Zygote **7** (May), pp 123–129. © 1999 Cambridge University Press Printed in the United Kingdom

Role of actin filaments in the hatching process of mouse blastocyst

Yong Pil Cheon[1,2], *Myung Chan Gye*[3], *Chung-Hoon Kim*[2], *Byung Moon Kang*[2], *Yoon Seok Chang*[2], *Sung Rye Kim*[4] *and Moon Kyoo Kim*[1]

Date submitted: 29.7.98. Date accepted: 3.9.98

Summary

Hatching has been suggested to occur as a result of protease-mediated lysis and the blastocoele tension. However, even if rupturing is initiated at multiple sites, interestingly only a single site is used for escape. This implies that there are several mechanisms involved in hatching. In this study, the involvement of actin filaments in mouse embryo hatching was examined. We treated mouse embryos with cytochalasin B for 12 h or 24 h at the morula, middle blastocyst, expanded blastocyst, lobe-formed blastocyst and hatching blastocyst stages, and measured the amount and distribution of actin filaments using a confocal microscope. At morula, middle blastocyst, lobe-formed blastocyst and hatching blastocyst stages embryonic development was completely arrested by cytochalasin B. However, when transferred to cytochalasin-B-free medium, the embryos resumed development and escaped the zona pellucida. In the expanded blastocysts development was almost completely inhibited by cytochalasin B, but rupturing occurred in some embryos. However, development stopped completely at the ruptured stage. Distribution of actin filaments was prominent at rupturing and hatching sites regardless of cytochalasin B treatment. The amount of actin filaments was prominent at hatching embryos compared with other developmental stages of embryos. These actin filaments were distributed intensively between the trophectodermal cells, and formed locomotion patterns. Taken together, these results suggest that not only tension and lytic enzymes are required to rupture, but the activity of actin filaments may have a crucial role in the process of hatching.

Keywords: Actin filament, Cytochalasin B, Hatching, Rupture, Zona pellucida (ZP)

Introduction

Activated oocytes undergo differentiation through the cleavage and polarisation induced by developmental stage-specific gene expression and the microenvironmental changes in the reproductive tract. Briefly, before implantation the blastocyst is either outside of the zona pellucida (ZP) (rat, pig, ruminants), still within the ZP (guinea pig, ferret, primates), or enveloped by glycoprotein coats that replace the ZP (rabbit, horse) (Guillomot *et al.*, 1993). The stage of pregnancy at which implantation occurs is different among species and is not related to the duration of gestation. However, the early phases of implantation are common to all species. The communication between trophectoderm and uterine epithelial cells is increased throughout hatching. During this process, both the embryo and the uterus prepare for implantation.

Hatching is regulated by uterine and/or embryonic factors. In hamster embryos hatching is dependent on the uterine environment (Gonzales & Bavister, 1995). However, pre-implantation mouse embryos are known to hatch, grow and differentiate well *in vitro* (Hsu, 1973, 1979; Wu *et al.*, 1981). This suggests that the hatching process of mouse embryos may be dependent not only on the uterine environments but also on embryonic control. One of mechanisms for loss of the ZP is enzymatic lysis by uterine and/or embryonic proteases (lytic enzyme, stripsin; Perona & Wassarman,

All correspondence to: Yong Pil Cheon, Department of Obstetrics and Gynecology, College of Medicine, University of Ulsan, Asan Medical Center, Seoul 138–040, Korea. Telephone: +82 2 224 5053. Fax: +82 2 224 5051. e-mail: ypcheon@www.amc.seoul.kr.
[1]Department of Biology, College of Natural Sciences, Hanyang University, Seoul 133–791, Korea.
[2]Department of Obstetrics and Gynecology, College of Medicine, University of Ulsan, Asan Medical Center, Seoul, Korea.
[3]Department of Biology, College of Natural Sciences, Kyonggi University, Suwon, Korea.
[4]Department of Medicine, Ewha Women's University, Seoul, Korea.

