Distributions of Mitochondria and the Cytoskeleton in Hamster Embryos Developed In Vivo and In Vitro

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Abstract: *To clarify the causes of low viability of hamster embryos following in vitro culture, the present study compared the distributions of mitochondria and the cytoskeleton in embryos grown in vivo and in vitro. Hamster 2- and 4-cell embryos were characterized by perinuclear clustering of mitochondria, the degree of which was almost the same for in vivo and in vitro embryos. In the cell cortex and cell-to-cell contact region, however, microfilaments were located less densely in in vitro embryos than in in vivo ones. The nucleus moved towards the apex of the blastomere at the late 8-cell stage, when embryos begin the process of compaction. The density of mitochondria seemed to increase in the cell-to-cell contact region during this cellular rearrangement. Mitochondria were concentrated at the perinuclear region of in-vivo 8-cell embryos, whereas they were diffused into the subcortical region of in-vitro 8-cell embryos. Such a diffusion pattern of mitochondrial distribution was also noted in the morulae and blastocysts grown in vitro. These results show that both mitochondrial translocation and cytoskeletal reorganization did not proceed normally in the hamster embryos cultured in vitro, probably resulting in decreased viability of these embryos.*

Key words: *Cytoskeleton, Hamster Embryo, Mitochondria*

Introduction

The ability to support *in vitro* development of preimplantation embryos is basic for recent animal biotechnology. However, hamster embryos are very sensitive to culture milieus, which may seriously affect their viability after embryo transfer (see review [1]). In particular, the energy substrate requirements for hamster embryo development *in vitro* are markedly different from those of mouse embryos, and mitochondrial metabolism during preimplantation development must still be resolved. Translocation of active mitochondria, being associated with energy production, has some functional correlation with successful preimplantation development [1–5]. Under unsuitable culture conditions, mitochondria are distributed inappropriately, resulting in early embryonic failure [2–5]. Maternal age also influences the loss of mitochondrial activity, leading to lower embryonic development and pregnancy rates in humans [6]. Our previous study revealed that redistribution of the cytoskeleton in hamster embryos occurs during the process of embryonic compaction and is accompanied by an outward migration of blastomere nuclei [7]. Furthermore, it has been reported that the cytoskeletal elements are involved in the mechanism of mitochondrial translocation in mouse [3, 8], hamster [4], and pig embryos [9]. The present study was undertaken to compare the mitochondrial and cytoskeletal distributions between *in vivo* and *in vitro* embryos in the hamster. Understanding the relationship between the organelle-cytoskeletal network and the subsequent development of early embryos will contribute to improvement of embryo culture systems for the hamster.

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Materials and Methods

Collection and culture of embryos

Golden hamsters (*Mesocricetus auratus*), 10–12 weeks old, were superovulated as described previously [7]. Embryos at the 2-cell to blastocyst stages were

collected from oviducts and/or uteri with hamster embryo culture medium-3 supplemented with 1.0 mM hypotaurine (HECM-3ht) [10] equilibrated with 10% CO_2, 5% O_2, and 85% N_2 at 37.5°C. Two-cell embryos were recovered at 5:00–6:00 p.m. on day 2 of pregnancy, 4-cell embryos were recovered at 1:00–2:00 a.m. on day 3, early 8-cell embryos were recovered at 11:00–12:00 a.m. on day 3, late 8-cell embryos were recovered at 4:00–6:00 p.m. on day 3, and morulae and blastocysts were recovered at 0:00–1:00 a.m. on day 4 [7, 11].

Two-cell embryos were cultured in HECM-3ht in a humidified atmosphere of 10% CO_2, 5% O_2, and 85% N_2 at 37.5°C, and their development was checked at the time points mentioned above. In some cases, the culture period was extended to obtain embryos at the stage expected. Two-cell embryos cultured for 5 h were referred to as *in vitro* 2-cells. The experimental design was approved by the Ethical Committee for Experimentation with Animals, Hirosaki University.

