Developmental potential of 2n/3n mixoploid mouse embryos produced by fusion of individual second polar bodies and blastomeres of 2-cell embryos.

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4   **Running title:** Development of mixoploid embryos

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2 **Abstract**

3   Using $2n/3n$ mixoploid mouse embryos produced by fusion of

4 individual second polar bodies (PB2s) with individual blastomeres of 2-cell

5 embryos, we examined the dynamics of PB2 nuclei in the host blastomeres

6 during mitosis, and followed the fate of the $3n$ cell line in the mixoploid

7 embryos. Most of the PB2 nuclei were synchronized with the cell cycle of the

8 host blastomeres, and all chromosomes were incorporated into a single

9 mitotic spindle. The majority of the mixoploid embryos developed to

10 blastocysts with $3n$ cells. In conceptuses at day 11.5 and day 18.5 of

11 gestation, $3n$ cells were recognized in both of the embryonic/fetal and the

12 placental tissues. When green fluorescent protein (GFP)-transgenic mice

13 were used as a donor of PB2, GFP-positive $3n$ cells were found in more than

14 40% of morulae and blastocysts, indicating that the PB2 genome can be

15 reactivated during the preimplantation stage. GFP-positive $3n$ cells were

16 non-randomly allocated in trophectoderm in blastocysts. These findings may

17 explain the production mechanism of $2n/3n$ mixoploid human embryos, that

18 is, PB2 is incorporated into one daughter blastomere during the early

19 cleavage period.

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2   **Introduction**

3   Mixoploidy is a numerical chromosomal anomaly that consists of

4   different ploidies. In humans, mixoploidy such as diploidy ($2n$)/triploidy ($3n$)

5   and diploidy ($2n$)/tetraploidy ($4n$) are relevant to malformation syndromes,

6   including mental retardation, truncal obesity and body/facial asymmetry

7   (Edwards et al., 1994; van de Laar et al., 2002). Mixoploidy also occurs in

8   early cleavage embryos of other mammalian species, including pigs (Hornak

9   et al., 2012), horses (Rambags et al., 2005), sheep (Coppola et al., 2007) and

10   cattle (King, 2008). Tatewaki et al. (1989) estimated the incidence of $2n/4n$

11   mixoploidy to be 8% in mouse blastocysts. Surprisingly, Bielanska et al.

12   (2002) reported that more than 80% of human blastocysts of patients

13   undergoing in vitro fertilization (IVF) were mixoploid, with complex

14   combinations of different ploidies.

15   The widely accepted mechanism underlying $2n/4n$ mixoploidy

16   formation is a failure in the cytokinesis of blastomere(s) of $2n$ embryos

17   during early cleavage divisions, because sex-chromosome constitution of

18   $2n/4n$ patients was exclusively XX and XXXX or XY and XXYY (Wilson et al.,

19   1988; Edwards et al., 1994; Alonso et al., 2002; Tözüm et al., 2005; Olgun-

20   Erdemir et al., 2010). On the other hand, potential mechanisms of $2n/3n$

21   mixoploidy include (1) delayed dispermy, in which a second spermatozoon

22   may be integrated into a daughter blastomere of a diploid embryo, (2)

23   incorporation of a second polar body (PB2) into a daughter blastomere, and

1. (3) chimerism due to the union of a $2n$ embryo and a $3n$ embryo. The other

2. plausible mechanism is postzygotic diploidization of diandric triploid

3. embryos, in which one of the paternal genomes may be eliminated from a

4. daughter blastomere (Golubovsky, 2003). Studies with genomic DNA from

5. $2n/3n$ patients and their parents have revealed that in most of the cases the

6. extra haploid set of $3n$ cell line is of maternal origin (Müller et al., 1993;

7. Daniel et al., 2001; van de Laar et al., 2002; Daniel et al., 2003; Brems et al.,

8. 2003; Rittinger et al., 2008; Boonen et al., 2011). Therefore, the

9. incorporation of a PB2 into a daughter blastomere may be more likely than

10. other scenarios. In this context, it should be considered that PB2s of human

11. embryos remain in the perivitelline space during early cleavages (Hertig et

12. al., 1956).

13.   Mouse PB2 can survive relatively long after fertilization without

14. undergoing apoptosis (Hino et al., 2013; Fabian et al., 2014). When a PB2

15. was artificially fused with one blastomere of a 2-cell embryo, the resultant

16. $2n/3n$ mixoploid embryos developed to the 4-cell stage, and the nucleus of

17. the PB2 resumed cell cycle in the host blastomere (Hino et al., 2013). These

18. findings suggest that the $3n$ cell line of PB2 origin in $2n/3n$ mixoploid mouse

19. embryos can participate in the subsequent embryonic development.

