Journal of Assisted Reproduction and Genetics (2012) 30巻1号:91～98.

Chromosomal stability of second polar bodies in mouse embryos

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Abstract

Purpose Incorporation of a second polar body (PB2) into one of the blastomeres has been considered as a causal mechanism underlying diploid/triploid mixoploidy in human. Using mouse model, we examined whether PB2s can participate in the formation of mixoploidy. *Methods* Uptake of BrdU was examined to determine DNA synthesis in PB2s up to 28 h after fertilization. PB2s from embryos at 4–6 (1-cell), 24 (2-cell), 48 (4-cell), and 72 h (morula) were fused with MII oocytes to induce premature chromosome condensation. Caspase and TUNEL assays were used to detect apoptotic PB2s at 24, 48, and 72h. PB2s were fused with one of the blastomeres of the 2-cell embryos to produce mixoploid embryos. *Results* DNA synthesis in the PB2s continued until 22 h after fertilization. At 4–6 h, nearly all of the PB2s showed G1-type chromosomes and there was no significant increase in chromosome damage. At 24, 48, and 72 h, S-type chromatin predominated. Little PB2s showed apoptotic response until 72 h. Regardless of the fusion with the PB2, more than 90% of the embryos developed to 4-cell stage, and over 80% of the resultant 4-cell embryos had

daughter blastomeres with a morphologically normal nucleus. Some of the daughter blastomeres displayed triploidy.

Conclusions The PB2 is viable for at least 72 h after fertilization, with slow progression through the cell cycle. Once the PB2 has been incorporated into a blastomere, the cell cycle of the PB2 might be synchronized with that of the host resulting in diploid/triploid mixoploidy.

Keywords

Second polar bodies, Chromosomal stability, Mixoploidy, Preimplantation genetic diagnosis

Introduction

In mammalian fertilization, a haploid set of maternal chromatids is extruded into the second polar body (PB2) from the oocyte shortly after penetration of a spermatozoon. In a mouse model, when the female pronucleus of recently fertilized eggs was replaced with their own PB2, the reconstructed eggs could develop to blastocysts (Evsikov and Evsikov 1994) and even to full term (Wakayama et al. 1997). Thus, the newly formed PB2 retains the ability to support embryonic development. Although the PB2 remains in the perivitelline space of the embryo during successive cleavages in several species including the human (Hertig et al. 1956), there has been little study on its longevity (Bartholomeusz 2003). There is no report on the developmental potential of aged PB2s in cleaving embryos. However, the persistence of the PB2 in human embryos might be relevant to the occurrence of congenital anomalies. Patients with diploid (2*n*)/triploid (3*n*) mixoploidy are characterized by mental and growth retardation, truncal obesity, facial and body asymmetry, skin pigmentation abnormalities, syndactyly and malformed low-set ears (Brems et al. 2003; Muller et al. 1993; Rittinger et al. 2008; van de Laar et al. 2002; Wulfsberg et al. 1991). DNA marker analysis of skin fibroblasts from such patients showed that the extra haploid set of chromosomes was of maternal origin. Therefore, it has been suggested that incorporation of the PB2 into one of the blastomeres of an early embryo is a likely cause of 2*n*/3*n* mixoploidy (Brems et al. 2003; Muller et al. 1993; Rittinger et al. 2008; van de Laar et al. 2002).

To determine whether this mechanism is involved in the formation of mixoploidy, it is fundamental to have better understanding of the genomic fate of the PB2. Here, we examined the time course of DNA synthesis, the incidence of chromosomal damage, and apoptotic response in the PB2s of mouse embryos from the 1-cell stage to the morula. Furthermore, PB2s were artificially fused with the blastomeres of 2-cell embryos to examine the

morphology and chromosomes of nuclei originating from the PB2 in the daughter blastomeres of the resultant 4-cell embryos.

Materials and Methods

Animals

$B6D2F_1$ (C57BL/6Cr × DBA/2Cr) mice, 6 weeks of age, were obtained from Japan SLC (Hamamatsu, Japan). The mice were kept in a light- and temperature-controlled room (14 h light/10 h dark; 23 ± 2 °C) and given *ad libitum* access to food and water. All experimental procedures conformed to the Guidelines for Animal Experiments of Asahikawa Medical University.

Chemicals

Organic and inorganic reagents for preparing culture media were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated.

Production of embryos

For generating oocytes, female mice were superovulated with an intraperitoneal injection of 10 IU eCG followed 48 h later by an injection of 10 IU hCG. Cumulus–oocyte complexes (COCs) were collected from the oviducts at 14–16 h after the hCG injection. The COCs were then treated with 0.02% hyaluronidase for 5 min to remove the cumulus cells. The cumulus-free MII oocytes were washed twice with modified CZB medium (mCZB) (Chatot et al. 1989) and kept in the medium at 37 °C under 5% CO_2 in air until use.

A dense mass of spermatozoa, squeezed out of the cauda epididymis of a male mouse with forceps, was introduced into TYH medium (Toyoda et al. 1971) and then incubated for 2 h at 37 °C under 5% CO_2 in air to allow motile spermatozoa to swim up.