Fluorescence observations

Active mitochondria were stained with rhodamine 123 (Rh123, 10 μg/ml, Sigma, St. Louis, MO, USA) for 15 min in HECM-3ht, as described previously [12], washed 3 times in HECM-3ht, mounted onto slide glasses, and imaged immediately after labeling. To assess the nuclear configuration and distribution of microtubules and microfilaments, the eggs were processed as reported previously [7]. After fixation in microtubule stabilization buffer, the samples were exposed overnight to primary monoclonal anti-β tubulin antibody (1:200; Sigma) at 5°C and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma) at 37°C for 2 h. After rinsing, the samples were stained with rhodamine-phalloidin (1:1000; Molecular Probes, Eugene, OR, USA) for microfilaments for 1 h and then stained for DNA with Hoechst 33342 (10 μg/ml) in mounting medium containing PBS and glycerol (1:1).

Fluorescence imaging

The samples were viewed under a fluorescent 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, and a U-MWU set (Olympus) was used for Hoechst. A cooled CCD video system (ImagePoint, Photometrics Ltd., Tucson, AZ, USA) was used to obtain images on a computer, and color adjustment was performed using the IPLab-Spectrum P software (Signal Analytics Corporation, Vienna, VA, USA).

Quantification of fluorescence intensity

To compare mitochondrial intensity among the specific stages, digital images were obtained at a constant exposure of 0.1 sec. The images of embryos stained with Rh123 (10–17 eggs for each stage) were analyzed by quantifying the average pixel intensities of the cytoplasm, according to a previous study [12]. The distribution density of microfilaments and microtubules was assessed by their fluorescence intensity. Briefly, embryos showing pixel intensity of more than 90% of the average intensity of *in vivo* embryos were referred to as having dense distribution of microfilaments and microtubules. As reported previously [7], blastomeres showing nuclear migration, the nuclei of which were located in the apical cytoplasm, were also recorded.

Statistical analyses

The mean mitochondrial intensities at different stages *in vivo* and *in vitro* were analyzed by ANOVA. Proportional data were analyzed by Chi-square test or Fisher's exact probability test.

Results

Distribution of active mitochondria and the cytoskeleton

A total of 421 embryos were analyzed for the distributions of their mitochondria (68 *in vivo* and 72 *in vitro* embryos) and cytoskeletons (135 *in vivo* and 146 *in vitro* embryos). In blastomeres of the 2-4-cell embryos grown *in vivo*, most mitochondria were accumulated in the perinuclear region, while few mitochondria were noted in the cell cortex (Figs. 1a, b). A similar distribution pattern was noted in *in vitro* embryos at the corresponding stages (Figs. 1a', b'), but microfilament staining was decreased in the cell cortex and cell-to-cell contact region of the *in vitro* embryos (Figs. 2a', b' vs. 2a, b). Surprisingly, the mitochondria of 2-cell embryos cultured for 5 hours had significantly decreased fluorescence intensity, approximately 10%, compared to the 2-cell embryos just after recovery (Table 1). A similar rate of decline was noted in the fluorescence intensity of *in vitro* early- and late-8-cell embryos compared with *in vivo* ones, but no significant difference was found at the 4-cell stage between *in vivo* and *in vitro* embryos (Table 1).

In the early 8-cell embryos grown *in vivo*, the cell cortex and perinuclear region showed intense mitochondrial staining (Figs. 1c-1, c-2). In *in vitro* embryos, however, mitochondria had extended into the

