Both Microtubules and Microfilaments Mutually Control the Distribution of Mitochondria in Two-Cell Embryos of Golden Hamsters

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Abstract: *The roles of microtubules and microfilaments on distribution of mitochondria were evaluated by using fluorescent staining in 2-cell embryos of golden hamsters with or without cytoskeletal assembly inhibitors. In 2-cell embryos without treatment (control), most mitochondria were accumulated at the perinuclear region, while some mitochondria were noted at the cell cortex. Microtubules were found around the nuclei, correlating with distribution of the mitochondria. In contrast, microfilaments were stained intensely beneath the cell membrane and especially at the cell-to-cell contact region. In most (82%) of embryos treated with nocodazole (an inhibitor of microtubule polymerization), mitochondria had extended into the subcortical (intermediate) region with varying degree, where they were aggregated in patches. After a treatment of cytochalasin D (an inhibitor of actin polymerization), distributional density of mitochondria decreased at the cell cortex, suggesting that mitochondria moved back around the nucleus. After a treatment of both inhibitors, the distribution pattern of mitochondria was almost similar to that observed after cytochalasin D treatment. Our results suggest that the translocation of mitochondria to the perinuclear region is mediated by microtubules, while the movement of mitochondria to the cell cortex is regulated by microfilaments. Microtubules and microfilaments may function as bidirectional anchors of mitochondria to the perinuclear region and to the peripheral region, respectively.*

Key words: *Hamster embryos, Mitochondria, Microtubules, Microfilaments*

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Introduction

Mitochondria are required to provide energy/metabolites to specific regions of oocytes/embryos, and their distribution is dramatically changed in the cytoplasm during oocyte maturation, fertilization, and early embryonic development in mice [1, 2], hamsters [3–5], pigs [6], cattle [7], goats [8] and humans [9]. Our previous study showed that distribution of mitochondria differed between *in vivo* and *in vitro* hamster embryos [5], suggesting that altered distribution of mitochondria may be one of the reasons for the low developmental ability of the embryos cultured *in vitro*. Cytoskeleton, such as microtubules and microfilaments, are involved in translocation of organelles, including mitochondria [1, 3, 6, 10]. In murine and porcine oocytes, it has been reported that translocation of mitochondria is mediated by microtubules, not by microfilaments [1, 6]. However, a study on hamster 2-cell embryos suggested that microfilaments play a role in the distribution of mitochondria [3, 11]. Therefore, the function of cytoskeleton on the mitochondrial distributions in mammalian oocytes and early embryos is still controversial. In the present study, we evaluated the role of microtubules and microfilaments in the distribution of mitochondria in hamster 2-cell embryos. The results show that both microtubules and microfilaments mutually control the distribution of mitochondria in 2-cell embryos.

Materials and Methods

Collection and culture of embryos

Female golden hamsters (*Mesocricetus auratus*), 8–12 weeks old, were superovulated on the day of post-

estrus discharge by pregnant mare serum gonadotropin (PMSG, Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan) with a weight-dependent manner [12], and mated with males in the evening 3 days later. Two-cell embryos were collected from the oviducts at 0800–0900 on day 2 of pregnancy and were cultured in hamster embryo culture medium 10 (HECM-10) [13] in a humidified atmosphere of 10% CO_2, 5% O_2 and 85% N_2 at 37.5°C. Some embryos were cultured in HECM-10 with a microtubule assembly inhibitor, nocodazole (100 μM, Sigma, St. Louis, MO, USA) and/or a microfilament assembly inhibitor, cytochalasin D (5 μM, Sigma) for 8-10 h under 10% CO_2, 5% O_2 and 85% N_2 at 37.5°C. The experimental design was approved by the Ethical Committee for Experimentation with Animals, Hirosaki University.

