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—Full Paper—

Hatching and Distribution of Actin Filaments in Mouse Blastocysts Whose Activities of Protein Kinase A were Suppressed by H-89

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Abstract. The role of actin filaments and contractions in hatching was determined in mouse blastocysts whose actin filament bundling abilities had been suppressed by H-89, an inhibitor of protein kinase A. The hatching rate of blastocysts developed from morulae in a medium containing H-89 at a concentration of 4.0 μM was 17.2%, which was significantly lower than the 76.7% of the control blastocysts developed from morulae in a medium without H-89. The rates of blastocysts starting hatching and forming a slit in the zona pellucida were significantly lower in H-89-treated blastocysts (84.4 and 21.9%) than in control blastocysts (100.0 and 90.6%). The lengths of time needed for slit formation in the zona pellucida and for completion of hatching were significantly longer in the H-89-treated blastocysts (27.4 and 43.3 h) than in the control blastocysts (6.5 and 18.8 h). Over the course of 32 h after blastocoel formation, the number of strong contractions was similar in the H-89-treated and control blastocysts, but the number of weak contractions was significantly fewer in the H-89-treated blastocysts (2.41 times) than in the control blastocysts (4.19 times). Although the distribution of actin filaments was similar in the H-89-treated and control blastocysts in the pre-hatching, hatching and post-hatching periods, the rate of H-89-treated blastocysts in which most trophectoderm cells possessed the fluorescence of actin filaments (12.7%) was significantly lower than the 95.0% of the control blastocysts in the pre-hatching period. These results suggest that actin filament-mediated movements of trophectoderm cells contribute to hatching by facilitating the protrusion of trophectoderm cells from a small hole in the zona pellucida and by enlarging the protrusion. We also suggest that the low hatching ability of the treated blastocysts is related to weak contractions with a low frequency and to strong contractions requiring a longer time for re-expansion.

Key words: Blastocyst hatching, Bundling of actin filament, Contraction, Protein kinase A, Time-lapse videomicrography

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It is generally accepted that hatching of mouse blastocysts is accompanied by regional dissolution of the zona pellucida by a trypsin-like proteinase synthesized in trophectoderm cells [1, 2] and that trophectoderm cells protrude from the dissolved hole of the zona pellucida [3–5]. A slit is then formed in the zona pellucida from the hole by enlargement of the protruding trophectoderm cells [3–5]. During hatching, the blastocyst repeatedly contracts, leading to enlargement of the slit, and then escapes from the zona pellucida [3–5].

Generally, it is known that most animal cell motility is induced molecularly by the involvement of actin and that actin filaments, one of the cytoskeletal proteins, play a role in cell movements [6, 7]. Using HeLa cells, Glenn and Jacobson [8, 9] have clarified that the activity of protein kinase C (PKC) triggers actin polymerization leading to cell spreading and that the action of protein kinase A (PKA) leads to bundling of polymerized actin (actin filaments) in the cortical cytoplasm, and this cytoskeletal organization correlates with cell motility. In blastocysts, it is also believed that trophectoderm cell motility is involved in hatching through the action of actin filaments [10, 11]. That is, actin filaments are distributed abundantly in mouse blastocysts during the hatching period compared with the before and after hatching periods and are densely packed, especially in the cortical cytoplasm of trophectoderm cells protruding from the zona pellucida [10]. Therefore, in mouse blastocysts treated with cytochalasin B (CB), which is the inhibitor of actin filament polymerization, the distribution of actin filaments changes [10], and changes also occur in contractions that increase the protrusion of trophectoderm cells from the zona pellucida and the protruded trophectoderm [11]. As a result, hatching is inhibited. Based on results concerning the distribution of actin filaments in mouse blastocysts treated with CB, Cheon *et al*. [10] suggested that dynamic polymerization of actin molecules in trophectoderm cells is essential for blastocyst hatching and that actin filament-mediated movements of trophectoderm cells play an important role in the hatching process of mouse blastocysts.

On the other hand, it is known that actin filaments mostly exist in the cytoplasm in the form of a bundle and that the bundling of actin filaments is promoted by PKA activated by cAMP [8, 9, 12]. It is therefore considered that in blastocysts whose PKA activity has been suppressed, hatching will be inhibited and changes will occur in the distribution of actin filaments. Nevertheless, there have been no observations of the hatching process or the distribution of actin filaments in blastocysts whose PKA activity has been suppressed in order to determine the role of actin filaments in hatching.