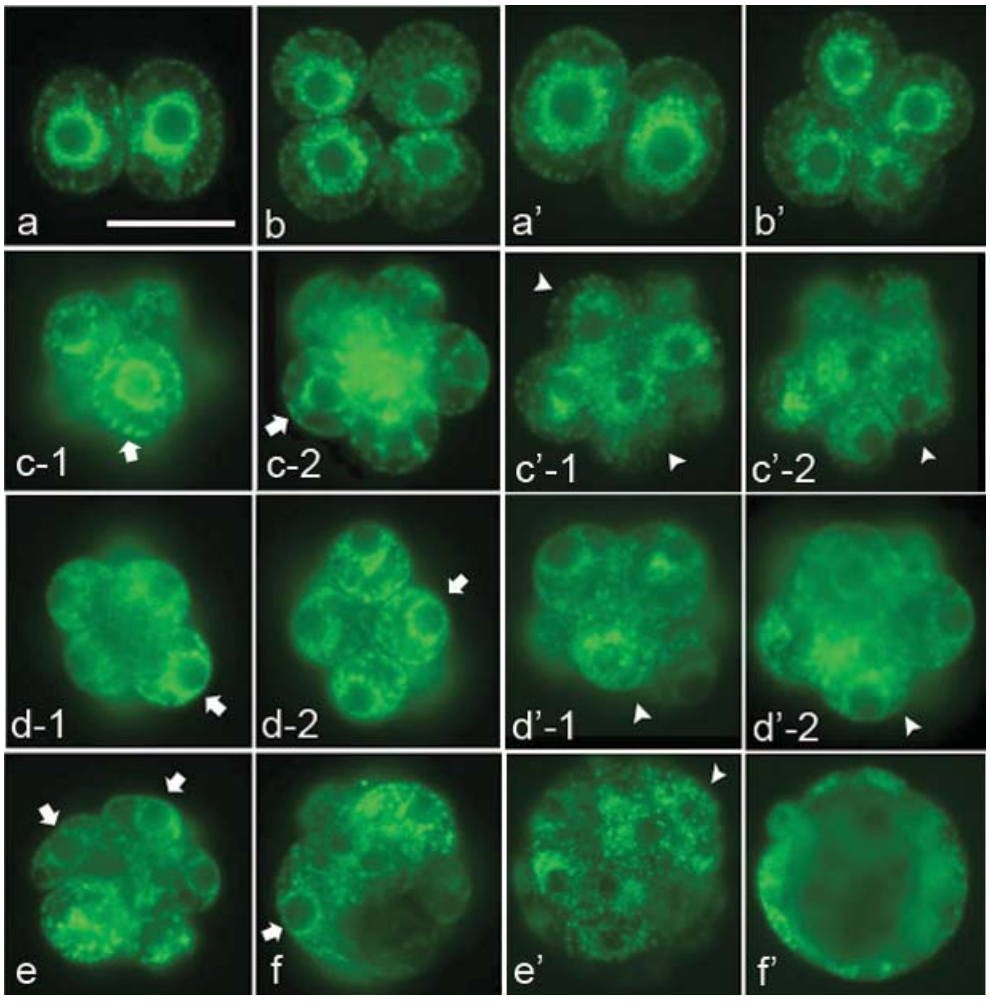

Fig. 1. Fluorescence micrographs showing the distribution of mitochondrial clustering in hamster embryos developed *in vivo* (a–f) and *in vitro* (a'–f'). The bar in (a) represents 50 μm for all micrographs. Two-cell embryos (a, a'); 4-cell embryos (b,b'); 3 blastomeres from an early 8-cell embryo *in vivo* (c-1); the other 5 blastomeres from the same embryo (c-2); 4 blastomeres from an early 8-cell embryo *in vitro* (c'-1); the other 4 blastomeres from the same embryo (c'-2); 4 blastomeres from a late 8-cell embryo *in vivo* (d-1); the other 4 blastomeres from the same embryo (d-2); 4 blastomeres from a late 8-cell embryo *in vitro* (d'-1); the other 4 blastomeres from the same embryo (d'-2); morulae (e, e'); blastocysts (f, f'). Two- and four-cell embryos are characterized by complete perinuclear clustering of active mitochondria with a thin cortical ring both *in vivo* (a, b) and *in vitro* (a', b'). In *in vivo* 8-cell embryos (c, d), the cortex became strongly stained with Rh123, and perinuclear clustering is still observed (arrows), whereas perinuclear mitochondria are dispersing into the middle layer in *in vitro* embryos, resulting in a wider distribution of mitochondria (c', d'). *In vitro* embryos display partial migration into the subcortical region (c', d', e'; arrowheads). The mitochondrial localization of morulae and blastocysts became difficult to evaluate because the signals overlapped each other due to increasing cell numbers. However, fluorescence intensity seems to decline slightly in *in vitro* embryos (e', f') compared to *in vivo* ones (e, f).

subcortical (intermediate) region at various degrees (Figs. 1c'–f'), showing a diffusion pattern for mitochondrial distribution after the 8-cell stage. In the late 8-cell stages onward, the nuclei of the blastomeres were located in the apex of the cells (Figs. 2d, d'), as reported previously [7]. In association with the outward migration of the nucleus, mitochondrial clusters also moved outwards (Figs. 1d, d'). But the percentage of