Fluorescent staining and imaging

A total of 193 embryos were used for fluorescence observations. Mitochondria were stained with rhodamine 123 (Rh123, 10 μg/ml, Molecular Probes, Eugene, OR, USA) for 15 min in HECM-10 as described previously [4, 5], washed 3 times in HECM-10, mounted on slide glasses and imaged immediately after labeling. To assess the distribution of microtubules and microfilaments, the embryos were processed as reported previously [14]. After fixation in a microtubule stabilization buffer, the samples were exposed to anti-β tubulin primary antibody (1:200; Sigma) at 37°C for 2 h, and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma) at 37°C for 1 h. After rinsing, the samples were stained with rhodamine-phalloidin (1:1000; Molecular Probes) for microfilaments for 30 min. The samples were viewed under a fluorescence microscope (BX-FLA, Olympus, Tokyo, Japan). A U-MNIBA filter set (Olympus) was used for Rh123 and FITC, a U-MWIB set (Olympus) was used for rhodamine. A cooled CCD video system (ImagePoint, Photometrics Ltd., Tucson, AZ, USA) was used to obtain images on a computer and color adjustment was performed by IPLab-Spectrum P software (Signal Analytics Corporation, Vienna, VA, USA).

Evaluation of perinuclear clustering of mitochondria

To test whether each inhibitor induces movement of mitochondria from the perinuclear region to the subcortical region, the widths of mitochondrial cluster from the nucleus in 4 subregions along radii (subregions; see Fig. 1) for each blastomere were quantitated (8 measurements/embryo) from the raw digital images. Overall means were calculated in each treatment and considered as an estimator for the extent of perinuclear clustering of mitochondria. The data were analyzed by ANOVA to evaluate the effect of each inhibitor.

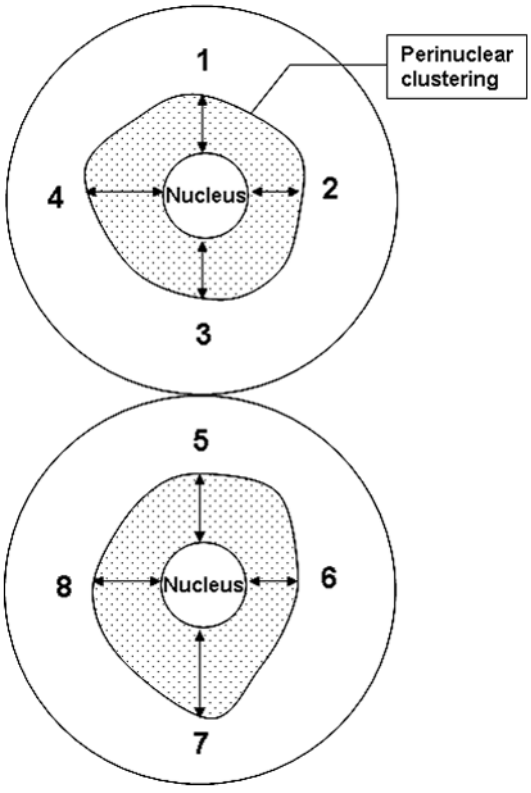

Fig. 1. Schematic diagram of a hamster 2-cell embryo indicating the positioning of the 8 subregions along the radii for analysis of perinuclear clustering of mitochondria (dotted area). The overall mean was considered as an estimate of the extent of perinuclear clustering of mitochondria.

Results

Rh123 staining of hamster 2-cell embryos (n=37) revealed that most mitochondria accumulated at the perinuclear region, while some mitochondria were noted at the cell cortex (Fig. 2a). Microtubules were found around the nuclei, and their distribution was very similar to that of mitochondria (n=38, Fig. 2b). Microfilaments were stained strongly at the cell-to-cell contact region and were found moderately beneath the cell membrane (n=38, Fig. 2c).

In the majority of embryos treated with nocodazole (82%, 23/28), mitochondria had extended into the

