* activation of oocyte revealed that the rearrangement in distribution of 16S rRNA in zygotes occurs independently of the presence of sperm components. Thus, the strontium-activated
haploid parthenotes showed a similar distribution pattern and dynamics of the 16S rRNA (Figure 4) to that of the zygotes obtained by fertilization in vivo (Figure 1B and 1C). While the second polar body extrusion was taking place, the distribution of 16S rRNA remained predominantly in the animal hemisphere (Figure 4A). This predominant animal hemisphere distribution was succeeded by a peri-pronuclear one on completion of the polar body extrusion (Figure 4B). The amount of 16S rRNA in parthenotes began to decrease by 6 hours after activation (Figure 4C), and the staining intensity was faint after first cleavage (Figure 4D).

**Effect of inhibitor for mitochondrial translation**

Chloramphenicol (CP) specifically inhibits prokaryotic translation and therefore serves as a specific inhibitor of mitochondrial translation in eukaryotes. Along with CP, cycloheximide (CH), a general inhibitor for translation, and á-amanitin (AM), a transcription inhibitor, were also applied to the late pronuclei stage zygote (around E0.75). In line with several other studies reporting that zygotic gene expression generally begins at 2-cell stage in mice [52–54], AM did not prevent the first cleavage, whereas CH prevented the first cleavage with the zygote in an elongated or ellipsoidal shape presumably arrested in the beginning of mitosis (Table 1). This indicates that de novo protein synthesis from maternally deposited mRNAs is required for completing the first cleavage.

While exposure of late zygote to CP did not prevent first cleavage (Table 1), it resulted in many 2-cell conceptuses showing unequal sized blastomeres (Figure 5A and 5B). Morphometric and statistical analysis clearly demonstrated that such size disparities were significantly larger than normal among 2-cell conceptuses (Figure 5C). About 28% of CP-treated 2-cell conceptuses underwent second cleavage when they were washed and transferred into fresh medium, but the majority became arrested either at 2-cell stage or during the second cleavage so as to yield 3-cell stages (Table 2). To exclude a possibility that the CP reduces energy production of the mitochondrion then affects development of the mouse conceptuses, living cell staining using an oxidation-sensitive, mitochondrion-specific fluorescent dye was carried out. Even after 24 hours treatment of CP, the treated 2-cell conceptus did not display any visual sign of the functional retardation of mitochondrion (Figure 5D and 5E). In longer term, impaired mitochondrial translation could eventually affect the respiratory function because all 13 mitochondrion-encoded proteins are indispensable to the electron transport chain and oxidative phosphorylation, the aberrant cleavage pattern of zygotes seems to be a direct result of failure of the mitochondrial translation in short term.

**DISCUSSION**

Contrary to the uniform and amorphous appearance of the mouse secondary oocyte except in the region of the MI spindle, certain gene products are localized within its cytoplasm [17,18]. Whether these products are related to the cleavage pattern of the zygote is not clear. This study shows that rRNAs derived from mitochondria are subcellularly distributed in the mouse oocyte and zygote implies that developmental strategies in mammals may have aspects in common with those of the oocyte and zygote. However, the occurrence of localized mtrRNAs in the mouse oocyte and zygote suggests that the developmental strategies in mammals may have aspects in common...
with other metazoa. While loss-of-function type experiments show that these mtrRNAs are crucial for segregation of germ cells in certain metazoa [56], the present study implicates them in establishing a proper first cleavage pattern in mice.

The mtrRNAs are acting through translational contribution

CP-treatment of zygotes reveals that the mtrRNA is acting through its own translational contribution rather than newly uncovered functionality of RNA like microRNA or ribozyme. In Drosophila, translation of a gene named “germ-cell less (gcl)”, whose loss-of-function leads to failure of pole cell formation, is known to be dependent on mtrRNA [57]. This rather unusual translation of nuclear genome-encoded mRNA by mtrRNA also takes place in...
mammalian sperm during capacitation [58] raising the possibility that similar translational activity might be initiated upon fertilization of mouse oocytes. In Drosophila, maternally deposited mRNA of gel is translated in association with posteriorly localized mtrRNA through which the cytoplasm of posterior pole becomes the germ plasm. In the case of mouse, inhibition of the mtrRNA's contribution to translation perturbs positioning of the first cleavage plane.