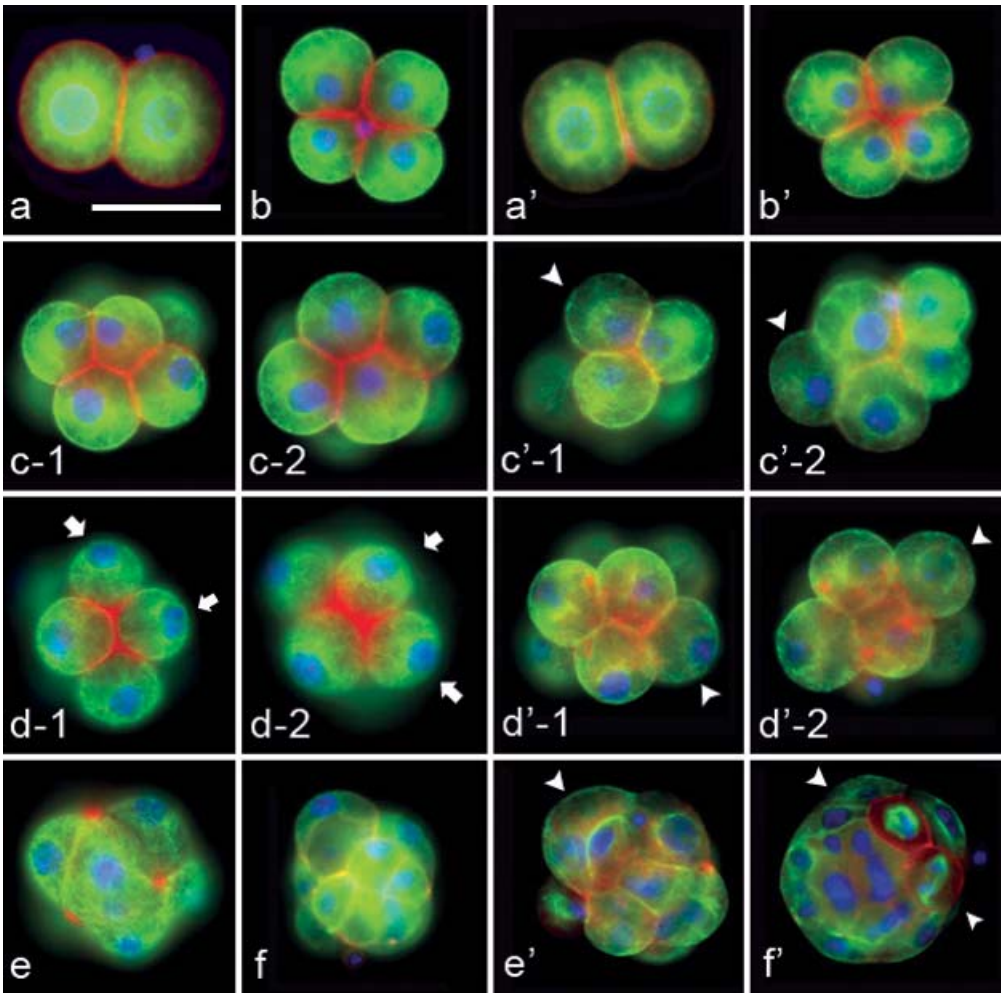

Fig. 2. Fluorescence micrographs showing the distribution of the nucleus and cytoskeleton of hamster embryos developed *in vivo* (a–f) and *in vitro* (a'–f'). The bar in (a) represents 50 μm for all micrographs. Microfilaments are red, microtubules are green, and nuclei are blue; yellow shows the distribution of microfilaments and microtubules. Two-cell embryos (a, a'); 4-cell embryos (b, b'); 4 blastomeres from an early 8-cell embryo *in vivo* (c-1); the other 4 blastomeres from the same embryo (c-2); 3 blastomeres from an early 8-cell embryo *in vitro* (c'-1); the other 5 blastomeres from the same embryo (c'-2); 4 blastomeres from a late 8-cell embryo *in vivo* (d-1); the other 4 blastomeres from the same embryo (d-2); 4 blastomeres from a late 8-cell embryo *in vitro* (d'-1); the other 4 blastomeres from the same embryo (d'-2); morulae (e, e'); blastocysts (f, f'). The nuclei of individual cells were located in the center region of the cytoplasm of 2-cell (a, a') and 4-cell (b, b') embryos and in most cells of the early 8-cell embryos (c, c'). As reported previously [7], outward migration of the nucleus of the blastomere is observed in the late 8-cell embryos (arrows). Such features are also noted in the outer cells of morulae (e, e') and blastocysts (f, f'). Decreased intensity in microfilaments and/or microtubules is frequently observed in the *in vitro* embryos from the 8-cell stage onwards (c', d', e', f'; arrowheads).