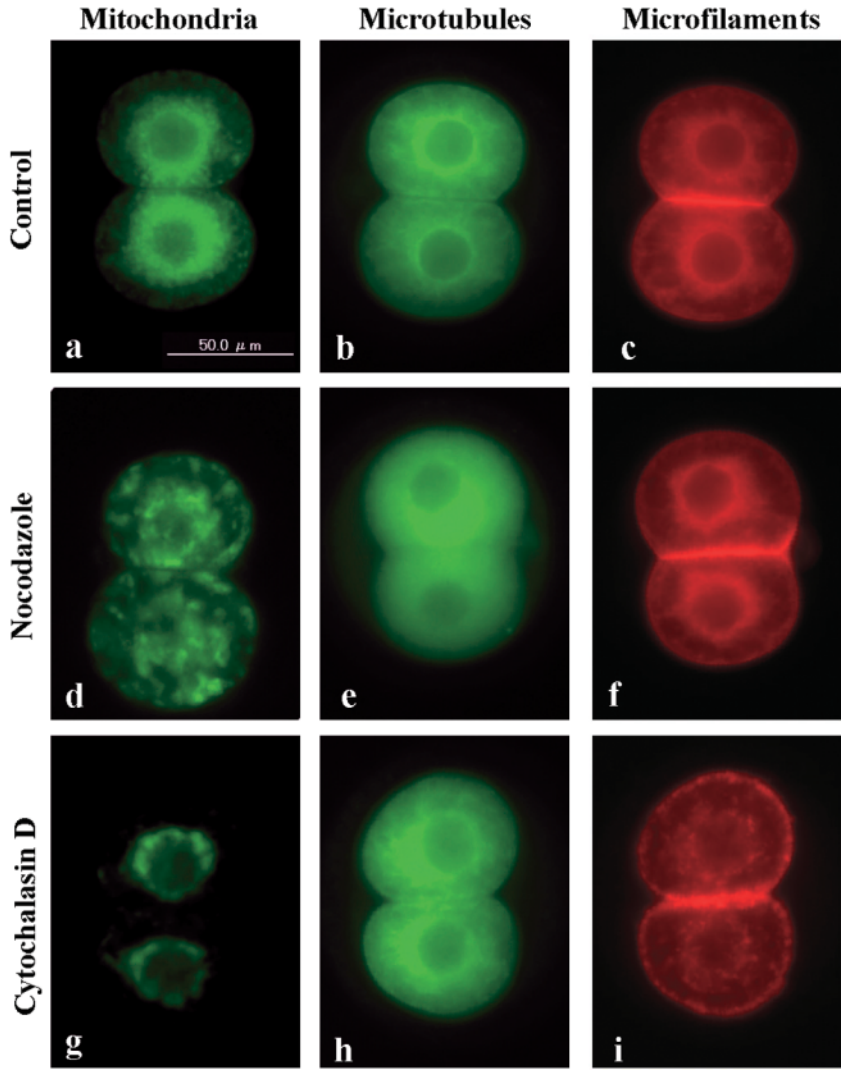

Fig. 2. Distributions of mitochondria, microtubules and microfilaments in hamster 2-cell embryos with or without nocodazole and/or cytochalasin D. The bar in (a) represents 50 μm for all micrographs. (a) A control embryo. Mitochondria are accumulated at the perinuclear region, while some mitochondria are noted in the cell cortex. (b) Microtubules are found around the nuclei. (c) Microfilaments locate with high density beneath the cell membrane and especially in the cell-to-cell contact region. (d) A nocodazole-treated embryo. Mitochondria have extended into the subcortical (intermediate) region. (e) Free tubulin stained evenly in the cytoplasm. (f) Microfilament organization is not affected by nocodazole. (g) A cytochalasin D-treated embryo. Distributional density of mitochondria at the peripheral region has decreased, and almost all mitochondria are seen only around the nuclei. (h) Microtubule organization is not altered by cytochalasin D. (i) Microfilaments are observed to be fragmented.

subcortical (intermediate) region with varying degree, where they were aggregated in patches (Fig. 2d). The remaining 5 embryos showed a perinuclear pattern similar to that seen in the controls. Microtubules had been disassembled, and free tubulin was stained evenly throughout the cytoplasm (n=17, Fig. 2e). Nocodazole did not affect the microfilament organization (n=17, Fig. 2f).

In contrast, cytochalasin D treatment decreased the distributional density of mitochondria at the peripheral

region, accordingly, almost all mitochondria were seen around the nucleus (n=21, Fig. 2g). Microfilaments were observed to be fragmented (n=17, Fig. 2i), but microtubules seemed to be unaffected by cytochalasin D (n=17, Fig. 2h). After combined treatment of both inhibitors, the distribution pattern of mitochondria (n=18) was similar to that observed in cytochalasin D-treated embryos, and changes in the microtubules and microfilaments resembled those described above (n=17, figure not shown).