Although the physiological role of contraction in blastocysts is unknown, contraction has been observed by time-lapse microcine-

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matography or time-lapse videomicrography in cultured blastocysts of rabbits [13], mice [14–20], rats [21], guinea pigs [22], hamsters [23, 24] and cattle [25–27], and it is considered to participate in the process of blastocyst hatching [15–20]. The authors [17] ascertained in mice that during the period up to 32 h after blastocoel formation, there is no difference in the number of weak contractions (less than 20% volume reduction) between blastocysts completely hatched and incompletely hatched, but the number of strong contractions (more than 20% volume reduction) is significantly fewer in completely hatched blastocysts than in incompletely hatched blastocysts. Furthermore, the authors have previously reported that when mouse blastocysts were treated with indomethacin [18], soybean trypsin inhibitor (STI) [19] or CB [11], with have the effect of inhibiting, respectively, the prostaglandin synthesis, trypsin-like proteinase activity and actin polymerization that are related to blastocyst hatching, the hatching rate significantly decreased after all treatments compared with untreated blastocysts, and either the number of weak contractions significantly decreased or the number of strong contractions significantly increased. From these findings, the authors suggested that of the contractions seen in blastocysts, weak ones are physiological and promote hatching, while strong contractions have the influence of inhibiting hatching. Therefore, it was inferred that the strength and frequency of contractions, which were thought to be associated with blastocyst hatching [11, 17–19], might be altered in blastocysts in which hatching is suppressed by an inhibitor of PKA.

In the present study, the hatching process, distribution of actin filaments and contraction were examined in mouse blastocysts whose actin filament bundling abilities had been suppressed by H-89, an inhibitor of PKA [28], and were compared with those of blastocysts cultured in a medium without H-89 in order to determine the role of actin filaments and contractions in blastocyst hatching.

Materials and Methods

Animals

Eighty female mature mice of the ICR strain were used in the present study. They were housed in autoclaved metal cages and were given a standard chow (MF, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room (24 C) under controlled lighting conditions (14L/10D; L: 0400 to 1800 h). They received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Niigata University Animal Care Committee). These females were intraperitoneally injected with 5 i.u. of PMSG (Serotropin®, Teikoku Hormone Manufacturing, Tokyo, Japan) and subsequently with 5 i.u. of hCG (Gonatropin®, Teikoku Hormone Manufacturing) 48 h later to induce superovulation. Immediately after the hCG injection, these females were mated with mature males of the same strain.

Determination of the H-89 concentration necessary to inhibit blastocyst hatching

In observing contraction of blastocysts, as in the present study, embryos should be cultured before development to the blastocyst stage because images of individual blastocysts over time in the period soon after blastocoel formation are necessary for analysis of contractions. Therefore, a concentration of H-89 at which the H-89 had no effect on the development of morulae to blastocysts, but significantly inhibited the completion of hatching in the resultant blastocysts, was first determined. Morulae were collected from oviducts and uteri of superovulated and mated females 72 h after the hCG injection and were cultured in M16 medium [29] containing H-89 (LKT Laboratories, St. Paul, MN, USA) at 1.0, 2.0, 3.0, 4.0 or 5.0 μM in a CO_2 incubator (5% CO_2 in air) at 37 C. The H-89 had previously been dissolved in DMSO. Morulae cultured in M16 medium containing 0.2% DMSO, but devoid of H-89, were used as controls.

Development of morulae to blastocysts and completion of hatching in the resultant blastocysts were observed after 24 and 100 h of culture, respectively.

Observation of the hatching process and contraction in blastocysts

In order to observe the hatching process and contraction in blastocysts, morulae were collected 72 h after the hCG injection and cultured in M16 medium without H-89 or in M16 medium containing H-89 at a concentration of 4.0 μM. These morulae were cultured in a CO_2 culture chamber (SK-1, Sankei, Tokyo, Japan; 5% CO_2 in air) equipped with an inverted microscope (Diaphot, Nikon, Tokyo, Japan) at 37 C. To analyze the hatching process and contraction of H-89-treated and control blastocysts, observations were performed on images taken at 4-sec intervals by a CCD color camera (Hitachi, Tokyo, Japan) connected to an inverted microscope and recorded by a time-lapse video cassette recorder (BR-9050, Victor Company of Japan, Yokohama, Japan).