Intracytoplasmic sperm injection (ICSI) was carried out as described by Kimura and Yanagimachi (Kimura and Yanagimachi 1995). In brief, a small amount of preincubated spermatozoa was transferred into Hepes-buffered mCZB (H-mCZB) containing 12% polyvinyl pyrrolidone (PVP). A single spermatozoon was drawn into an injection pipette attached to a piezo impact drive unit (Prime Tech, Tsuchiura, Japan). The sperm head was freed from its tail by applying piezo pulses to the neck region. The sperm heads were injected individually into fresh MII oocytes (Kuretake et al. 1996). The injected oocytes were transferred into mCZB at 37 °C under 5% CO_2 in air for further culture.

Assessment of DNA synthesis in PB2s

To evaluate the ability of PB2s to synthesize their own DNAs, uptake of 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich, Saint Louis, MO, USA) into PB2 nuclei were examined as described by Adenot et al. (Adenot et al. 1997) with some modifications. After embryos had been cultured in mCZB for different times after fertilization, they were transferred into mCZB containing 10 μM BrdU for 30 min. The embryos were fixed with 3.7% paraformaldehyde (PFA) in Dulbecco's phosphate-buffered saline (D-PBS, pH 7.4) containing 0.5 N NaOH for 15 min. The fixed embryos were washed three times in D-PBS containing 2% bovine serum albumin (BSA) and 0.1% Triton X-100 (2% BSA/0.1% TX-100 solution). They were incubated for 30 min in prewarmed (37 °C) D-PBS containing 10% BSA and 0.2% Triton X-100 and then treated with anti-BrdU monoclonal antibodies (BioLegend, San Diego, CA, USA) diluted with 2% BSA/0.1% TX-100 solution (1:100 dilution) for 1 h at 37 °C. After washing three times in 2% BSA/0.1% TX-100 solution, the embryos were treated with an anti-mouse IgG (whole molecule)–FITC-conjugated antibody (Sigma-Aldrich) diluted with 2% BSA/0.1% TX-100 solution (1:400 dilution) for 1 h at

37 °C in the dark. The PB2 nuclei were counterstained with propidium iodide (PI) or 4′-6-diamidino-2-phenylindole (DAPI) for fluorescence microscopy.

Induction of premature chromosome condensation (PCC) in PB2 and chromosome analysis

To analyze chromosomes of the PB2s, PCC was artificially induced by fusion of a PB2 with a MII oocyte. For this purpose, MII oocytes were cryopreserved in advance by vitrification according to Nakao et al. (Nakao et al. 1997). Briefly, oocytes were collected from superovulated females and treated with the cryoprotectant cocktail 2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol. The oocytes were plunged into liquid nitrogen and stored until used for cell fusion. They were then thawed and washed with prewarmed (37 °C) phosphate-buffered medium (Whittingham 1974) and incubated in mCZB for 30 min at 37 °C under 5% CO_2 in air for recovery. Prior to cell fusion, they were treated with 0.2–0.5% protease for 5–10 min to digest the zona pellucida.

Embryos at 4–6 h (1-cell), 24 h (2-cell), 48 h (4-cell), and 72 h (morula) after fertilization were used as PB2 donors. The zonae pellucidae were digested as described above. Removal of the PB2s from the embryos and cell fusion were performed with a micromanipulator. A coverslip attached to the bottom of a perforated slide glass (Matsunami Glass, Osaka, Japan) was used as an operating chamber. The embryos and the oocytes were transferred into individual droplets (3 μl) of H-mCZB on the same chamber. When the PB2s were removed from the embryos at 4–6 and 24 h after fertilization, cytochalasin B (5 μg/ml) was added to the droplet to detach them. As shown in Figure 1, the embryo was held on a holding pipette and each PB2 was drawn gently into a fine glass pipette (25–30 μm diameter). A small amount of Sendai virus (SV; GenomONE™-CF; Ishihara Sangyo Kaisha, Osaka, Japan) in another droplet of H-mCZB was aspirated into the same pipette. Then the PB2 was released from the pipette together with the SV to attach it to the oocyte. The oocytes fused

successfully with the PB2s were cultured for 2 h in mCZB at 37 °C under 5% CO_2 in air to induce PCC in the PB2 nuclei.

For chromosome analysis of the PB2s, the fused oocytes were placed in hypotonic solution (10:10:1 mixture of distilled water, mCZB and 30% fetal bovine serum, FBS) for 20 min at room temperature. Chromosome slides were made by the gradual-fixation/air-drying method (Mikamo and Kamiguchi 1983). The slides were stained with 2% Giemsa (Merck Japan, Tokyo, Japan) in phosphate-buffered solution (pH 6.8) for 8 min. After conventional chromosome analysis, the slides were stained by C-banding to detect dicentric chromosomes and acentric fragments (Tateno et al. 2000).

Analysis of apoptosis in PB2

For detecting caspase activity in the PB2s, a carboxyfluorescein-based fluorochrome-labeled inhibitors of caspases (FLICA) apoptosis detection kit (Immunochemistry Technologies, Bloomington, MN, USA) was used in accordance with the manufacturer's instructions. At 24, 48, and 72 h after fertilization, whole embryos were incubated at 37 °C for 1 h in FLICA solution containing a fluorochrome inhibitor of poly-caspase. The embryos were washed three times in the kit's wash buffer and fixed in 3.7% PFA diluted with D-PBS. After being washed thoroughly, the fixed embryos were put on a glass slide and mounted with mounting medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, CA, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed using *in situ* cell death detection kits (Roche Diagnostics, East Sussex, UK) to detect DNA fragmentation associated with apoptosis. Whole embryos at 24, 48, and 72 h after fertilization were fixed in 3.7% PFA in D-PBS for 1 h and washed with D-PBS supplemented with 0.3% BSA for blocking. The embryos were further washed three times in

the blocking solution and then permeabilized by treatment with 0.5% Triton X-100 for 1 h. The embryos were incubated at 37 °C for 1 h in enzyme solution containing fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase. The embryos were washed with the blocking solution and mounted on a glass slide with mounting medium containing PI or DAPI. As positive controls, 2-cell embryos were treated with 100 μM cycloheximide (BioVision, Mountain View, CA, USA) in mCZB for 96 h to induce apoptosis.

