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Time-Lapse Videomicrographic Observations of Parthenogenetic Mouse Embryos during Early Development

Sueo Niimura[1]* and Nana Futatsumata[2]

[1]Faculty of Agriculture, and
[2]Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan

Abstract: *The process of early development in diploid parthenogenetic mouse embryos from the 2-cell to the blastocyst stages was observed by time-lapse videomicrography, and was compared with that in fertilized embryos. Parthenogenetic 2-cell embryos cleaved and developed to 8-cell embryos after 23.0 hrs of culture. Transformation of blastomeres occurred at the 8-cell stage: namely, outer blastomeres were flattened. The embryos compacted at the morula stage, and then developed to blastocysts after 54.0 hrs of culture. A slit in the zona pellucida was formed 42.7 hrs after blastocyst formation, and trophectoderm cells protruded out of the zona pellucida through the slit. Protrusion of trophectoderm cells from the zona pellucida could arise from either side, polar trophectoderm or mural trophectoderm. After 7.0 hrs, the blastocysts completely escaped from the zona pellucida in either state, expansion or contraction. Fertilized 2-cell embryos showed morphological changes similar to those of parthenogenetic embryos and developed to blastocysts after 51.9 hrs of culture. Fertilized blastocysts took a significantly shorter time, 31.1 hrs, to start hatching, but required a significantly longer time, 23.8 hrs, to complete hatching, compared with parthenogenetic blastocysts. Hatching patterns of fertilized blastocysts were consistent with those of parthenogenetic blastocysts, except that hatching began with protrusion of trophectoderm cells from small holes in zonae pellucidae of fertilized blastocysts. From these results, it was confirmed that the developmental ability of early diploid parthenogenetic embryos prepared by the treatment with ethanol and cytochalasin B is comparable to that in fertilized embryos.*

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*To whom correspondence should be addressed.

Parthenogenesis is the phenomenon in which an oocyte begins to develop by activation without sperm penetration. Artificial induction of this phenomenon is thought to be useful in clarifying the mechanisms of oocyte activation and to investigate the role of sperm in embryo development [1]. Although parthenogenetic embryos are prepared in many mammals by various methods, all attempts to obtain newborns from such embryos have failed [1, 2].

It has also been reported that parthenogenetic embryos have a low potential of development in vitro [1, 3–5]. Namely, parthenogenetic embryos are known to cleave at a slower rate, compared with fertilized embryos, regardless of the methods employed to prepare them. Therefore, it is inferred that the process of early development differs between parthenogenetic embryos and fertilized embryos. Recently, ethanol treatment has been widely used to prepare parthenogenetic embryos [6–11], but there have been no reports with regard to the process of early development in those embryos.

In the present study, therefore, developmental changes in morphology and size and time required for development from the 2-cell stage to the blastocyst stage were observed in parthenogenetic mouse embryos by time-lapse videomicrography, and the results were compared with those obtained from fertilized embryos.

Materials and Methods

Animals

Ninety-five mature female mice of the ICR strain used in the present study were kept in a room at 24°C and fed *ad libitum*. They were superovulated with 5 i.u. PMSG (Serotropin, Teikoku Hormone Manufacturing Co. Ltd., Japan), and with 5 i.u. hCG (Gonatropin, Teikoku Hormone Manufacturing Co. Ltd.) injected 48 hrs later.

About one third of the superovulated female mice were mated with ICR males of proven fertility.

Preparation of 2-cell embryos

To induce parthenogenesis, unfertilized oocytes recovered from superovulated female mice at 14 hrs after the hCG injection were immersed in M2 medium [12] containing 100 μM EDTA-2Na (EDTA-M2 medium) and 7% ethanol for 7 min at room temperature, and then they were transferred into EDTA-M2 medium containing cytochalasin B (Sigma Chemical Co., USA) at 5 μg/ml for 6 hrs at 37°C. The treated oocytes were immersed in EDTA-M2 medium containing 0.1% hyaluronidase (Sigma Chemical Co.) to remove the cumulus cells, and then they were cultured in EDTA-M2 medium for 22 hrs in a CO_2 incubator (5% CO_2 in air) at 37°C.

As controls, sperm-penetrated oocytes were collected from superovulated and mated female mice at 14 hrs after the hCG injection, and cultured in EDTA-M2 medium for 28 hrs.