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press

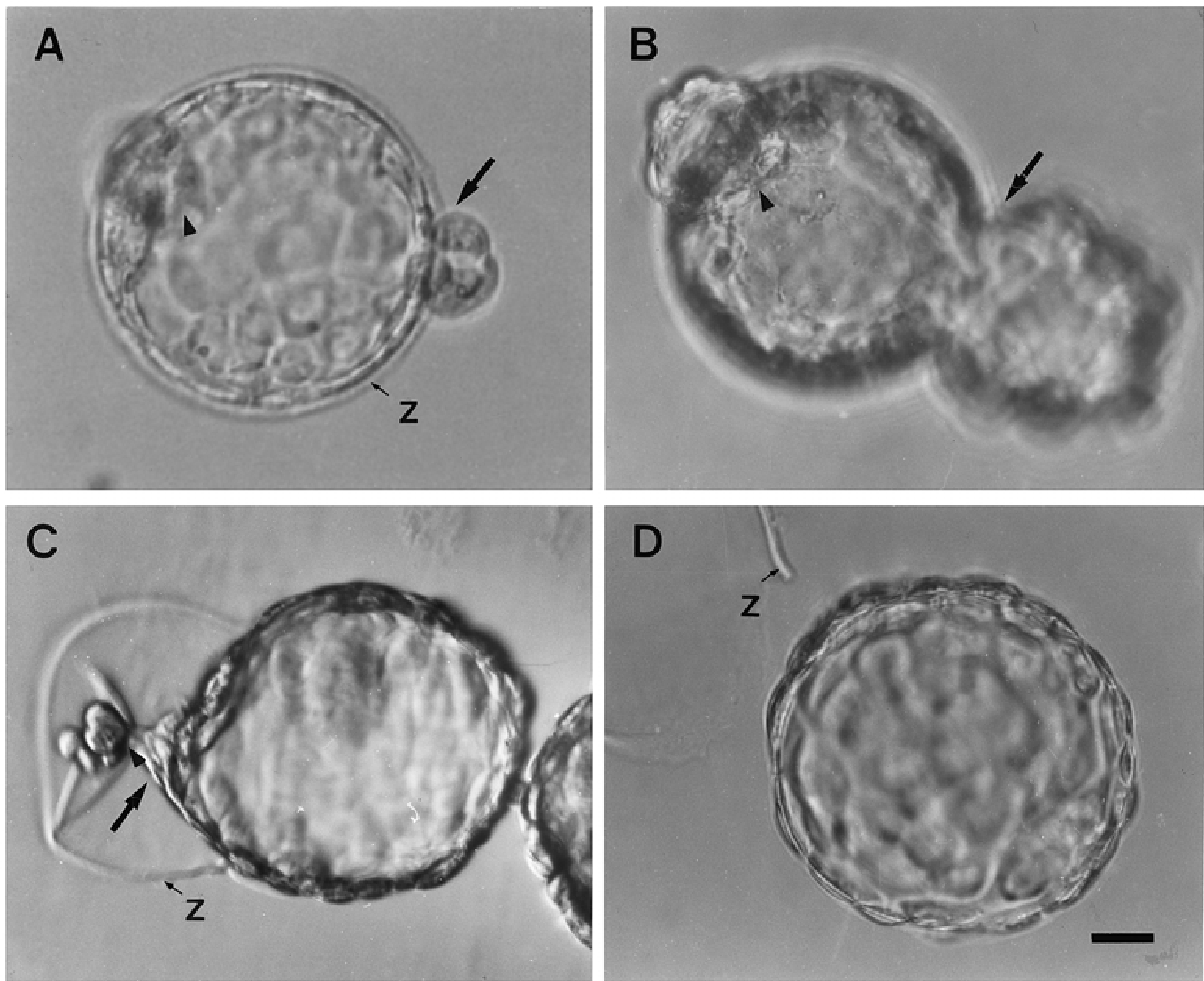

Figure 1 (A–D) Hatching of mouse blastocyst observed by light microscopy. The zona pellucida is distended by the expanding blastocyst and ruptured in two places. The blastocyst is extruded through one of these ruptures, but hatches through only one rupture (arrows indicate the hatching site; arrowheads show the ruptured site not involved in hatching). *z*, zona pellucida. Scale bar represents 20 μm.

1986; Yamazaki & Kato, 1989). The other important factor is blastocoele tension, which is increased by Na/K$^+$-ATPase of trophectoderm (Mintz, 1962; Cole, 1967; Gordon & Dapunt, 1993; Schiewe *et al.*, 1995). After escape from the ZP, cellular contacts are not observed between the trophoblast and the uterine epithelium. The blastocyst positions itself in the uterus for a variable period after hatching. The non-random orientation of the blastocyst represents a biological constant of a given species (McLaren, 1982; Sawada *et al.*, 1990; Guillemot *et al.*, 1993).