Nuclear morphology and ploidy of blastomeres fused with PB2

Two-cell embryos at 17–19 and 27–28 h after fertilization were treated with 0.5% protease or acidic Tyrode's solution (pH 2.5) for up to 10 min to digest the zonae pellucidae. The zona-free embryos were transferred into a droplet of calcium-free mCZB in a manipulating chamber. The PB2s were detached from the embryos and attached to one of the blastomeres of the original embryos. Cell fusion was performed as described above. The PB2-fused embryos were cultured in mCZB at 37 °C under 5% CO_2 in air until they reached the 4-cell stage. These embryos were fixed in 3.7% PFA in D-PBS for 1 h and then washed three times in D-PBS containing 0.3% BSA. The embryos were put on glass slides and covered with mounting medium with DAPI to examine nuclear morphology.

To observe the metaphase chromosomes of the daughter blastomeres originating from the fused blastomeres, the embryos were transferred into a droplet of mCZB containing 0.005 μg/ml vinblastine sulfate and cultured further. When the nuclear membrane of the daughter blastomeres disappeared, the embryos were then placed in a hypotonic solution (1:4 mixture of 1% sodium citrate and 60% FBS) for 20 min at room temperature. Chromosome spreads of the embryos were made as described (Mikamo and Kamiguchi 1983).

Statistical analysis

Percentile data except for those of the apoptosis assays were transformed into arcsine values for statistical analysis. When data were compared between two different groups, one-way analysis of variance (ANOVA) followed by Student's *t*-test or the Aspin–Welch method was used. For multiple comparisons, one-way ANOVA and the Tukey–Kramer method were used. Percentile data for the apoptosis assays were analyzed using Fisher's exact test. Statistical significance was accepted at $p<0.05$.

Results

DNA synthesis

Uptake of BrdU into nuclei was estimated in both PB2s and blastomeres at various times after fertilization (Fig. 2). Data were obtained using 20–30 embryos at 6–24 h after fertilization. At 26 h and 28 h after fertilization, 11 and 13 embryos were used, respectively. At 6 h after fertilization, 46% of the PB2s had taken up BrdU in their nucleus, while 92% of 1-cell embryos had BrdU-positive pronuclei (Fig. 3a, a′). Two hours later, the uptake of BrdU was observed in more than 90% of the PB2s. One-cell embryos had completely stopped uptake of BrdU into their pronuclei by 12 h after fertilization. The second uptake of BrdU into the blastomeric nuclei of 2-cell embryos was observed at 18–24 h after fertilization. On the other hand, the PB2s showed a continuity of DNA synthesis beyond the first cell cycle of the embryos (Fig. 3b, b′, c, c′). PB2s never entered this second cell cycle by 28 h after fertilization.

PCC and chromosome analysis

PB2s were successfully fused with MII oocytes, independently of their age (Table 1). Those obtained from 1-cell embryos at 4–6 h after fertilization overwhelmingly displayed G1-type chromosomes (Fig. 4a), so they were available for chromosomal analysis. Of 134

PB2s prepared, 116 were karyotyped. One (0.8%) was hypohaploid, and two (1.7%) had a break and an achromatic lesion, respectively. On the other hand, more than 90% of the PB2s obtained from 2-cell embryos at 24 h, 4-cell embryos at 48 h and morulae at 72 h after fertilization showed S-type chromatin (Fig. 4b). This type of chromatin completely precluded chromosomal analysis. Although a few PB2s of 4-cell embryos exhibited G2-type PCC chromosomes (Fig. 4c), they were not good enough to estimate the incidence of chromosome aberrations in PB2s at this embryonic stage.

Apoptosis

Viability of the PB2s obtained from embryos at 24, 48, and 72 h after fertilization was evaluated by both caspase and TUNEL assays, because the great majority of the PB2s at these times showed S-type chromatin. As shown in Table 2, 3% (2/70) of the PB2s obtained from 2-cell embryos at 24 h after fertilization had caspase activity (Fig. 5a, a′). The frequency increased slightly in a time-dependent manner, but this was not statistically significant. On the other hand, the TUNEL assay detected no apoptotic activity in the PB2s obtained from the embryos at any time after fertilization. In the positive control, the TUNEL assay clearly found apoptotic PB2s following treatment with 28.135 μg/ml cycloheximide for 4 days (Fig. 5b, b′).