Observation of early development

The process of early development was observed in both parthenogenetic 2-cell embryos and fertilized ones cultured in M16 medium [13] using a CO_2 culture chamber (SK-1, Sankei, Japan; CO_2 5% in air) equipped with an inverted microscope (DIAPHOT, Nikon Corporation, Japan). The changes in morphology and diameter of cultured embryos and the time intervals of their developmental events were examined on the images which were taken by a CCD color camera (Hitachi Electronic Co., Japan) connected to an inverted microscope and a time-lapse video cassette recorder (Victor Co., Japan). The analyses were done during the period from the 2-cell stage to completion of blastocyst hatching.

Statistical analysis

Statistical analysis of the time intervals of developmental events and diameters of embryos was performed using one-way analysis of variance. The number of blastocysts with different hatching patterns was statistically analyzed by Chi-square test.

Results

Process of early development and time required for development in parthenogenetic and fertilized embryos

In the developmental process of 17 parthenogenetic embryos which completed hatching, the embryos at the 2-cell stage (Fig. 1-a) developed into the 3-cell stage (Fig. 1-b) after 11.0 hrs of culture, the 4-cell stage (Fig. 1-c) after 11.8 hrs, the 5-cell stage (Fig. 1-d) after 21.0 hrs, the 6-cell stage (Fig. 1-e) after 21.7 hrs, the 7-cell stage after 22.3 hrs, and into the 8-cell stage (Fig. 1-f) after 23.0 hrs. These 8-cell embryos were composed of only round blastomeres. The transformation of blastomeres, flattening of outer blastomeres, occurred at the 8-cell stage (Fig. 1-g). The embryos compacted at the morula stage (Fig. 1-h), and then formed a cavity and developed into the blastocyst stage (Fig. 1-i) after 54.0 hrs of culture (Table 1). The blastocysts repeated contraction and expansion (Fig. 2-a) during the expanded blastocyst stage from 7.0 hrs after blastocoel formation. A relatively large-sized slit was formed in the zona pellucida by expansion of embryos 42.7 hrs after blastocoel formation. Trophectoderm cells of the blastocysts protruded out of the zona pellucida through the slit (Fig. 2-b, Table 1), and the blastocysts started hatching. Protrusion of trophectoderm cells from the zona pellucida could arise from either mural trophectoderm or polar trophectoderm, but more often from the mural trophectoderm (77%, Fig. 2-b). In blastocysts starting hatching, trophectoderm continuously escaped from the zona pellucida (Fig. 2-c), and hatching was completed after 7.0 hrs (Fig. 2-d, Table 1). Blastocysts completed hatching in either state, expansion or contraction (Fig. 2-d), but more often in expansion (53%). None of parthenogenetic blastocysts, in which protrusion had occurred from the polar trophectoderm, completed hatching in the state of contraction.

In the developmental process of 10 fertilized embryos which completed hatching, the embryos at the 2-cell stage developed into the blastocyst stage through a process similar to that of the parthenogenetic embryos. Namely, 2-cell embryos developed to 8-cell embryos composed of only round blastomeres after 28.9 hrs of culture, and the outer blastomeres of the embryos became flattened at the 8-cell stage. The embryos compacted at the morula stage, and developed to blastocysts after 51.9 hrs of culture (Table 1). The time needed to reach each stage from the 3-cell stage until the morula stage did not differ between parthenogenetic and fertilized embryos. The blastocysts derived from fertilized oocytes repeated contraction and expansion during the expanded blastocyst stage from 6.2 hrs after blastocoel formation. Differing from parthenogenetic blastocysts, a small hole formed in the zona pellucida, and the blastocysts began to hatch by protruding a portion of trophectoderm cells through the hole (Fig. 2-e). The time until the onset of hatching after blastocoel formation was significantly shorter in fertilized blastocysts (31.1 hrs) than in parthenogenetic blastocysts (42.7 hrs, Table 1). Protrusion of trophectoderm cells from the hole in the zona pellucida occurred in