blastomeres showing nuclear migration was significantly higher in *in vivo* embryos than in *in vitro* embryos (94%, n=33 vs. 57%, n=37; P<0.01). After the 8-cell stage, the proportion of embryos showing a dense distribution of microfilaments or microtubules decreased more significantly *in vitro* than *in vivo* (Table 2).

In morulae and blastocysts under the conventional fluorescent microscope used in this study, it became difficult to identify the distribution of mitochondria and the cytoskeleton within each blastomere because the

Table 1. Fluorescence intensity of mitochondria in the hamster embryos*

Developmental Stage	*In vivo*		*In vitro*	
	No. of embryos	Mean ± SEM	No. of embryos	Mean ± SEM
2-cell	12	193.8 ± 4.8^{A}	10**	173.1 ± 5.0^{B}
4-cell	11	173.9 ± 2.4	13	171.1 ± 4.5
Early 8-cell	13	199.3 ± 8.7^{a}	12	177.9 ± 4.9^{b}
Late 8-cell	11	208.5 ± 3.1^{A}	13	187.7 ± 3.1^{B}

*The morula and blastocyst stages were not evaluated because the signals overlapped. **The 2-cell embryos were cultured for 5 h after recovery. Values with different superscripts are significantly different between *in vivo* and *in vitro* embryos ($p<0.05$ between a and b; $p<0.01$ between A and B).

Table 2. Percentage of embryos showing a dense distribution of the cytoskeleton*

Developmental Stage	*In vivo*			*In vitro*		
	No. of embryos	MFs	MTs	No. of embryos	MFs	MTs
2-cell	24	92	96	21	81	90
4-cell	26	85	92	33	88	79
Early 8-cell	32	84^{a}	100^{A}	35	57^{b}	69^{B}
Late 8-cell	33	94^{A}	97^{A}	37	49^{B}	30^{B}

*The morula and blastocyst stages were not evaluated because the signals overlapped. Embryos showing pixel intensity of more than 90% of the average intensity of *in vivo* embryos were referred to as having dense distribution for microfilaments (MFs) and microtubules (MTs). Values with different superscripts are significantly different between *in vivo* and *in vitro* embryos ($p<0.05$ between a and b; $p<0.01$ between A and B).

Table 3. Developmental schedule of hamster embryos grown *in vivo* and cultured in HECM-3ht

Developmental stage	*In vivo*	*In vitro*	
	Hours PEA*	Hours PEA*	No. (%) of embryos developed**
4-cell	43–44	44	149 (100)
Early 8-cell	54–55	55	146 (98.0)
Late 8-cell	60–61	61	144 (96.6)
Morula	64–65	68	131 (87.9)
Blastocyst	68–69	72	56 (37.6)

*Post-egg activation [11]. **Culture was initiated from 2-cell embryos. Times given for developmental stages are when approximately 50% of the embryos were at the stage shown.

fluorescence signals overlapped each other. At these stages, therefore, only the outer cells of the embryos, which were not overlapped, were able to be evaluated. Perinuclear clustering of mitochondria became obscure at the morula or later stages (Figs. 1e, f).

Fluorescence observations for different components within each blastomere revealed that microtubules were found around the nuclei, and the distribution of the mitochondria was somewhat correlated with that of the microtubules. Furthermore, some active mitochondria were found in the cell-to-cell contact region of *in vivo* embryos (Figs. 1c-2, 1d-1, d-2), where microfilaments were located in a high density, especially around the time of compaction (Figs. 2c-1, 2d-1, d-2). Decreased intensity of the cytoskeleton and diffused distribution of mitochondria were more frequently noted in *in vitro* embryos (Figs. 1c'–f', 2c'–f') than *in vivo* ones (Figs. 1c–f, 2c–f).