Nuclear morphology and ploidy in blastomeres of 4-cell embryos that had been fused with PB2s at the 2-cell stage

Fusion of a PB2 with one blastomere of a 2-cell embryo was carried out at 17–19 h and 27–28 h after fertilization. These times corresponded to G1–early S and G2 phases of the second cell cycle of the blastomeres, respectively (see Fig. 2). Regardless of the time of the cell fusion, more than 90% of the embryos developed to the 4-cell stage. However, the

nuclear morphology of the blastomeres of the resultant 4-cell embryos was influenced by the timing of cell fusion. In those fused at 17–19 h after fertilization, 84% (41/49) of embryos had morphologically normal nuclei. In contrast, there was a significant increase of embryos with abnormal nuclei (73%; 37/51) when the cell fusion was carried out at 27–28 h after fertilization. The abnormalities mainly consisted of micronuclei, extra nuclei, and beaded micronuclei situated between two daughter blastomeres (Fig. 6). When the daughter blastomeres originated from the fused blastomere of 2-cell embryos were karyotyped at the metaphase of the third cell cycle, they were both found to have a triploid ($3n = 60$) karyotype (Fig. 7).

Discussion

Uptake of BrdU was found in approximately half of the PB2s at 6 h after fertilization and in nearly all of the PB2s at 8 h after fertilization. This process was largely synchronized with that of the pronuclei. Subsequently, in contrast to the quick disappearance of pronuclei showing BrdU uptake, the BrdU-positive PB2s decreased gradually in number and disappeared at 24 h after fertilization. The disappearance of BrdU uptake in the PB2s did not indicate necessarily that the PB2s had entered the G2 phase of the cell cycle, because the PCC analysis revealed that the PB2s at 24 h after fertilization had S-type chromatin. Abramczuk and Sawicki (Abramczuk and Sawicki 1975) measured the DNA content of embryos fertilized *in vivo* by Feulgen staining and found that the DNA content (C) of the PB2 started to increase at 8 h after fertilization and reached 1.4 C by 22 h after fertilization. Moreover, they reported that the DNA content of the PB2 never reached 2 C until 32 h after fertilization. Howlett and Bolton (Howlett and Bolton 1985) also reported that DNA content of the PB2 of embryos fertilized *in vitro* increased from 1 C to 1.7 C between 8 and 18 h after fertilization and never reached 2 C. Although the mouse strain and methodology used in the

present study differ from those of the previous studies, it would be likely that DNA synthesis in the PB2s was incompletely finished. Such incomplete DNA synthesis in the PB2s might have been caused by the lack of mRNAs and proteins necessary for DNA synthesis. Cellular activities of 1-cell embryos are dependent on maternal mRNAs and proteins accumulated during oogenesis (Telford et al. 1990). The cytoplasmic mRNAs and proteins of the PB2 might be insufficient to maintain DNA synthesis. Nevertheless, we found a few PB2s showing G2-type PCC chromosomes at 48 h after fertilization. These PB2s might have had sufficient cytoplasmic mRNAs and proteins to carry out DNA synthesis, because enlarged PB2s were extruded occasionally. Whether artificially produced large PB2 can enter the G2 phase remains to be investigated.

Chromosome complements in first polar bodies and PB2s reflect those in MI oocytes and MII oocytes. Hence, as a noninvasive method, preimplantation genetic diagnosis (PGD) using polar bodies has been available for human genetic disease or aneuploidy (Verlinsky et al. 1996). Analysis of PCC revealed that the incidence of chromosome aberrations in the PB2s at 4–6 h after fertilization was similar to that of 1-cell embryos prepared as control experiments in our previous studies using the same chromosomal technique and the same mouse strain (Hino et al. 2010; Tateno and Kamiguchi 2007). Therefore, PCC analysis of the PB2 at 4–6 h after fertilization (pre-DNA replication) might be appropriate for human PGD.

Interestingly, mouse PB2s hardly underwent any apoptosis until 72 h after fertilization when the embryos developed to morulae, as seen in blastomeres of early cleavage stage embryos (Fabian et al. 2007; Kamjoo et al. 2002). The cell cycle checkpoint machinery, in which DNA damage is assessed and apoptosis is induced if DNA damage is beyond repair, is absent or restricted in mouse early cleavage embryos (Toyoshima 2009). Shimura et al. (Shimura et al. 2002) found that mouse 1-cell embryos derived from irradiated spermatozoa could start DNA synthesis at the normal time but that the duration of DNA synthesis was

extended. They suggested that the S checkpoint functioned but that the G1/S-phase checkpoint did not exist in this model. Moreover, Yukawa et al. (Yukawa et al. 2007) reported that the G2/M checkpoint functions insufficiently in mouse 1-cell and 2-cell embryos because over 80% of embryos irradiated at the G2 phase were able to develop to the next stage without undergoing DNA repair, though cleavage was considerably delayed. Early cleavage stage mouse embryos did not undergo apoptosis even when they were treated with the apoptosis inducer actinomycin (Fabian et al. 2007). In the present study, a poor apoptotic response of the PB2 was observed when they were treated with cycloheximide (28.135 μg/ml). The PB2s had to be treated with this agent for 4 days to induce apoptosis responses that were clearly detectable by Caspase and TUNEL assays. Because the PB2 is extruded after fertilization, it might have the same cytological characteristics as embryos in this regard.