The size disparity between blastomeres of the 2-cell conceptus is not significant in non-treated or vehicle control culture unless morphometric analysis is performed, when a small minority shows visible size difference under a microscope. These unequally cleaved 2-cell conceptuses however develop normally till at least the blastocyst stage. In contrast, a high proportion of CP-treated 2-cell conceptuses arrested at 2- or 3-cell stage regardless of whether they showed obvious differences in blastomere size. The mechanism whereby the first cleavage plane is established in the mouse zygote is as yet unknown, but seems to tolerate fluctuation in its positioning. As the CH-treated zygote arrested during the first cleavage, most of protein components, which are essential for the mitosis, should be dependent on de novo global cytoplasmic translation. Mitochondrial translation, on the other hand, accounts for less than 2% of total translation of the mouse oocytes and zygotes [59], small number of unidentified protein factors, which are required for proper establishment of the cleavage plane, might rely on the mtrRNA to their translation. Some manipulation experiments on the mouse zygotes, such as removing either animal or vegetal hemisphere region, suggest that localized factors at animal hemisphere direct the mitotic spindle and therefore define the plane of first cleavage [60]. As the 16S rRNAs are predominantly distributed at the animal hemisphere of MII oocytes and zygotes, the mtrRNA could be one such factor. Hence, maternally deposited mRNAs and their mtrRNA-dependent translation might be involved in stabilizing the position of the first cleavage plane and the proper segregation of the cytoplasmic components of the zygote.

A series of studies shows that the mouse pronuclear zygotes are bilaterally symmetrical and this bilateral symmetry extends to the blastocyst [27]. This bilateral symmetry relates neither to the sperm entry point nor the pronuclear distribution, supporting the assumption that the morphological significance might be a reflection of intrinsic organization of the oocyte [61]. It has been claimed that the entry point of sperm determines the prospective first cleavage plane of mouse zygote [62], but this has been challenged by other findings arguing that the position of first cleavage plane has been specified intrinsically [31,63] or epigenetically [64–66] rather than determined by an external cue. That inhibition of a maternal process such as mitochondrial translation leads to disruption of zygote cleavage, and haploid parthenotes show similar distribution pattern and dynamics of the mtrRNAs to the zygote further support the possibility of pre-patterning of the mouse oocytes.

A transmission electron microscopy showed that the CP treatment does not affect the amount of ribosomes in both cytoplasm and mitochondria of the mouse conceptuses [32]. Taken together with present result showing the CP treatment has no impact on the respiratory function of mitochondria, the failure of mitochondrial translation should be responsible for the aberrant cleavage. Which genes are dependent on mtrRNA for
translation in mouse oocytes and zygotes is an intriguing question that needs to be addressed.

The mtrRNAs are extra-mitochondrial

ATP6 and Cox1 expression were not detectable from MII oocyte to 8-cell morula either because the ISH used here was not sensitive enough to detect their transcripts or the DIG-labeled probes were unable to penetrate into the mitochondria. A previous study using blot hybridization showed that the amount of mtrRNAs and Cox1 mRNA remains low until just before the second cleavage and increase at least 25 times thereafter by the early blastocyst stage [39]. The study also revealed that the actual number of molecules of Cox1 mRNA in 8-cell morula is more than one-fifth and one-tenth to that of mtRNA in MII oocyte and 8-cell morula respectively, and the amount of mitochondrial mRNAs is about 10% of total amount of poly(A)+ RNAs at the 8-cell morula stage [39]. Assuming that there are such abundant transcripts in the 8-cell morula requiring plentiful energy to sustain growth, it is unlikely that the ISH is not sensitive enough to detect mRNAs of ATP6 and Cox1. In fact, an ISH by antisense probe to the mEMK, which is nuclear genome-encoded single copy gene, successfully revealed its expression dynamics around mouse fertilization, as previously reported using RT-PCR [46]. Whereas an ISH by antisense probe to the Cox1 mRNA in 8-cell morula, ATP6 and Cox1, failed to give staining on the MII oocyte and 8-cell morula by this ISH (data not shown). These results support the view that the ISH methodology used was able to detect RNAs in the cytoplasm but not inside the mitochondria of whole mount tissue.

Sense probes of 16S rRNA (Figure S2) and other mitochondrial genes (data not shown) gave either background level staining intensity (Figure S2A) or no gold colloidal particle deposition (Figure S2C), indicating that the rRNA antisense staining did not come from the hybridization between RNA probes and mitochondrial genomic DNAs, even if the probes penetrated into the mitochondria. Since the transcription of mitochondrial DNA is bidirectional, it also indicates that no detectable hybridization took place between the probes and pre-processed transcripts. In line with several other reports using mitochondrion-specific fluorescent dye [48,49], anti-mHSP70 immunofluorescence showed relatively even distribution of mitochondria throughout the cytoplasm of MII oocytes. These results contradict one particular observation employing a combination of living oocyte laser confocal microscopy and mitochondrion-specific fluorescent dye, in which an animal hemisphere-predominant polarized distribution of non-spindle mitochondria has been reported [67]. The author attributed the contradiction to a superior ability of the confocal microscopy over a conventional microscopy for viewing entire oocyte. However, it also might be possible that these non-spindle mitochondria have selective membrane permeability to the dye in a living condition, hence the strong fluorescent. Interestingly, the polarized distribution of mitochondria in the living oocyte is strikingly similar to that of 16S rRNAs reported here. As the distributions of mitochondria and 16S rRNAs do not coincide, a likely explanation for this discrepancy would be that either the ISH used here detects mTRNAs in a subset of mitochondria, which increase membrane permeability and localize animal hemisphere, or the mTRNAs are translocated from mitochondria, which has been found to be the case in Drosophila [12,13], sea urchin [15] and Xenopus [16]. The notion of extra-mitochondrial mTRNAs in the mouse oocyte is further ensured by the combination of ISH and SEM. The method employed here for the ISH-SEM was a pre-embedding hybridization and thus, it would be fewer gold colloidal particles inside endoplasmic organelle if the DIG-labeled probes have difficulties to penetrate membrane of the organelle. That is the exact case in terms of the mitochondria of MII oocytes. Moreover, the ISH-SEM also reveals the existence of cytoplasmic mTRNAs, which are distributed in certain area of the oocyte but not all over the oocyte.