In pigs it was observed that the ZP was distended by the expanding blastocyst and ruptured in several places. The blastocyst was found to be extruded through one of these ruptures (Guillomont *et al.*, 1993). In the mouse, hatching is initiated at multiple sites, but all extrusion occurs through one of the initiation sites (Fig. 1). The total number of cells and the number of trophectodermal cells in expanded, hatching and recently hatched embryos are the same (Kim *et al.*, 1996; Cheon, 1997).

Cellular structure and polarity are mediated by the cytoskeleton, and cell movements depend on labile structures constructed from actin filaments (Alberts *et al.*, 1994). An actin filament is a polar structure with two structurally different ends: a relatively inert and slow-growing minus end (pointed end) and a faster-growing plus end (barbed end). The leading edge of a moving cell contains actin filaments that are continuously polymerised and are, therefore, very sensitive to cytochalasin B (Cooper, 1987; Forscher & Smith, 1988).

Lytic enzymes and blastocoele tension may not completely explain escape of the blastocyst from the ZP (hatching). Therefore, in this study we tested the role of actin filaments and actin-mediated movement during hatching.

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press

Materials and methods

Animals and embryo collection

ICR mice were maintained on a 14 h light and 10 h dark cycle under standard vivarium conditions, and supplied with food and water *ad libitum*. Seven-week-old virgin female mice were superovulated with 5 IU of pregnant mares' serum gonadotrophin (PMSG, Sigma) followed by 5 IU of human chorionic gonadotrophin (hCG, Sigma) 48 h later. After administration of the hCG, one female was placed in a cage with one stud male, and checked for a copulation plug the following morning.

Mated females were killed by cervical dislocation at 72 h post-hCG injection and the oviduct and uterus were dissected out. Embryos at the early morula stage were collected by flushing with 0.4% bovine serum albumin (BSA) containing Biggers–Whitten–Whittingham (BWW) medium. Only healthy embryos were chosen for culture.

Culture of embryos and treatment with cytochalasin B

Embryos were cultured in 10 µl of BWW medium under mineral oil (Sigma), and incubated for 72 h. Embryos at various developmental stages, i.e. morula, middle blastocyst, expanded blastocyst, lobe-formed embryo and early hatching embryos, were collected depending on the time schedule. The embryos were cultured in a humidified atmosphere of 5% CO_2, 95% air.

To study cell movement, embryos were treated with cytochalasin B (Sigma, 5 µg/ml) for 12 h or 24 h, and then transferred to plain medium containing 0.4% BSA and cultured for 72 h. Embryonic development was checked under a Hoffman modulated contrast inverted microscope (Olympus IX-70).

Cell fixation, immunocytochemical staining and confocal laser scanning microscopy

For actin staining, embryos at each stage were fixed at room temperature in 3.75% (v/v) formaldehyde and 0.1% Triton X-100 (in phosphate-buffered saline (PBS), pH 7.4) for 1 min and fixed further for 45 min in 3.75% formaldehyde–PBS. The fixed embryos were transferred to 0.25% Triton X-100 (in PBS, pH 7.4) and incubated for 15–30 min, and stored at 4 °C in PBS in 0.1% BSA until used. For actin staining, embryos were transferred to PBS with 0.1% Triton X-100 and further incubated for 15–30 min, then incubated for 30–60 min in PBS with 0.2% BSA and rhodamine–phalloidin (cat. no. R-415, Molecular Probes, USA). Rhodamine–phalloidin binds specifically to F-actin. Embryos were washed in PBS with 3% BSA and 0.1% Tween-20 for 30 min, and then mounted on a microscope slide.

The samples were observed under an Olympus fluorescence microscope (Olympus BX 40) and a confocal laser scanning microscope (Carl Zeiss, ISM 410). For quantification of the actin filaments, intensity of fluorescence was analysed in embryos at different stages of blastocyst development. The status of blastocyst hatching was also examined in the presence or absence of cytochalasin B. Chi-squared test was used in statistical analysis, and a P value less than 0.5 was considered significant.