Our results on fusion of a PB2 with a blastomere in 2-cell embryos showed that the synchronization of the cell cycle between both cells plays a key role in the formation of 2*n*/3*n* mixoploid embryos. In the resultant 4-cell embryos, the incidence of daughter blastomeres with morphologically normal nuclei was higher when PB2–blastomere fusion was done at the S phase or earlier in the cell cycle, than when fusion was done at the G2 phase. Wakayama et al. (Wakayama et al. 1997) demonstrated that 1-cell embryos developed successfully to blastocysts when the female pronucleus was replaced by the PB2 nucleus of the same or younger embryo than when it was replaced by the PB2 nucleus of an older embryo. Furthermore, Verlinsky et al. (Verlinsky et al. 1994) reported that when replacing the female pronucleus with the PB2 nucleus, the lag of the PB2 cell cycle behind that of the normal blastomeres caused structural chromosomal aberrations. Here, we observed 4-cell embryos with micronuclei, extra nuclei, and beaded micronuclei sitting between two daughter blastomeres. These abnormalities might be caused by the forced separation of chromatid pairs at cytokinesis before the completion of DNA synthesis in nuclei originating from the PB2s.

In conclusion, we found that the PB2s remain in the S phase of the cell cycle until 72 h after fertilization without undergoing apoptosis. Furthermore, we demonstrated that 2*n*/3*n* mixoploid embryos could be produced artificially by fusing the PB2 with one of the blastomeres in 2-cell embryos. This suggests that such 2*n*/3*n* mixoploid embryos have the potential to develop further. Further investigation as to how 2*n*/3*n* mixoploid embryos can develop to term will contribute to better understanding of this potential cause of human congenital anomalies.

Acknowledgements

This study was supported by a Grant-in-Aid for Young Scientists (B) (24791678) from the Japan Society for the Promotion of Science, a grant from the Akiyama Foundation and a grant from the Promotion of Science Program, Asahikawa Medical University.

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Figure legends

Figure 1.

Fusion of a PB2 with a MII stage oocyte. (a) A zona-free 2-cell embryo with a PB2 held on a holding pipette. (b) Aspiration of the PB2 into a fine glass pipette. (c, d) Attachment of the PB2 to a MII oocyte using SV. (e) Fusion of the PB2 with a MII oocyte. The fused PB2 is identified by a small bulge (arrow) in the cortex. An arrowhead indicates the MII spindle. Bar = 50 μm.

Figure 2.

Comparison of the uptake of BrdU between PB2s and blastomeres of embryos at various times after fertilization.

Figure 3.

Detection of BrdU uptake in PB2s and blastomeres of embryos. (a) A 1-cell embryo at 6 h after fertilization showing FITC fluorescence in two pronuclei and in a PB2 nucleus. (b) A 1-cell embryo at 14 h after fertilization showing the FITC-positive PB2 nucleus. There is no FITC fluorescence in the pronuclei. (c) A 2-cell embryo at 20 h after fertilization showing nuclei of both blastomeres. The PB2 nucleus has no FITC fluorescence. (a′), (b′), and (c′) are the counterstained images of (a), (b), and (c), respectively. Arrows indicate the PB2s. Bar = 30 μm.

Figure 4.

Induction of PCC in PB2s following fusion with MII oocytes. (a) G1 chromatid spread in a PB2 collected from a 1-cell embryo at 4–6 h after fertilization. (b) S-type chromatin in a PB2 of a 2-cell embryo at 24 h after fertilization. (c) G2 chromosome spread from the PB2 of a 4-cell embryo at 48 h after fertilization. Bar = 10 μm.

Figure 5.

Apoptosis in PB2s of 2-cell embryos. (a) Caspase assay and (b) TUNEL assay. (a′) and (b′) are the counterstained images of (a) and (b), respectively. Bar = 30 μm.

Figure 6.

Morphological analysis of blastomeric nuclei of 4-cell embryos in which a PB2 was artificially fused with one blastomere at the 2-cell stage. (a) Four daughter nuclei with a morphologically normal shape. (b) Two abnormal daughter nuclei. One at the lower left is accompanied by an extra nucleus. There is a chain of chromatin beads between the extra nucleus and another daughter nucleus at the upper right. Bar = 20 μm.

Figure 7.

Chromosome preparation of an embryo in which a PB2 had been fused artificially with one of the blastomeres at the 2-cell stage. (a) The upper four daughter blastomeres originated from a blastomere that did not undergo cell fusion. They had already reached the fourth cleavage metaphase. The other two daughter blastomeres originated from a blastomere that had undergone cell fusion. One of these displayed a third cleavage metaphase spread (framed), the other remained at interphase. (b) Chromosomal spread at higher magnification of the framed region in (a). Bar = 30 μm.

Table 1. Induction of premature chromosome condensation (PCC) in second polar bodies (PB2s) by cell fusion with MII stage oocytes

Time (h) of PB2 sampling after fertilization (embryo stage)	No. of PB2s:		No. of PB2s examined	Types of PCC and frequency (%)		
	manipulated (No. experiments)	fused successfully with MII oocytes (%)		G1	S	G2
4–6 (1-cell)	153 (7)	146 (95.4)	135	134 (99.3)	1 (0.7)	0
24 (2-cell)	70 (3)	65 (93)	55	0	55 (100)	0
48 (4-cell)	72 (3)	67 (89)	66	0	62 (94)	4 (6)
72 (morula)	50 (2)	43 (86)	41	0	41 (100)	0

Table 2. Assays of apoptosis in second polar bodies (PB2s)

Time (h) after fertilization	Method	No. of PB2s analyzed (No. experiments)	No. (%) of apoptotic PB2s
24	Poly-caspase	70 (3)	2 (3)
	TUNEL	70 (3)	0
48	Poly-caspase	66 (3)	4 (6)
	TUNEL	67 (3)	0
72	Poly-caspase	40 (2)	3 (8)
	TUNEL	39 (2)	0

Figure 1.