In mice and humans, the 16S rRNAs are translocated into the sperm nucleus from mitochondria during spermatogenesis [68], establishing a precedent for their presence extra-mitochondrially in mammals in certain circumstances. In Drosophila, the extra-mitochondrial mTRNAs are translocated onto the surface of the polar granule, and this translocation is regulated under the posterior group genes [55], especially under oskar (osk) [69]. The polar granule itself is composed of osk protein together with products of caska (caska) and tudor (tud), other major members of the posterior group genes [70]. An exact mechanism underlying the polar granule formation is as yet unknown [4], a common assumption is that an active translocation and aggregation of the components including osk, vas and tud is completed during oogenesis then mTRNAs are translocated and associated with the granules in early embryogenesis [12].

However another interesting possibility becomes apparent. Transmission electron microscopy of the mouse Graafian follicle revealed that cristae-containing electron-dense bodies, which morphologically resemble the germinal bodies of other metazoa, are derivatives of mitochondria [71]. These derivatives lack an outer membrane and are gradually condensing into electron-dense bodies. It is also suggested that the transformed mitochondria might be precursors or platforms of the germinal body-like structure [72]. In any case, degradation or lack of outer membrane seems reasonable explanation for how DIG-labeled probes easily access to the mTRNAs. It also might explain the similarity of distribution pattern between the mitochondria in a living oocyte [67] and the mTRNAs in this report. Elucidating whether the electron-dense bodies are actually attaching the mTRNAs on their surface as well as bona fide composition and function of these cytoplasmic mitochondrial translation machinery are obvious next steps to be taken.

SUPPORTING INFORMATION

Figure S1 Expression and distribution of mitochondrial RNAs and mEMK in the mouse oocytes and zygotes. Distribution of two mitochondrial rRNAs and expression of two mitochondrial genes along with nucleus-encoded mEMK are examined by ISH in the MII oocytes and various stages of zygotes. The ISH staining intensity of 12S rRNA (A) is weaker than that of 16S rRNA (B) in the MII oocyte and zygote. ATP6 (C) and Cox1 (D) expression are undetectable during these stages. To illustrate a differential expression level of 16S rRNA and Cox1, 8-cell stage conceptuses are shown on fourth column. Transcript of mEMK (E) are ubiquitous in the MII oocyte and zygote then diminished after the first cleavage. Durations of colour reaction: 30 minutes for mTRNAs, 2 hours for mitochondrial mRNAs and 1 hour for mEMK. Bar = 25 μm

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Figure S2 16S rRNA sense probe ISH for negative control. Sense (A, C) and antisense (B, D) probes of 16S rRNA were applied to the MII oocytes and visualized via either alkaline phosphatase (AP) substrate BCIP/NBT (A, B) or gold colloidal particle conjugated anti-DIG antibody (C, D). For AP colour reaction, the hybridized and AP conjugated anti-DIG antibody applied samples were embedded in agarose then incubated in the substrate solution in a same well for 35 minutes. Bar = 10 μm
Table S1  Mean size and size difference of paired 2-cell stage blastomeres. This table is the raw data set to Figure 5C. Late pronuclei stage zygotes (around E0.75) were cultured for 24 hours in presence of either 200 μg/mL of chloramphenicol (CP200) or 1% of DMSO as vehicle control (vehicle). The cleaved 2-cell conceptuses were selected, fixed and photographed. Outlines of each blastomere are traced on the microphotographs then converted to the areas using ImageJ software. Pair values of the area are processed to calculate “Mean size of paired blastomeres (Mean Size)” and “Size difference between paired blastomeres (Size Diff)” and presented. Unit: μm²

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Author Contributions
Conceived and designed the experiments: YN. Performed the experiments: YN. SI. Analyzed the data: YN. SI. Contributed reagents/materials/analysis tools: YN. SI. Wrote the paper: YN.

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