Results

Effects of cytochalasin B

To study the role of actin filaments in hatching, embryos at different stages of blastocyst development were treated with cytochalasin B for 12 h. Embryonic development was completely inhibited, but the external morphology was not altered by cytochalasin B in morula-stage embryos. After transfer to cytochalasin-B-free medium, most of the embryos developed to blastocysts. Some of them ruptured (17.2%) and escaped the ZP (2.8%). Hatching rate in the cytochalasin-B-treated group was lower than that of controls (rupture, 17.2% vs 79%; hatched, 2.8% vs 48.8%; Fig. 2). In middle blastocysts, even though the external morphology remained normal, development was arrested (199%). However, they were able to resume development in cytochalasin-B-free medium. Also, the hatching rate of treated embryos was lower than that of controls (rupture, 37.2 vs 79%; hatched, 8.6% vs 55.6%; Fig. 2). In expanded blastocysts development was arrested and most of the embryos remained in an expanded state. However, in some embryos, rupture of the ZP occurred, but development of those embryos stopped at this stage. Those embryos transferred to cytochalasin-B-free medium developed and escaped from the ZP (rupture, 93.8% vs 98.7%; hatched, 75% vs 89.5% in treated and control embryos respectively; Fig. 2). In lobe-formed embryos or hatching embryos development was arrested and some embryos showed shrunken forms. However, when those embryos were transferred to cytochalasin-B-free medium, hatching was resumed and embryos escaped the ZP (Fig. 2).

To clarify the effects of cytochalasin B depending on the period of treatment, we cultured embryos in cytochalasin B containing BWW for 24 h. At all stages the results were similar to the 12 h treatment groups (Fig. 3). When embryos were transferred to medium without cytochalasin B they resumed development and escaped the ZP. However, the recovery time was longer than that of embryos treated for 12 h and the degeneration rate was higher (Fig. 3).