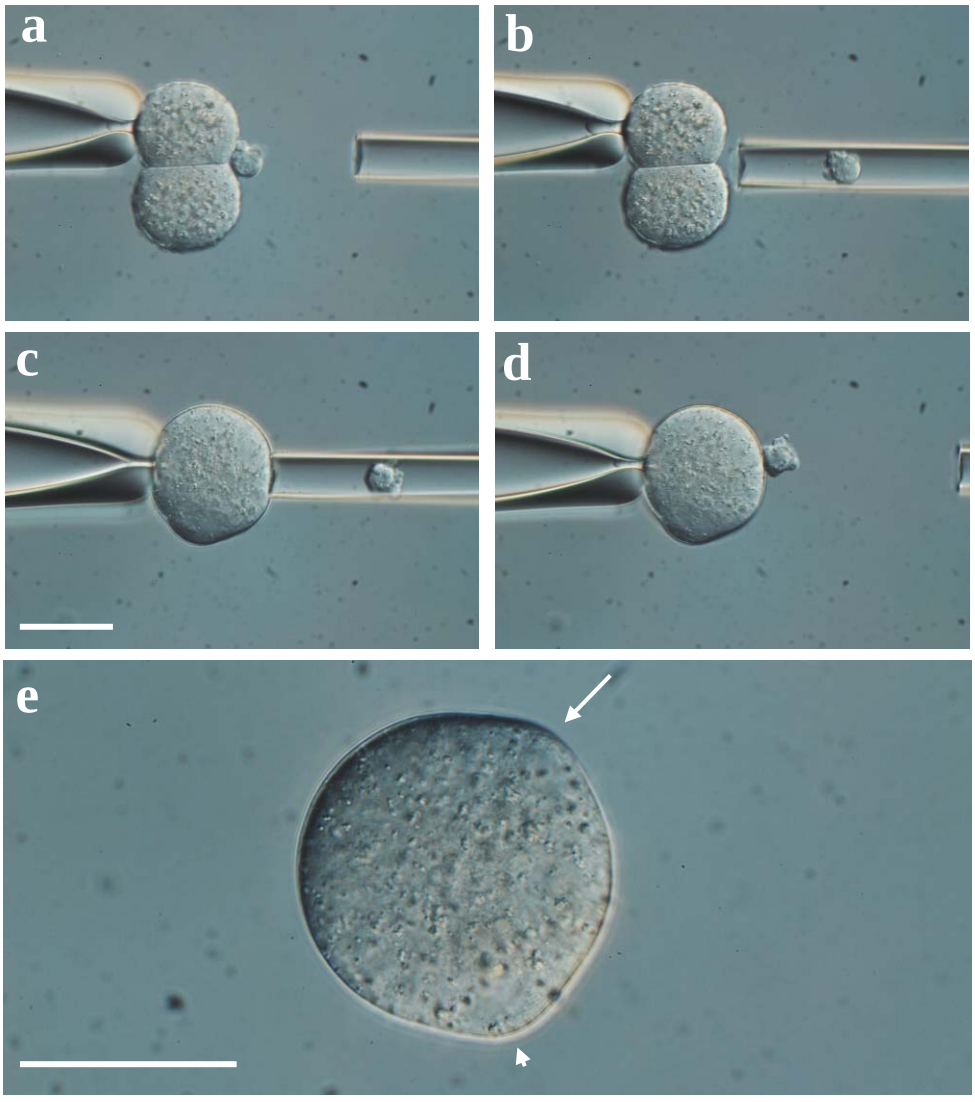

Figure 2.

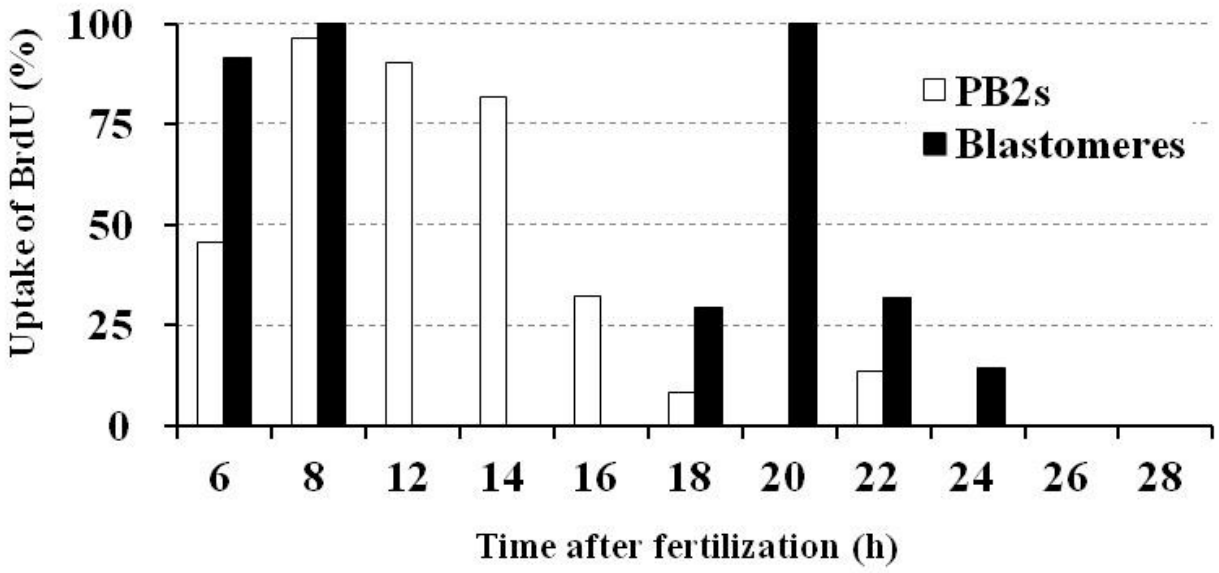
Uptake of BrdU (%)
100
75
50
25
0
6
8
12
14
16
18
20
22
24
26
28
Time after fertilization (h)
PB2s
Blastomeres