Developmental schedule of hamster embryos

Table 3 shows the timing of development of the hamster embryos grown *in vivo* and *in vitro*. The time scale was presented as hours post-egg activation (PEA) and was based on the estimated time after sperm penetration according to Bavister *et al.* [11]. A total of 149 embryos recovered at 2-cell stage were cultured.

All embryos (100%) developed to the 4-cell stage after 7–8 h of culture, namely 43–44 h PEA, to the early 8-cell stage 54–55 h PEA, and to the late 8-cell stage 60–61 h PEA. Thus, embryo development to the 8-cell stage was very similar between *in vivo* and *in vitro* embryos. More than 95% of embryos reached the late 8-cell stage. However, *in vitro* development was retarded around compaction; *in vivo* embryos developed to the morula stage 64–65 h PEA, whereas *in vitro* embryos developed to this stage 68 h PEA. Afterward, 87.9% of the embryos reached the morula or later stages and 37.6% developed into blastocysts. Once embryos reached the morula stage, no more delays were noted in formation of the blastocoele. Therefore, embryonic development was delayed at least 4 hours preferentially from the late 8-cell to morula stages under the culture condition used in this study.

Discussion

Our previous study showed that mitochondria in the ooplasm intensified their activities progressively from the germinal vesicle stage to the first metaphase stage and reorganized dramatically during maturation and fertilization in hamster oocytes [12]. The present study clearly demonstrated that the mitochondrial localization of embryonic cells changed after the 8-cell stage. At the late 8-cell stage, hamster embryos were characterized by concentration of mitochondria in the cell-to-cell contact region and perinuclear region in the *in vivo* embryos, but this was not clear in the *in vitro* embryos. Such heterogeneity in mitochondrial distribution may be associated with energy production/utilization for compaction. *In vitro* culture may change mitochondrial localization in the cytoplasm of hamster embryos, especially after the 8-cell stage, where the distributional density of microfilaments and microtubules is reduced. Furthermore, the percentage of blastomeres showing nuclear migration significantly decreased in *in vitro* embryos compared to *in vivo* ones (57% vs. 94%, $P<0.01$). This observation suggests that the nucleus migrates from the central cytoplasm to the apical cytoplasm around the late 8-cell stage [7], but some failure occurred in the mechanism of nuclear migration *in vitro*. Of particular interest was the observation that *in-vitro* embryonic development was delayed from the late 8-cell to morula stages, namely around the time of compaction. This suggests that the process of compaction may require increased energy production in a particular region of each blastomere and that culture conditions that disrupt energy production in the embryo may retard the compaction process. Previous studies have shown that changes in culture conditions can alter the organization or structure of mitochondria in the oocytes or embryos of hamsters [4, 13–15], mice [2, 3], and cattle [16, 17]. It has been reported that exposure of hamster 2-cell embryos to suboptimal culture conditions (addition of glucose and phosphate, which disrupts development) causes dispersion of mitochondria away from the nuclei [5, 13, 14]. The observations of the present study revealed that a similar dispersion of mitochondria was noted from the 8-cell stage onwards for hamster embryos cultured in HECM-3ht.

Translocation of mitochondria in oocytes/embryos is associated with reorganization of the cytoskeleton. However, experiments in which the cytoskeleton was disrupted with inhibitors have presented confusing results. Some researchers suggest that microfilaments play a role in the translocation of mitochondria [4], whereas others indicate that microtubules play a role in the translocation [8, 9]. In our preliminary observations of hamster 2-cell embryos, a treatment of cytochalasin D (a microfilament disruptor) caused mitochondria to disappear from the cortical cytoplasm, and a treatment of nocodazole (a microtubule disruptor) caused the breakup of perinuclear clustering of mitochondria. Therefore, both microfilaments and microtubules may be mutually related to spatial organization of the mitochondria in the cytoplasm of embryos. Further studies are needed to understand how mitochondrial reorganization is controlled during early embryonic development.

In conclusion, a combination of translocation of mitochondria and reorganization of the cytoskeletal networks may be involved in the developmental program of cytoplasmic alterations, and this type of organelle-cytoskeletal network was not maintained normally under the culture conditions used in this study. These findings may offer some explanation for low viability of hamster embryos cultured *in vitro*.

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