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press

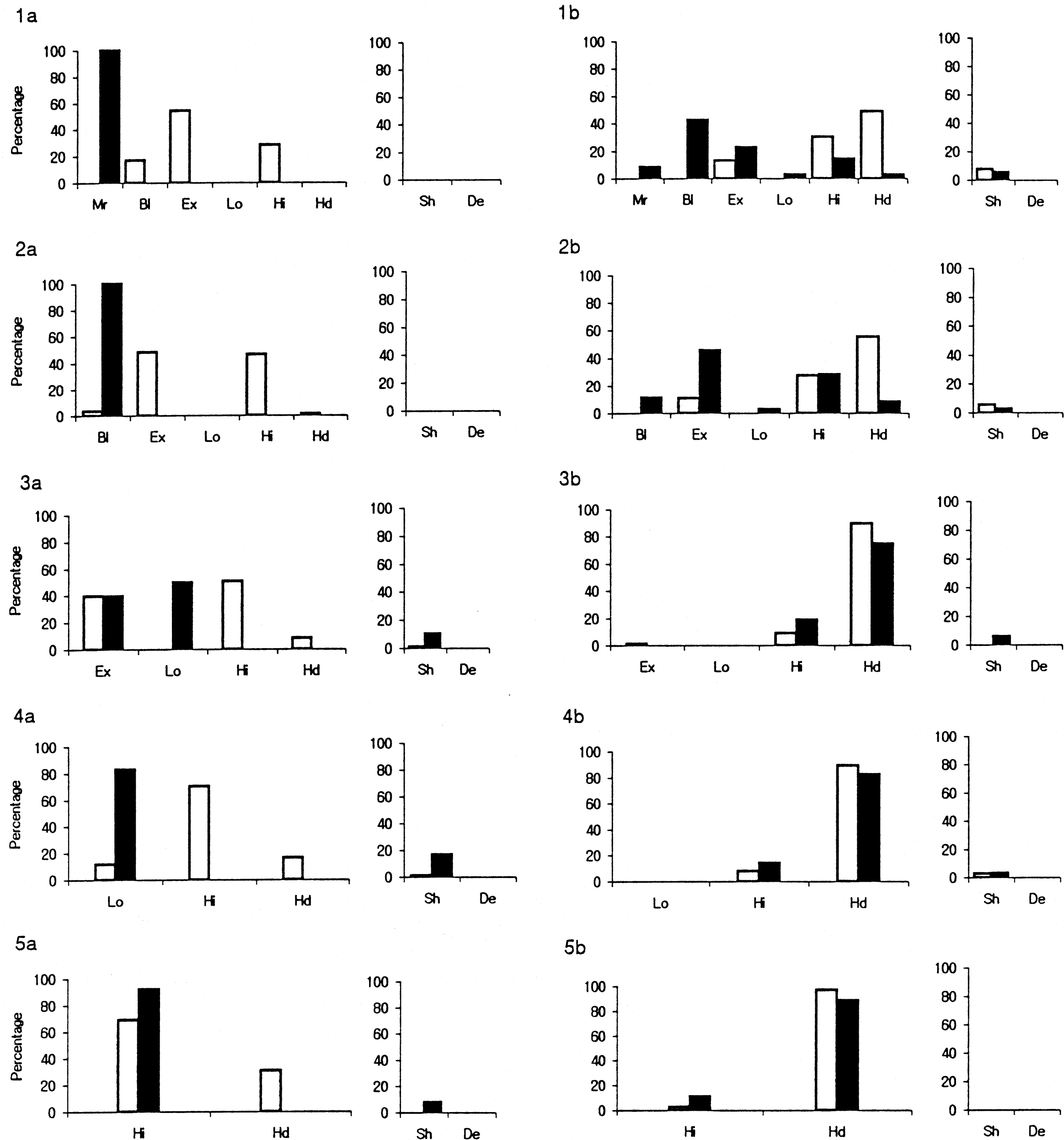

Figure 2 Effects of cytochalasin B on the development of mouse embryos. Embryos were cultured for 12 h in a medium containing 5 μg/ml cytochalasin B (*a*) and then cultured in cytochalasin-B-free medium for 72 h (*b*). 1, morula; 2, middle blastocyst; 3, expanded blastocyst; 4, blastocyst forming lobe; 5, hatching blastocyst. Open columns, control; black columns, cytochalasin B present for 12 h. Mo, morula; Bl, middle blastocyst; Ex, expanded blastocyst; Lo, blastocyst forming lobe; Hi, hatching blastocyst; Hd, hatched blastocyst; Sh, shrunken; De, degenerated.

Distribution and relative quantities of actin filament

The distribution of actin filament in embryos cultured in BWW medium was investigated with scanned plane-face views and reconstructed three-dimensional views using a confocal laser scanning microscope. Plate 1 (facing p. 126) shows micrographs of the distribution of actin filaments in optical sectioning images and reconstructed three-dimensional images during hatching. In the three-dimensional images actin filaments were found to be present at cell–cell contact areas and some blastomeres in a particular area showed more intensive signals (Plate 1 1b, 2b, 3b, 4b, filled arrow). In the optically sectioned images actin filaments were densely localised at the trophectodermal cell surface and cell–cell contact areas (Plate 1 1a, 2a, 3a, 4a). Intensive signal was localised particularly in the vicinity of the hatching site. In contrast the site