Figure 3.

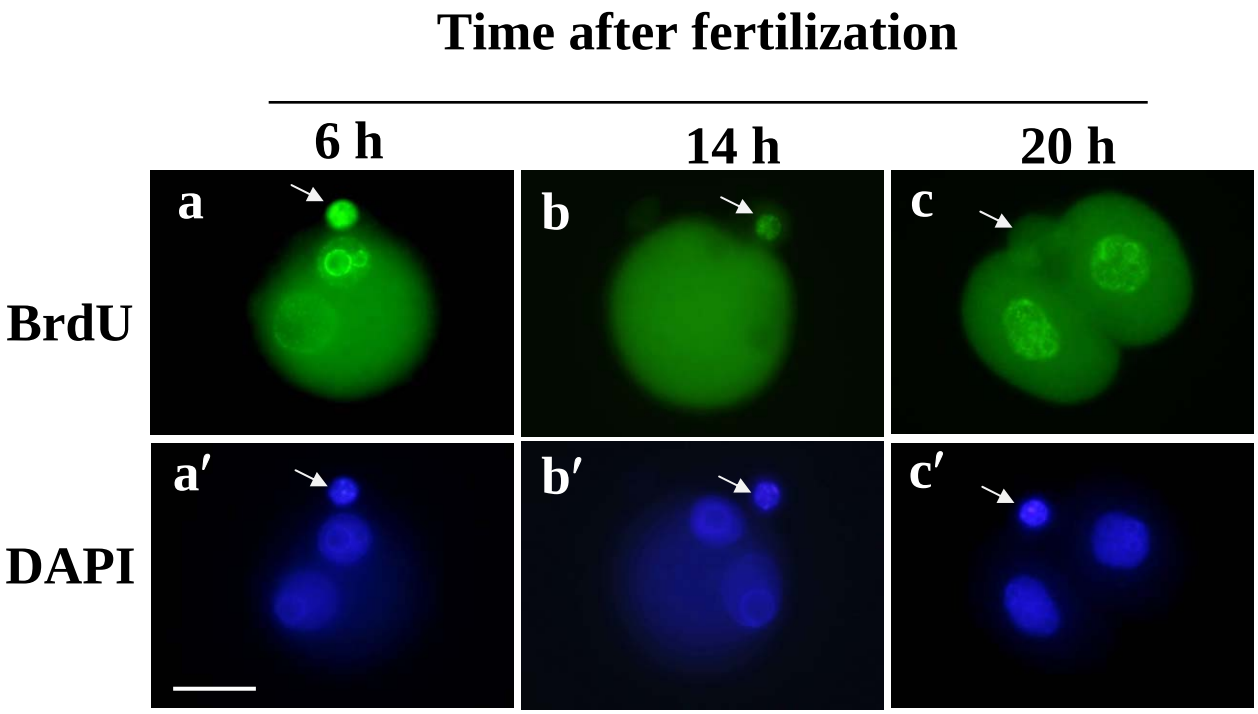

Figure 4.

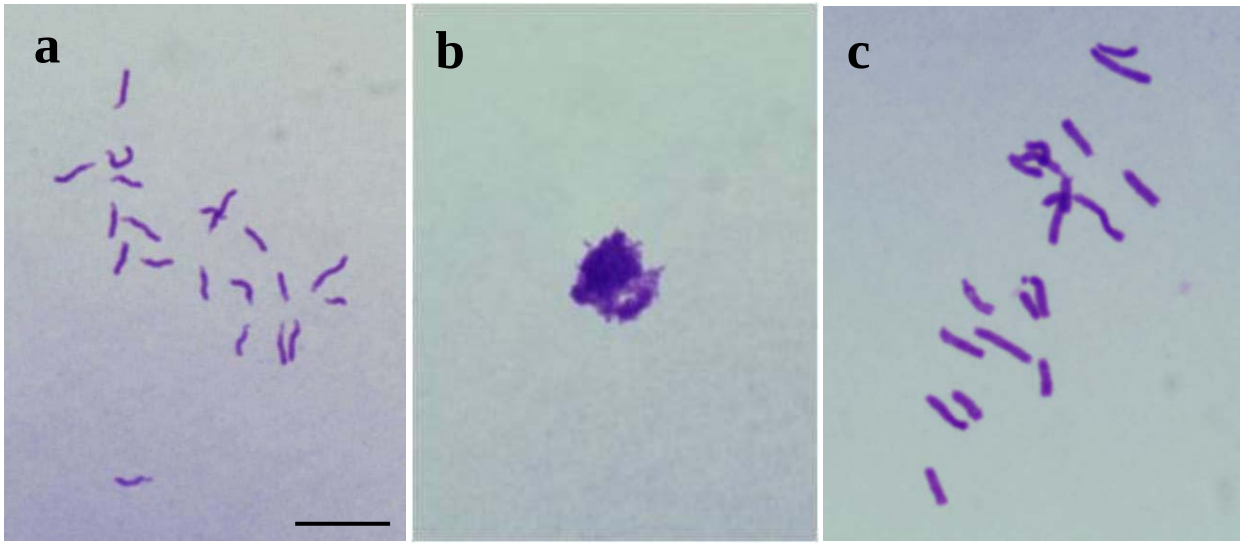

Figure 5.