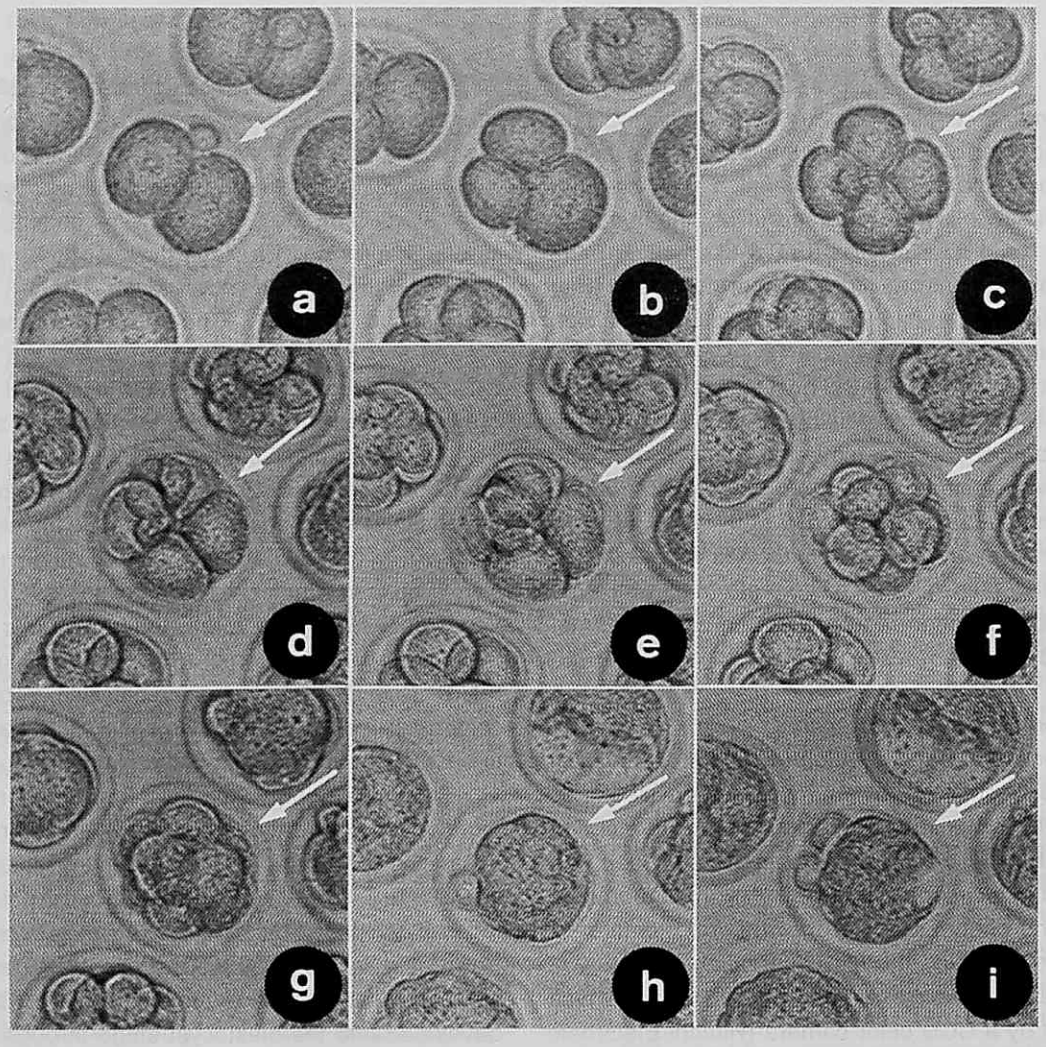

Fig. 1. Time-lapse videomicrographs showing the early development of a parthenogenetic mouse embryo (arrows) from the 2-cell stage to the blastocyst stage. × 200. (a) 2-Cell embryo. (b) 3-Cell embryo. (c) 4-Cell embryo. (d) 5-Cell embryo. (e) 6-Cell embryo. (f) 8-Cell embryo composed of only round blastomeres. (g) Transformed 8-cell embryo. Outer blastomeres were flattened. (h) Compacted morula. (i) Early blastocyst with a small cavity.

Table 1. Time intervals of developmental events in mouse embryos

Embryos	No. of embryos examined	No. of observations repeated	From the 2-cell stage to blastocyst formation	From the blastocyst formation to blastocyst hatching	From the onset to completion of blastocyst hatching	Total (From the 2-cell stage to the completion of blastocyst hatching)
Parthenogenone	17	11	54.0 ± 2.0 hrs*[a]	42.7 ± 2.5 hrs[a]	7.0 ± 1.6 hrs[b]	103.8 ± 2.5 hrs[a]
Fertilized	10	4	51.9 ± 1.3 hrs[a]	31.1 ± 4.6 hrs[b]	23.8 ± 5.2 hrs[a]	106.8 ± 2.4 hrs[a]