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press

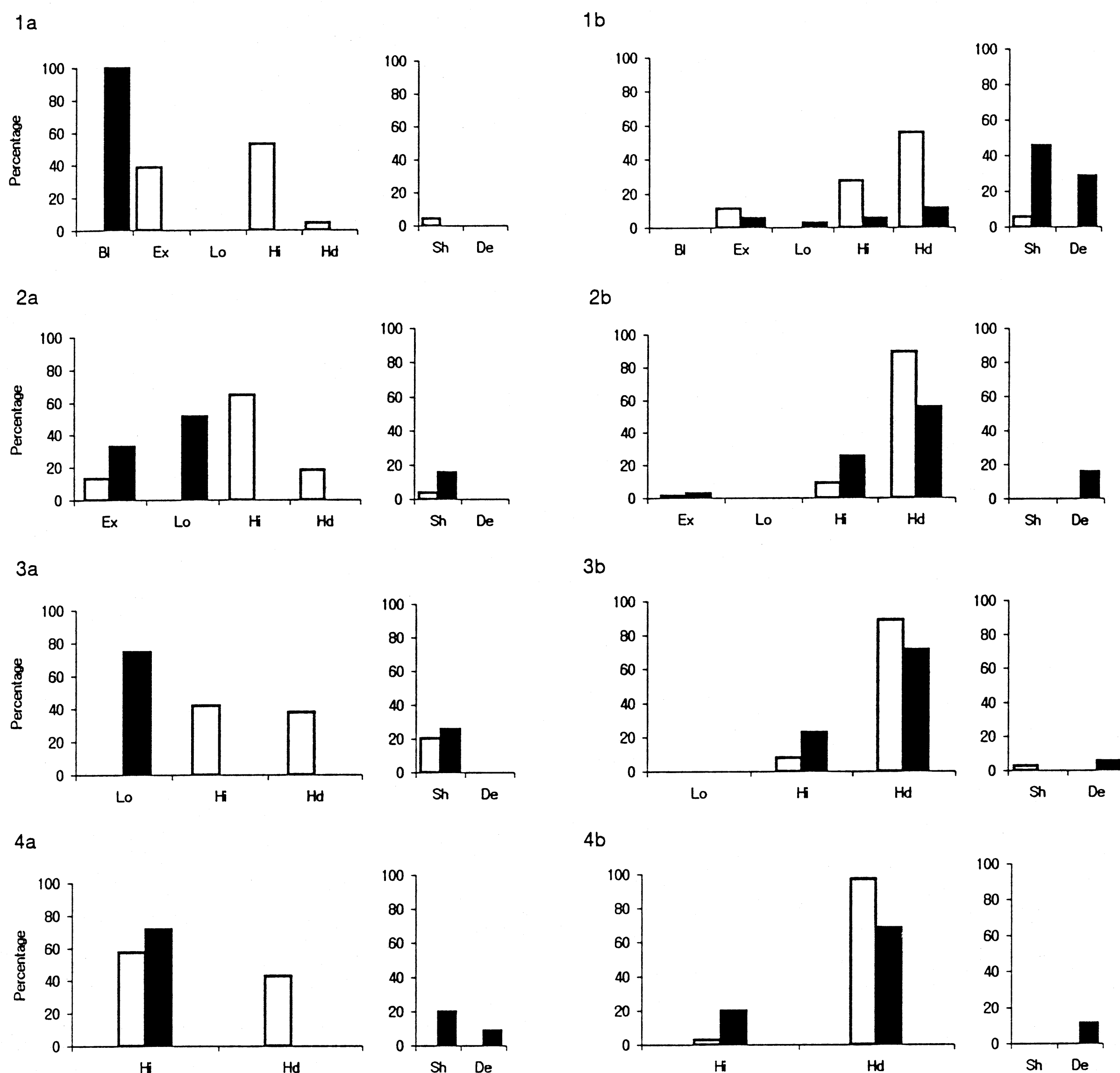

Figure 3 Effects of cytochalasin B on development of mouse embryos. Embryos were cultured for 24 h in medium containing 5 µg/ml cytochalasin B (*a*) and then cultured in cytochalasin-B-free medium for 72 h (*b*). 1, middle blastocyst; 2, expanded blastocyst; 3, blastocyst forming lobe; 4, hatching blastocyst. Open columns, control; black columns, cytochalasin B present for 24 h. Bl, middle blastocyst; Ex, expanded blastocyst; Lo, blastocyst forming lobe; Hi, hatching blastocyst; Hd, hatched blastocyst; Sh, shrunken; De, degenerated.

opposite the hatching point showed a relatively weak signal (Plate 1 2b, 3b, 4b, open arrow). In the cytochalasin-B-treated hatching embryos the actin filament distribution on the three-dimensional image showed similar patterns to those in the non-treated blastocysts (Plate 1 4a, 4b). In the three-dimensional images the density of actin filaments was weak in trophectodermal cells located opposite the hatching site (Plate 1 2b, 3b). This phenomenon was also observed in the cytochalasin-B-treated hatching embryos (Plate 4 4b). The actin filament distribution in the two-dimensional images showed a locomotion pattern (Plate 4, 3a, 4a). Density of actin filaments at the locomotion-like areas disappeared, as shown in Plate 1 4a, and trophectodermal cells were more oval than in non-treated embryos (Plate 1 4a).

The relative quantity of actin filaments in embryos (area × intensity) was dramatically increased when the blastocyst initiated hatching (Table 1). The intensity in hatching embryos was higher than that in other stages of embryos (Table 1).