The hatching process was observed in the images of both H-89-treated and control blastocysts until 100 h after culture.

The analyses of contractions were performed until 32 h after blastocoel formation, according to the method described in our previous reports [9, 17–19]. The degree of contraction was evaluated as follows: the volume of each blastocyst was calculated from its diameter on a display and measured with a micrometer according to the method of Hurst and MacFarlane [30]. The percentage of volume reduction at the time of contraction from the volume before contraction was classified as weak when the percentage was less than 20% (2.0 to 19.9%) and classified as strong when the percentage was 20.0% or more. Since blastocysts are not completely spherical in shape, the mean value for the short and long axes of each embryo was used as the diameter of the embryo.

The lengths of time needed for contraction and re-expansion were analyzed in images for all weak and strong contractions recorded for 32 h after blastocoel formation in both the H-89-treated and control blastocysts.

Demonstration of actin filaments

Morulae were collected 72 h after the hCG injection and cultured in M16 medium without H-89 or in M16 medium with 4.0 μM H-89. These morulae were cultured in a CO_2 incubator (5% CO_2 in air) at 37 C. After culture, blastocysts at the stages of expanded (pre-hatching), hatching and post-hatching were collected and fixed in PBS (pH 7.4) [31] containing 3.7%

Table 1. Effects of H-89 on the development of mouse morulae and on hatching of the resulting blastocysts

Concentrations of H-89 (μM)	No. of morulae cultured	No. (%) of morulae developed into blastocysts	No. (%) of blastocysts that completed hatching
0.0	31	30 (96.8)[a]	23 (76.7)[a]
1.0	30	26 (86.7)[a]	11 (42.3)[b]
2.0	30	29 (96.7)[a]	12 (41.4)[b]
3.0	32	28 (87.5)[a]	8 (28.6)[bc]
4.0	34	29 (85.3)[a]	5 (17.2)[cd]
5.0	33	20 (60.6)[b]	1 (5.0)[d]

The development of morulae to blastocysts and completion of blastocyst hatching were observed after 24 and 100 h of culture, respectively. Values with different superscripts in the same column are significantly different ($P<0.05$).

formaldehyde at room temperature for 30 min. They were then rinsed in a PBS and immersed in a PBS containing 0.25% Tween-20 (Bio-Rad Laboratories, NY, USA) for 5 min at room temperature. They were subsequently rinsed in a PBS again and then immersed in 100 μl PBS containing 16.5 ng phallacidin (Molecular Probes, Carlsbad, CA, USA) at room temperature for 20 min. A treated blastocyst was placed in the center of 4 Vaseline spots on a slide. A cover slip was then carefully placed on the Vaseline spots and pressed gently to anchor the embryo in between the cover slip and the slide. Observation was carried out under a reflected-light fluorescence microscope (Optiphot, Nikon).

Statistical analysis

The rate of development of morulae to blastocysts and the rate of hatching of the resulting blastocysts were statistically analyzed by the Chi-square test. The incidence of blastocysts in which most trophectoderm cells possessed fluorescence of actin filaments was also statistically analyzed by the Chi-square test. The number of contractions, length of time needed by blastocysts to contract and re-expand and length of time needed by blastocysts to hatch were statistically analyzed by one-way analysis of variance. A value of $P<0.05$ was considered to be statistically significant.

Results

Blastocyst formation and hatching

When morulae were cultured in media containing H-89 at 1.0, 2.0, 3.0 and 4.0 μM, 85.3 to 96.7% of the embryos developed to blastocysts, showing no difference from the developmental rate (96.8%) of the control morulae cultured in a medium without H-89 (Table 1). The rate of development to blastocysts from morulae cultured with H-89 at 5.0 μM was 60.6%, which was significantly lower than that in the control morulae. The hatching rates of blastocysts developed from morulae in a medium containing H-89 at 1.0 to 5.0 μM were 5.0 to 42.3%, which were all significantly lower than the 76.7% rate of the control blastocysts developed from morulae in a medium without H-89. In view of these results, the medium containing 4.0 μM H-89 was used in subsequent experiments because H-89 did not affect the development of morulae to blastocysts and had its maximum inhibitory effect on hatching of the resultant blastocysts at this concentration.