Overall mean of the widths of the perinuclear clustering of mitochondria was 5.7 $\pm$ 0.2 μm in control embryos (n=17). However, the values were reduced significantly to 3.2 $\pm$ 0.1 μm (n=12) and 3.0 $\pm$ 0.1 μm (n=16) by treatment with cytochalasin D and both inhibitors, respectively (P<0.01). The nocodazole-treated embryos were not evaluated, because the perinuclear clustering was disrupted and mitochondria were scattered in the subcortical region in 82% of embryos treated with nocodazole.

Functional roles of microtubules and microfilaments in the translocation of mitochondria are expressed schematically in Fig. 3 in relation to the effects of nocodazole and cytochalasin D.

Discussion

Hamster 2-cell embryos are characterized by the perinuclear clustering of mitochondria, which continues until the 8-cell stage and then becomes obscure at the morula or blastocyst stages [5]. Mitochondria play an important role in supplying energy directly and rapidly to the nuclei for DNA replication and transcription [15]. The present study showed that the distribution of the microtubules was correlated with that of the mitochondria. Additionally, perinuclear clustering of the mitochondria was disrupted by nocodazole treatment, and unbounded mitochondria spreaded and/or aggregated into patches at random in the subcortical region. These results suggest that the accumulation of mitochondria in the perinuclear region is maintained by microtubules (see Fig. 3A).

The results of previous studies [5, 16, 17] and the present study show that the microfilaments located with high density beneath the cell membrane. It has been reported that the density of peripheral microfilaments was reduced during oocyte aging in pigs [16]. Webb *et al.* [18] observed that the peripherally-located meiotic spindle moved to the inner cytoplasm in aging mouse oocytes. The present study revealed that cytochalasin D treatment decreased mitochondria in density at the cell cortex; thus almost all mitochondria were seen around the nucleus. Treatment with both cytochalasin D and nocodazole gave a result similar to that observed after cytochalasin D treatment alone. It is well known that microfilaments regulate some cortical events, such as cortical granule migration, spindle orientation and polar body extrusion [19], suggesting that the cortical microfilaments may function as an anchor to the cell cortex and are required for those events. Our results suggest that the movement of mitochondria to the cell cortex is regulated by microfilaments. Therefore, destruction of microfilament organization in the cell cortex may have resulted in mitochondria moving back around the nucleus from the peripheral region (see Fig. 3B).

In the present study, the results suggest that both microtubules and microfilaments are involved in the distribution of mitochondria in hamster 2-cell embryos. Both cytoskeletal components are included in axonal transport of mitochondria [20]. In the axons, long-distance and fast transport of mitochondria requires microtubules, and mitochondria can also move along microfilaments in short-range movement [20]. Moreover, Calarco [21] has reported that the stabilization of microfilaments by jasplakinolide prevents mitochondrial relocation during mouse oocyte maturation. These observations suggest that the space-time reorganization of mitochondria may require interaction between microtubules and microfilaments. Translocation of organelles may be associated with the activity of specific motor proteins [20]. Kinesin and dynein motors drive along microtubules, and myosin acts as the actin-based motor. Further studies are needed to clarify the mechanism of mitochondrial translocation in the cytoskeletal network, including the function and regulation of the motor proteins during oocyte maturation, fertilization, and early embryonic development. Since mitochondrial functions can be a good marker for viability of embryos [22], understanding the mechanism of translocation of mitochondria in the ooplasm will provide useful information to improve embryo culture in mammals.

In conclusion, microtubules and microfilaments are mutually related in the configuration of mitochondria in hamster 2-cell embryos. Microtubules may function as the anchor of mitochondria at the perinuclear region, while microfilaments may play a role as the anchor of mitochondria at the peripheral region.