Discussion

It is known that uterine/embryonic proteinase and blastocoelic tension are factors involved in hatching (Cole, 1967; McLaren, 1970; Sawada *et al.*, 1990; Cohen

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press

Table 1 The relative quantities of actin filament and its intensity

Developmental stage of embryo	Quantities (area × intensity)	Intensity
Middle blastocyst	1.00 ± 0.00	120.89 ± 6.38
Expanded blastocyst	2.53 ± 0.94	227.70 ± 9.27
Blastocyst in the middle of hatching	2.97 ± 0.90	240.03 ± 5.98
Hatched blastocyst	3.05 ± 1.17	200.97 ± 3.74

Date are expressed as mean ± SD (n = 5).
Middle blastula stage was selected for actin filament densitometry comparison (as 1) and compared with other embryo stages.

& Feldberg, 1991; Gordon & Dapunt, 1993; Yamazaki *et al.*, 1994; Turpeenniemi-Hujanen *et al.*, 1995). In addition, it has been postulated that the growth or mitotic division of the trophectodermal cells may be another factor in hatching. The total number of cells and the number of trophectodermal cells in expanded, hatching and hatched embryos did not differ (Kim *et al.*, 1996; Cheon, 1997). Therefore, the growth of mitotic division may not be an additional factor for hatching in the mouse blastocyst. By analysing the confocal laser microscopic images (Plate 1), we observed that the sizes of the trophectodermal cells did not differ from each other. Furthermore, we observed by scanning electron microscopy that while the ZP of porcine blastocyst is ruptured in several places the blastocyst is extruded through only one of these holes. Light microscopic observation of mouse blastocyst that had multiple ruptures also showed that the blastocyst is extruded through one of them. This is the same phenomenon as was seen with the porcine embryos (Fig. 1). Therefore, we hypothesised that physical force is not the only factor involved in hatching.

In general, it is thought that microspikes and lamellipodia are generated by local actin polymerisation at the plasma membrane and that such actin polymerisation can rapidly push out the plasma membrane without tearing it (Alberts *et al.*, 1994). The leading edge of a moving cell contains actin filaments that are continually polymerising. Polarisation of most animal cells is maintained by microtubules and microfilaments. In moving cells, highly dynamic behaviour of actin filaments at the leading edge is crucial for locomotion. The cytochalasins are fungal products that prevent actin from polymerising by binding to the plus end of the filaments. As seen in Fig. 1, or reasoning from the porcine case, it can be postulated that actin-mediated movement may be related to hatching. In our results the development of embryos was reversibly blocked by cytochalasin B. When blastocysts were cultured for more than 24 h in cytochalasin-B-containing medium, developmental arrest was seen, which is consistent with previous results (not shown). It strongly suggests that the arrest phenomenon does not originate from the duration of culture.

In expanded blastocysts rupture of the ZP (12 h, 50%; 24 h, 51.4%) occurred, but development was arrested. However, embryos transferred to cytochalasin-B-free medium resumed development and escaped the ZP. This suggests that lytic enzymes and blastocoelic tension are responsible for rupture of the ZP but not for escape from it.

The distribution of actin filaments in hatching embryos was similar in three-dimensional images regardless of the cytochalasin B treatment. However, in two-dimensional images the patterns of actin filaments were different between treated and untreated embryos. The distribution of actin filaments at the edge between trophectodermal cells was the same, but the pattern was different. In untreated embryos actin filaments continuously polymerised near the edge and formed elongation patterns, but in treated blastocysts these were not found. The amount of actin filaments in hatching embryos is higher than that in expanded blastocysts or lobe-formed blastocysts. As the total number of cells and the number of trophectodermal cells do not differ among expanded, hatching and hatched blastocysts, we selected the expanded stage as the primary base for comparison between embryos derived from hatching blastocysts. The size of a hatching blastocyst is 1.17 times greater than that of an expanded blastocyst (not shown). Consequently, when cytochalasin B inhibited the polymerisation of actin filaments, it is suggested that this resulted in the disappearance of locomotion patterns and inhibited development and hatching.

It is clear that lytic enzymes and blastocoelic tension are factors involved in hatching but not in the escape of the blastocyst from the ZP. It is now feasible to suggest that dynamic actin polymerisation is essential for hatching, and that actin-filament-mediated movement might be crucial in the process of hatching in mouse embryos.