*Mean ± S.E. The numbers with different superscripts within the same column are significantly different ($P<0.05$).

either polar trophectoderm or mural trophectoderm, but more often in the mural trophectoderm (80%, Fig. 2-e). Trophectoderm continuously escaped from the zona pellucida and the blastocyst completed hatching. Hatching was completed in the state of expansion in 9 blastocysts (90%, Fig. 2-f) and in the state of contraction in 1 blastocyst (10%). None of the blastocysts, in which protrusion had occurred from the polar trophectoderm, completed

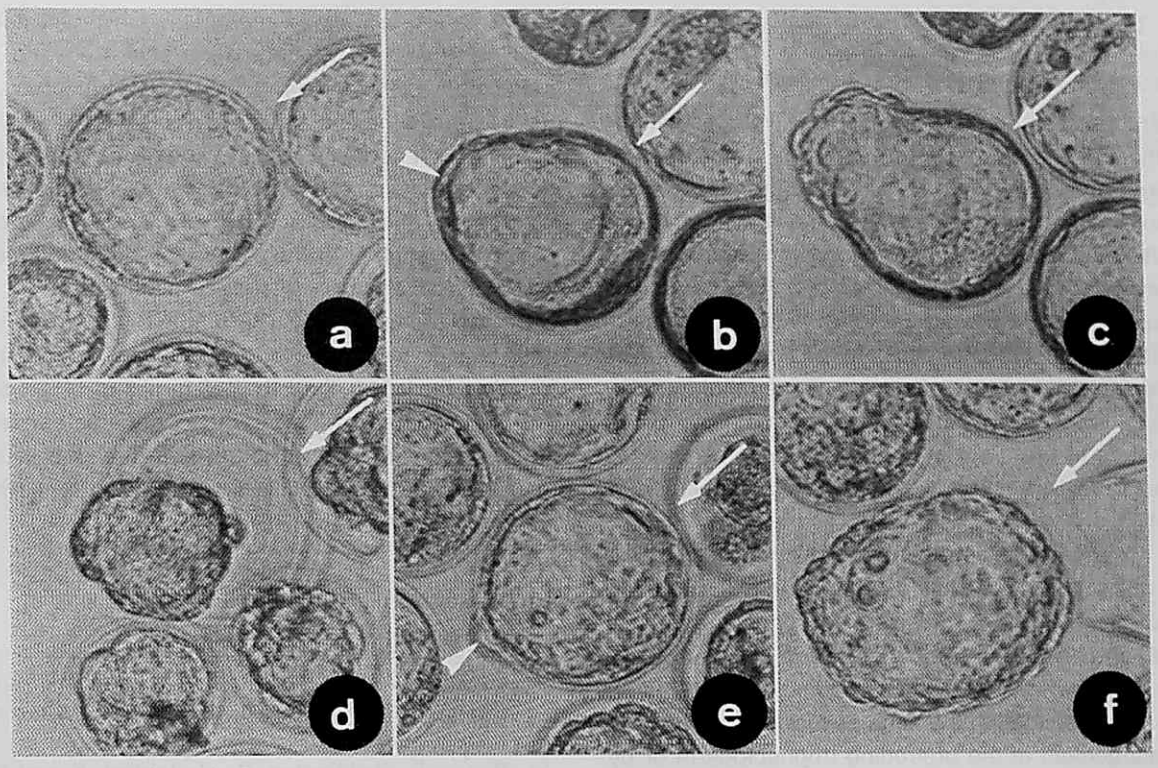

Fig. 2. Time-lapse videomicrographs showing the process of hatching of parthenogenetic and fertilized mouse blastocysts (arrows). × 200. (a) An expanded parthenogenetic blastocyst. (b) A parthenogenetic blastocyst at the onset of hatching. A slit (arrowhead) was formed in the zona pellucida facing the mural trophectoderm. (c) A parthenogenetic blastocyst during hatching. Mural trophectoderm cells protruded out of the zona pellucida through the slit. (d) A parthenogenetic blastocyst at the completion of hatching. The embryo completed hatching in a state of contraction. (e) A fertilized blastocyst at the onset of hatching. A small hole (arrowhead) was formed in the zona pellucida facing the mural trophectoderm. (f) A fertilized blastocyst at the completion of hatching. The embryo completed hatching in a state of expansion.