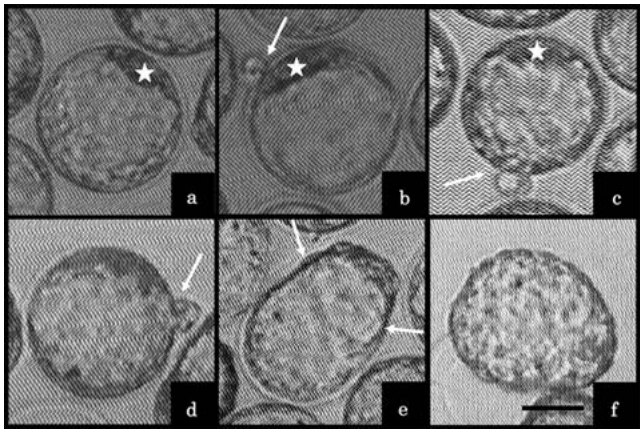

Fig. 1. Time-lapse videomicrographs of control mouse blastocysts developed from morulae in a medium without H-89. Asterisks show the inner cell mass. Scale bar indicates 50 μm. (a) Before hatching. (b) Protrusion of cells (arrow) from a small hole in the zona pellucida occurs at the embryonic polar trophectoderm. (c) Protrusion of cells (arrow) from a small hole in the zona pellucida occurs at the abembryonic polar trophectoderm. (d) Protrusion of cells (arrow) from a small hole in the zona pellucida occurs at the mural trophectoderm. (e) A slit (arrows) formed by enlarging a small hole is seen in the zona pellucida. (f) The blastocyst completely escapes from the zona pellucida.

Process of blastocyst hatching

In 32 control blastocysts (Fig.1a) developed from morulae in a medium without H-89, a small hole formed in the zona pellucida of all the blastocysts 30.2 h after blastocoel formation (Fig.1b–d), and these blastocysts started hatching by protruding trophectoderm cells through the hole. Blastocysts with a small hole in the zona pellucida increasingly protruded trophectoderm cells through the hole, and 29 blastocysts (90.6%) formed a slit in the zona pellucida by enlarging the hole 6.5 h after the start of hatching (Fig.1e). In the blastocysts that formed a slit in the zona pellucida, trophectoderm cells continuously escaped through the slit, and 25 blastocysts (78.1%) completed hatching 18.8 h after the start of hatching (Fig.1f). On the other hand, 84.4% (27/32) of H-89-treated blastocysts also protruded trophectoderm cells from a small hole in the zona pellucida 27.3 h after blastocoel formation, and these blasto-

Table 2. Numbers of contractions in cultured mouse blastocysts

Blastocysts	No. of blastocysts examined	Degree of contraction	
		Weak	Strong
Control	32	$4.19 \pm 0.31^{a*}$	0.81 ± 0.13^{a}
H-89-treated	32	2.41 ± 0.33^{b}	0.66 ± 0.13^{a}

*Mean ± S.E. The observed blastocysts were developed from morulae in M16 medium containing H-89 at a concentration of 4.0 μM (H-89-treated) or in M16 medium without H-89 (control). The degree of contraction was divided into two based on the percentages of volume reduction of the blastocysts: less than 20%, weak: more than 20%, strong. The number of contractions was counted for 32 h after blastocoel formation. Values with different superscripts in the same column are significantly different ($P<0.05$).