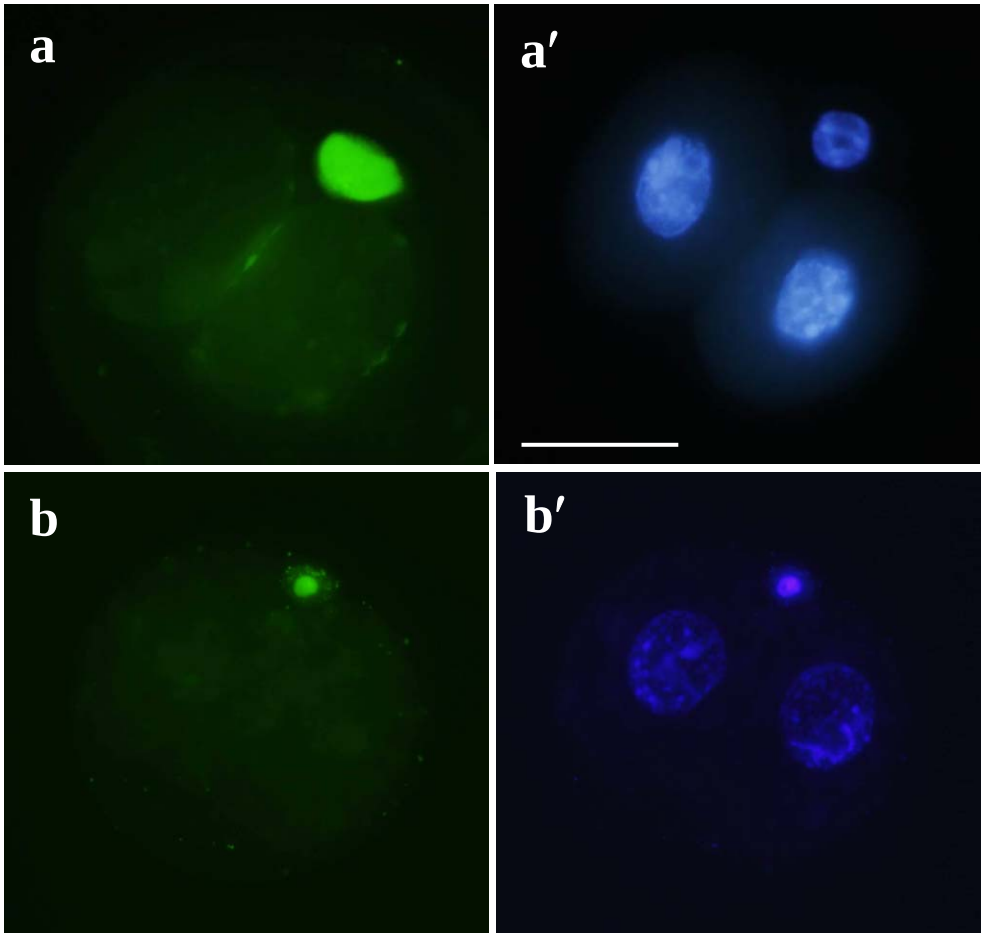

Figure 6.

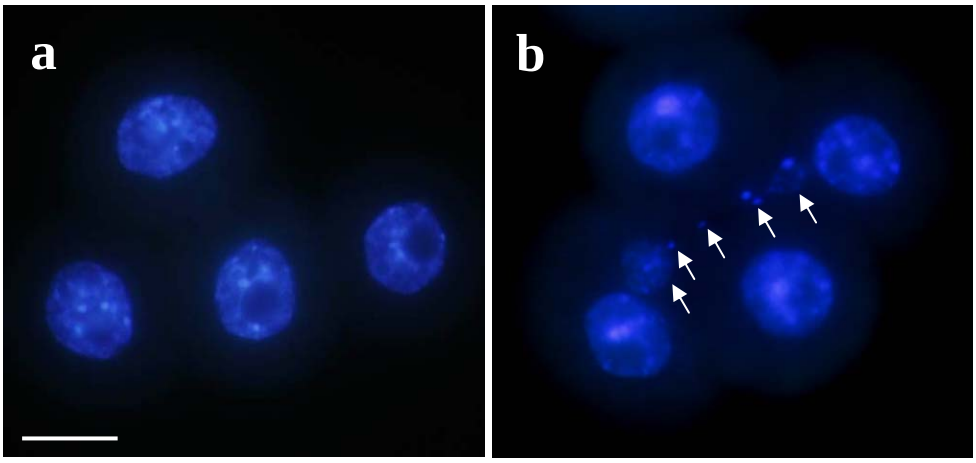

Figure 7.