Table 2. Changes in diameters of mouse embryos during early development

Embryos	No. of embryos examined	Developmental stages					
		2-Cell	4-Cell	8-Cell	Morula	Blastocyst	
						At the time of formation	At the time of hatching
Parthenogenone	17	92.4 ± 1.2 μm*[a]	93.5 ± 1.0 μm[a]	93.5 ± 1.0 μm[a]	93.2 ± 1.0 μm[a]	95.0 ± 1.0 μm[a]	130.9 ± 2.5 μm[a]
Fertilized	10	90.7 ± 0.8 μm[a]	91.5 ± 1.1 μm[a]	90.8 ± 1.1 μm[a]	90.6 ± 0.9 μm[a]	91.0 ± 1.4 μm[b]	108.1 ± 3.3 μm[b]

*Mean ± S.E. The numbers with different superscripts within the same column are significantly different ($P<0.05$).

hatching in a state of contraction. The time needed to complete hatching was significantly longer (23.8 hrs, Table 1) than that of parthenogenetic blastocysts. The ratio of blastocysts hatching from the polar trophectoderm to those from the mural trophectoderm and the ratio of blastocysts completing hatching in expansion to those in contraction did not significantly differ between parthenogenetic and fertilized blastocysts.

Size of diameters in parthenogenetic and fertilized embryos

Developmental changes in the diameters of parthenogenetic and fertilized embryos, including the zona pellucida, are shown in Table 2. Although the diameters did not differ between parthenogenetic and fertilized embryos until the morula stage, parthenogenetic blastocysts were significantly larger than fertilized blastocysts.

Discussion

The process of early development was observed in parthenogenetic embryos prepared by treatment with ethanol and cytochalasin B, using time-lapse videomicrography in the present study. It was confirmed for the first time that morphological changes from 2-cell embryos until blastocoel formation and the time needed for such development did not differ between parthenogenetic and fertilized embryos. In the fertilized embryos of the present study, it was also confirmed that morphological changes until blastocoel formation and the diameters of the blastocyst stage are consistent with those of previous reports with fertilized mouse embryos [14–17].

It is generally accepted that blastocyst hatching begins with regional dissolution of the zona pellucida by a trypsin-like enzyme synthesized in trophectoderm cells [2, 18] and protrusion of trophectoderm cells out of the zona pellucida through the dissolved hole [19–21]. And then a slit is formed in the zona pellucida from the hole by enlargement of the protruding trophectoderm or by blastocyst expansion [19–21]. After that, the blastocyst repeats active contractions, leading to the enlargement of the slit, and then escapes from the zona pellucida [19–21]. The hatching mode of fertilized blastocysts in the present study did not differ from that of fertilized blastocysts in previous reports [19–21]. On the other hand, the mode of initiation of hatching in parthenogenetic blastocysts observed in the present study differed from that of fertilized blastocysts; namely, it was clarified that a slit appeared in the zona pellucida, and trophectoderm cells protruded from the slit to start hatching. Ethanol treatment used to induce parthenogenesis has been confirmed in mice to reduce the solubility of the zona pellucida to α-chymotrypsin [22]. Therefore, the zona pellucida of parthenogenetic embryos observed in the present study would also be hardened. This seems to explain the longer time until the onset of hatching and different initial mode of hatching in parthenogenetic blastocysts, compared with fertilized blastocysts. The longer time until the start of hatching was thought to be a cause of the larger diameters in parthenogenetic blastocysts. The time taken to complete hatching was significantly shorter in parthenogenetic blastocysts than in fertilized blastocysts in the present study. The shorter time observed in parthenogenetic blastocysts was thought to be due to the direct formation of a relatively large-sized slit in the zona pellucida without hole formation.

It was also confirmed in parthenogenetic blastocysts that protrusion of trophectoderm cells from the zona pellucida, which represents the onset of hatching, can occur from either polar or mural trophectoderm, and hatching can be completed in either a state of contraction or expansion. The incidence of parthenogenetic blastocysts showing each one of the above hatching patterns in each hatching stage was also confirmed to be comparable to that of fertilized blastocysts.

In the present study, the diameter of parthenogenetic blastocysts at the time of blastocoel formation was found to be larger than that of fertilized blastocysts. This seemed to be related to the longer time needed by parthenogenetic embryos to develop to blastocysts, although no significant difference was detected between parthenogenetic and fertilized embryos.

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