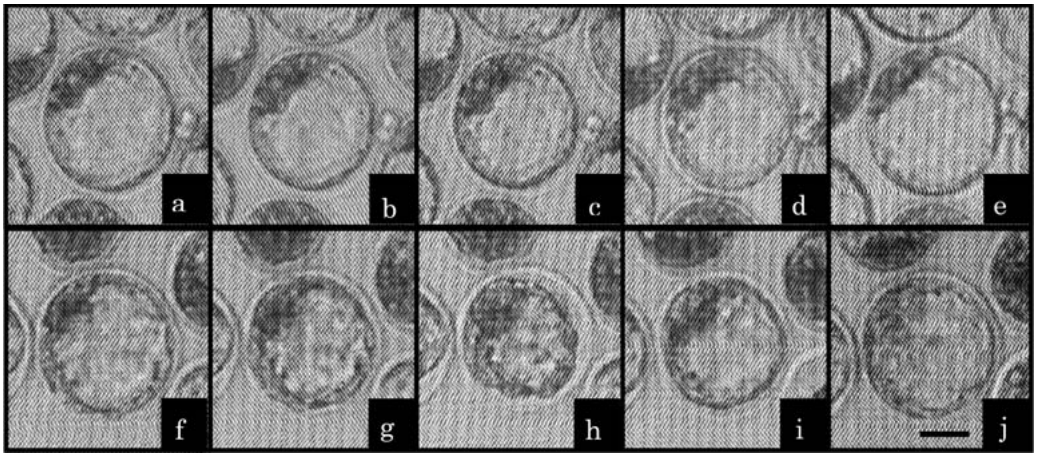

Fig. 2. Time-lapse videomicrographs of control mouse blastocysts showing a weak contraction (a–e) and a strong contraction (f–j). The blastocysts showing the weak and strong contractions required a maximum of 8 min (a to c) and 46 min (f to h) to contract, and 72 min (c to e) and 171 min (h to j) to expand to the size before contraction, respectively. Scale bar indicates 50 μm. (a, f) Before contraction. (b, g) During contraction. (c, h) At the time of contraction. (d, i) During re-expansion. (e, j) At the time of re-expansion.

cysts started hatching. Of these 27 H-89-treated blastocysts, only 7 formed a slit in the zona pellucida by enlarging the hole 27.4 h after the start of hatching, and 4 blastocysts completed escape through the slit 43.3 h after the start of hatching.

The rate of blastocysts with protrusion of trophectoderm cells from a small hole in the zona pellucida and that of blastocysts with a slit in the zona pellucida formed by enlarging the protrusion of trophectoderm cells were significantly lower in H-89-treated blastocysts (84.4 and 21.9%) than in control blastocysts (100.0 and 90.6%). In both H-89-treated and control blastocysts, protrusion of cells from zonae pellucidae occurred in the polar, abembryonic polar or mural trophectoderm, and there were no significant differences in the rates of H-89-treated and control blastocysts with protrusion from any of these cells. The lengths of time needed for small hole formation in the zona pellucida after blastocoel formation and for completion of hatching after slit formation in the zona pellucida in H-89-treated blastocysts (27.3 and 14.9 h) were similar to those in the control blastocysts (30.2 and 11.8 h). However, H-89-treated blastocysts took a significantly longer time for slit formation in the zona pellucida after the start of hatching (27.4 h) and for completion of hatching after its start (43.3 h) than control blastocysts (6.5 and 18.8 h), respectively.

Number of contractions

Both H-89-treated and control blastocysts began to contract and re-expand repeatedly from 7.6 or 10.3 h after blastocoel formation, respectively. The numbers of contractions until 32 h after blastocoel formation are shown in Table 2. The number of weak contractions (Fig. 2a–e) was significantly fewer in the H-89-treated blastocysts (2.41 times) than in the control blastocysts (4.19 times), whereas the number of strong contractions (Fig. 2f–j) did not differ between the H-89-treated and control blastocysts.

As shown in Table 3, the mean lengths of time required for weak contractions and for re-expansions following weak contractions were similar for both the H-89-treated and control blastocysts. In the strong contractions, the mean length of time required for contractions was similar for both the H-89-treated and control blastocysts, whereas that for re-expansion was significantly longer in the H-89-treated blastocysts than in the control blastocysts.

Table 3. Length of time needed by cultured mouse blastocysts to contract and re-expand

Blastocysts	Weak contraction			Strong contraction		
	No. of contractions examined	Time required for contraction (min)	Time required for re-expansion (min)	No. of contractions examined	Time required for contraction (min)	Time required for re-expansion (min)
Control	134	6.6 ± 1.48[a]*	69.6 ± 4.33[a]	26	39.0 ± 12.53[a]	184.5 ± 17.14[b]
H-89-treated	77	8.4 ± 4.03[a]	77.0 ± 6.79[a]	21	29.6 ± 12.10[a]	259.4 ± 32.31[a]

*Mean ± S.E. The observed blastocysts were developed from morulae in M16 medium containing H-89 at a concentration of 4.0 μM (H-89-treated) or in M16 medium without H-89 (control). Values with different superscripts in the same column are significantly different ($P<0.05$).

Distribution of actin filaments

Fluorescence indicating the presence of actin filaments was observed in the cytoplasm of trophectoderm and inner-cell-mass cells in both the H-89-treated (Fig. 3a, e, g) and control blastocysts (Fig. 3c) and was especially strong in the peripheral cytoplasm of trophectoderm cells (Fig. 3a, c, e, g). In blastocysts during the hatching period, actin fluorescence was much brighter in protruded trophectoderm cells in the region of a small hole or slit in the zona pellucida (Fig. 3e).