Acknowledgements

This work was supported in part by the Ministry of Education (BSRI 96–4437) in Korea. We thank Korea

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press

Basic Science Institute for their help in observation with the confocal microscope.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. (1994). The cytoskeleton. In *Molecular Biology of the Cell*, 3rd edn, eds B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson, pp. 788–863. New York and London: Garland Publishing.

Cheon, Y. P. (1997). The hatching regulation factors of the mouse embryos. PhD thesis, Hanyang University, Seoul, Korea.

Cohen, J. & Feldberg, D. (1991). Effects of the size and number of zona pellucida openings on hatching and trophoblast outgrowth in the mouse embryo. *Mol. Reprod. Dev.* **30**, 70–8.

Cole, R. J. (1967). Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst. *J. Embryol. Exp. Morphol.* **17**, 481–90.

Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105**, 1473–8.

Forscher, P. & Smith, S. J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* **107**, 1505–16.

Gonzales, D. S. & Bavister, B. D. (1995). Zona pellucida escape by hamster blastocysts *in vitro* is delayed and morphologically different compared with zona escape *in vivo*. *Biol. Reprod.* **52**, 470–80.

Gordon, J. W. & Dapunt, U. (1993). A new mouse model for embryos with a hatching deficiency and its use to elucidate the mechanism of blastocyst hatching. *Fertil. Steril.* **59**, 1296–301.

Guillomot, M., Fléchon, J.-E. & Leroy, F. (1993). Blastocyst development and implantation. In *Reproduction in Mammals and Man*, ed. C. Thibault, M. C. Levasseur & R. H. F. Hunter, pp. 387–412. Paris: Ellipses.

Hsu, Y. C. (1973). Differentiation *in vitro* of mouse embryos to the stage of early somite. *Dev. Biol.* **33**, 403–11.

Hsu, Y. C. (1979). *In vitro* development of individually cultured whole mouse embryos from blastocyst to early somite stage. *Dev. Biol.* **68**, 453–61.

Kim, E. Y., Kim, S. E., Yoon, S. H., Park, S. P., Chung, K. S. & Lim, J. H. (1996). ICM–trophectoderm cell numbers of mouse IVF/IVC blastocysts. *Kor. J. Fertil. Steril.* **23**, 25–32.

McLaren, A. (1970). The fate of the zona pellucida in mice. *J. Embryol. Exp. Morphol.* **23**, 1–19.

McLaren, A. (1982). The embryo. In *Reproduction in Mammals*, vol. 2, ed. C. R. Austin & R. V. Short, pp. 1–25. Cambridge: Cambridge University Press.

Mintz, B. (1962). Experimental study of the developing mammalian egg: removal of the zona pellucida. *Science* **138**, 594.

Perona, R. M. & Wassarman, P. M. (1968). Mouse blastocysts hatching *in vitro* by using a trypsin-like proteinase associated with cells of mural trophectoderm. *Dev. Biol.* **114**, 42–52.

Sawada, H., Yamazaki, K. & Hoshi, M. (1990). Trypsin-like hatching protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. *J. Exp. Zool.* **254**, 83–7.

Schiewe, M. C., Hazeleger, N. L., Sclimenti, C. & Balmaceda, J. (1995). Physiological characterization of blastocyst hatching mechanisms by use of a mouse antihatching model. *Fertil. Steril.* **63**, 288–94.

Turpeenniemi-Hujanen, T., Feinberg, R. F., Kauppila, A. & Puistola, U. (1995). Extracellular matrix interactions in early human embryos: implications for normal implantation events. *Fertil. Steril.* **64**, 132–8.

Yamazaki, K., Suzuki, R., Hojo, E., Kondo, S., Kato, Y., Kanioka, K., Hoshi, M. & Sawada, H. (1994). Trypsin-like hatching enzyme of mouse blastocysts: evidence for its participation in hatching process before zona shedding of embryos. *Dev. Growth Differ.* **36**, 149–54.

Wu, T. C., Wan, Y. J. & Damjanov, I. (1981). Positioning of inner cell mass determines the development of mouse blastocysts *in vitro*. *J. Embryol. Exp. Morphol.* **65**, 105–17.

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press

https://doi.org/10.1017/S0967199499000477 Published online by Cambridge University Press