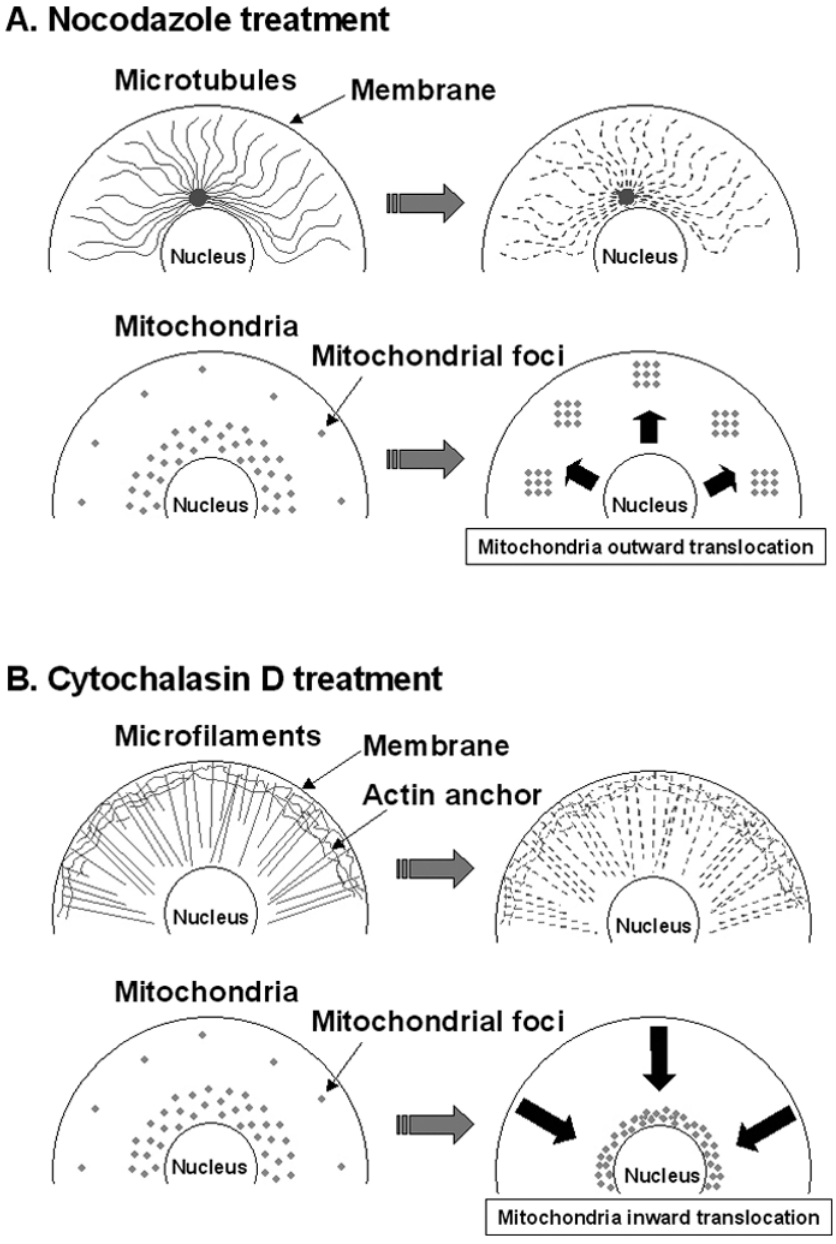

Fig. 3. Schematic diagram showing the effect of nocodazole and cytochalasin D on the distribution of microtubules, microfilaments and mitochondria in a hamster 2-cell embryo. (A) Effect of nocodazole on the distribution of microtubules (upper panel) and mitochondria (lower panel). After nocodazole treatment, the perinuclear anchor and transition track of microtubules are broken (changing from solid line to broken line); thus, mitochondria move outward. The distribution pattern of mitochondria has changed from the perinuclear clustering pattern (left panel) to the diffusion and aggregation pattern (right panel). (B) Effect of cytochalasin D on the distribution of microfilaments (upper panel) and mitochondria (lower panel). After cytochalasin D treatment, the cortical actin anchor is broken (changing from solid line to broken line); thus, peripheral and perinuclear mitochondria move back around the nucleus.

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