In the hatching and post-hatching periods, most trophectoderm cells of both the H-89-treated and control blastocysts possessed fluorescence of actin filaments (Fig. 3e, g), and the rates of blastocysts in which most trophectoderm cells possessed fluorescence of actin filaments were similar for the H-89-treated and control blastocysts (Table 4). In the pre-hatching period, however, the rate of H-89-treated blastocysts (12.7%) was significantly lower than the 95.0% of the control blastocysts, and the H-89-treated blastocysts had many trophectoderm cells devoid of fluorescence of actin filaments (Fig. 3a).

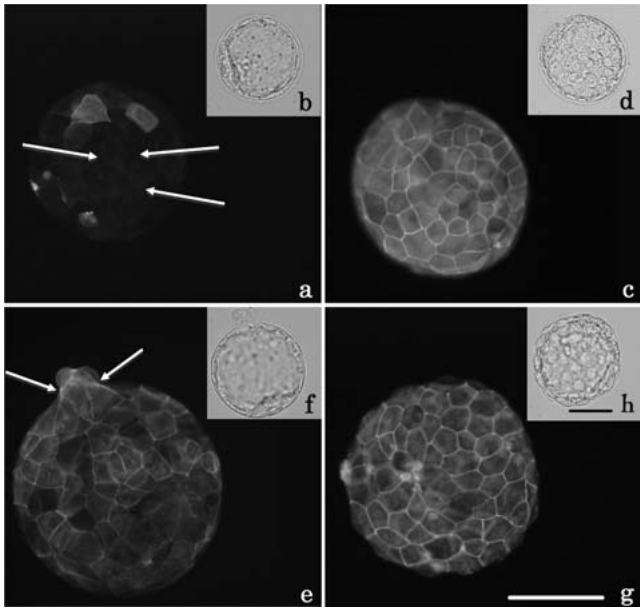

Fig. 3. Fluorescent micrographs (a, e, g) of mouse blastocysts developed from morulae in a medium with H-89 and a fluorescent micrograph (c) of a mouse blastocyst developed from a morula in a medium without H-89 (control) and stained with phallacidin. Light micrographs (b, d, f, h) of mouse blastocysts taken by fluorescent microscope (a, c, e, g). Scale bars indicate 50 μm. (a, b) A blastocyst at the expanded stage (pre-hatching period). Most trophectoderm cells (arrows) in the blastocyst are devoid of fluorescence indicating the presence of actin filaments. (c, d) A blastocyst at the expanded stage (pre-hatching period). Most trophectoderm cells in the blastocyst possess fluorescence of actin filaments. (e, f) A blastocyst in the hatching period. Most trophectoderm cells in the blastocyst possess fluorescence of actin filaments. The fluorescence of actin filaments is much brighter in protruded trophectoderm cells (arrows) from the zona pellucida. (g, h) A blastocyst in the post-hatching period. Most trophectoderm cells in the blastocyst possess fluorescence of actin filaments. The fluorescence of actin filaments is strong in the peripheral cytoplasm of each trophectoderm cell.

Discussion

Recently, it has been suggested that actin filament-mediated movements of trophectoderm cells [10] and contractions [11, 15–20] play important roles in the hatching of mouse blastocysts. It is known that actin filaments mostly exist in the cytoplasm in the form of a bundle, and PKA plays a role in the bundling of actin filaments [8, 9, 12]. Therefore, it is considered that in blastocysts whose PKA activity has been inhibited, hatching will be suppressed and changes will occur in the hatching process and contraction.

In the present study, it was clarified that the rates of H-89-treated blastocysts starting hatching, forming a slit in the zona pellucida and completing hatching were all significantly lower than those of control blastocysts and that the lengths of time needed for slit formation in the zona pellucida and for completion of hatching were significantly longer in H-89-treated blastocysts than in control blastocysts. From these findings, it was considered that in blastocysts in which the bundling of actin filaments has been inhibited, actin filament-mediated movements of trophectoderm cells required to form a small hole and a slit in the zona pellucida were inhibited, and as a result, hatching could not be completed. Therefore, the former findings in CB-treated blastocysts [11], together with the results of the present study with regards to the hatching process of H-89-treated blastocysts, strongly suggest that both the polymerization of actin and bundling of actin filaments are essential for blastocyst hatching and that actin filament-mediated movements of trophectoderm cells contribute to hatching by facilitating the protrusion of trophectoderm cells from a small hole in the zona

Table 4. The incidence of cultured mouse blastocysts in which most trophectoderm cells possessed fluorescence of actin filaments

Blastocysts	Hatching periods		
	Pre-hatching	Hatching	Post-hatching
Control	95.0 (38/40)[a]*	82.9 (34/41)[a]	92.0 (23/25)[a]
H-89-treated	12.7 (7/55)[b]	68.9 (31/45)[a]	75.0 (9/12)[a]

*The percentage of blastocysts with the numbers in parentheses. The observed blastocysts were developed from morulae in M16 medium containing H-89 at a concentration of 4.0 μM (H-89-treated) or in M16 medium without H-89 (control). Values with different superscripts in the same column are significantly different ($P<0.05$).

pellucida and by enlarging the protrusion for slit formation in the zona pellucida.

In the present study, we investigated the contraction of blastocysts whose hatching abilities were suppressed by H-89 and found that the number of weak contractions was significantly fewer than in control blastocysts. The time required for re-expansion following weak contractions in blastocysts treated with H-89 was comparable to that in control blastocysts, but it was significantly longer following strong contractions compared with control blastocysts. On the other hand, in mouse blastocysts in which hatching ability was suppressed by CB, it has been reported that the number of strong contractions was larger than in control blastocysts, but the number of weak contractions was no different from that seen in control blastocysts [11]. It was also demonstrated that the time required for re-expansions following weak and strong contractions in CB-treated blastocysts did not differ from that of control blastocysts [11]. Although it has remained unclear how actin filament-mediated movements of trophectoderm cells are related to contractions of blastocysts, it is suggested that the bundling of actin filaments has a greater role in contraction and re-expansion.

In mouse blastocysts whose hatching ability was suppressed by CB [11] or STI [19], it has been reported that small holes were formed in the zona pellucida by protrusion of trophectoderm cells, but these small holes did not expand in all the blastocysts, so no slits were formed, and as a result, hatching could not be completed. Also in the present study, significantly fewer H-89-treated blastocysts formed small holes in the zona pellucida, and there were remarkably few that formed slits in the zona pellucida. As a result, most blastocysts were clearly prevented from hatching. This suggests that in blastocysts in which the bundling of actin filaments has been inhibited, contractions required to form a slit in the zona pellucida are suppressed, and hatching cannot be completed. The effects of CB [11], STI [19] and H-89 on contractions also differ, but slits are not formed in the zona pellucida of most blastocysts treated with any of these agents, so it is considered that formation of a slit in the zona pellucida by contractions is essential for hatching.

It is generally thought that contraction of vascular endothelial cells is physiologically induced through activation of myosin light chain kinase (MLCK) by calcium-calmodulin and phosphorylation of MLC by activated MLCK [32–34]. It has also been suggested in mouse blastocysts that MLCK plays a role in physiological (weak) contraction and that permeability increases between trophectoderm cells due to contraction of trophectoderm cells by the action of both actin filaments and myosin filaments having a light chain phosphorylated by MLCK; this has been suggested to causes an efflux of blastocoelic fluid, leading to blastocyst contraction [35]. In the present study, we suggested that the interaction in actin and myosin filaments is affected in blastocysts treated with H-89 and that this is the reason for reduced number of weak contractions in the treated blastocysts. It was also confirmed in the present study that the time required for re-expansion following strong contractions is significantly longer in blastocysts treated with H-89 than in control blastocysts. Considering the effect of H-89 on blastocyst contractions, the low ability of hatching in H-89-treated blastocysts is closely related to weak contractions that are less frequent because weak contractions are known to promote hatching, as described above [11, 17–19]. Also, the low hatching ability in H-89-treated blastocysts is thought to be due to strong contractions, which require a much longer time for re-expansion.

Cyclic adenosine monophosphate-dependent PKA is considered to be a major signal transduction enzyme in essentially all eukaryotic cells that regulates many cell functions including energy metabolism, gene transcription, proliferation, differentiation and secretion in addition to contractility and motility [36]. Therefore, the low hatching ability in H-89-treated blastocysts seems to be caused by not only their low motility but also their defects in cellular functions. However, the relationship between cellular functions other than cell motility and hatching ability in H-89-treated blastocysts remains unknown. This issue should be further studied.

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