20.   This study was undertaken to understand the fate and genomic activities

21. of the $3n$ cell line in the $2n/3n$ mixoploid mouse embryos during pre- and

22. post-implantation periods.

23.

1   **Materials and methods**

2   *Animals*

3   In this study, hybrid mice BDF1 (C57BL/6Cr × DBA/2Cr) were used

4   as donors of 2-cell embryos and PB2s to produce mixoploid embryos. GFP-

5   transgenic C57BL/6-TgN (act-EGFP) OsbC14-Y01-FM131 mice (+/+) were

6   used as donors of PB2s to visualize genomic expression, and ICR (CD1) mice

7   were used as surrogate mothers of embryos. These mice, 6 weeks of age,

8   were obtained from Japan SLC (Hamamatsu, Japan), and used in the

9   experiments at 8–12 weeks of age.

10   The mice were kept in a light- and temperature-controlled room (14-h

11   light/10-h dark; 23°C ± 2°C) and given *ad libitum* access to food and water.

12   All experiments were carried out according to the Guidelines for Animal

13   Experiments of Asahikawa Medical University.

14

15   *Chemicals*

16   Organic and inorganic reagents were purchased from Nacalai Tesque

17   Inc. (Kyoto, Japan), unless otherwise stated.

18

19   *Production of embryos by in vitro fertilization*

20   Throughout this study, the *in vitro* fertilization technique was used to

21   fertilize oocytes. Spermatozoa obtained from the cauda epididymis of BDF1

22   male mice were put into a droplet (200 μl) of Toyoda-Yokoyama-Hosi (TYH)

23   medium (Toyoda et al., 1971) under paraffin oil (Merck KGaA, Darmstadt,

1 Germany), and incubated for 2 h at 37°C under 5% CO_2 in air to induce

2 capacitation. Capacitated spermatozoa were transferred into a droplet (200

3 µl) of TYH medium to adjust the final sperm concentration to 50−100

4 cells/µl.

5   Superovulation was induced in BDF1 and GFP-transgenic mice by

6 intraperitoneal injection of 7.5 IU of equine chorionic gonadotropin and 7.5

7 IU of human chorionic gonadotropin (hCG) at 48-h intervals. Cumulus-

8 oocyte complexes (COCs) were obtained from the oviducts at 14−16 h after

9 hCG injection. Immediately, they were put into the droplet (200 µl) of TYH

10 medium containing capacitated spermatozoa, and incubated at 37°C under

11 5% CO_2 in air. Two hours later, the COCs were treated with 0.02%

12 hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) for 5 min to completely

13 remove cumulus cells. After being washed three times in Chatot-Ziomek-

14 Bavister medium (Chatot et al., 1989) supplemented with 5.55 mM D-

15 glucose (mCZB), the oocytes were cultured in fresh mCZB at 37°C under 5%

16 CO_2 in air. Six to 8 h later, monospermic eggs with two pronuclei and a

17 second polar body (PB2) were selected under a dissecting microscope with a

18 magnification of 40×, and further cultured up to the 2-cell stages.

19

20 *Fusion of PB2 with blastomere*

21   Fusion of PB2 with blastomere was carried out according to Hino et al.,

22 (2013). Briefly, 2-cell embryos at 17−18 h after fertilization were treated

23 with 0.5% protease (1,000,000 tyrosine units/g; Kaken Pharmaceutical Co.,

Ltd, Tokyo, Japan) for 5–10 min to digest the zona pellucida. The PB2 was

aspirated with a fine pipette and fused with one blastomere using Sendai

virus (GenomONE™-CF; Ishihara Sangyo Kaisha, Osaka, Japan) to produce

$2n/3n$ mixoploid 2-cell embryos. The embryos were cultured in mCZB at

37ºC under 5% CO_2 in air for a further 75 h to check the development to

blastocysts.

Immunocytological observation of spindle

After blastomeres had fused with PB2s and reached the first metaphase,

they were processed for visualization of the spindle and chromosomes.

Blastomeres were placed in acidic Tyrode's solution (pH 2.5; Sigma-Aldrich)

for 20–30 min, and permeabilized with 1% triton X-100 (Sigma-Aldrich) in

Dulbecco's phosphate-buffered saline (D-PBS, pH 7.4) for 1 h at room

temperature. After washing with D-PBS containing 0.3 % bovine serum

albumin (BSA; Fraction-V, Sigma-Aldrich) (D-PBS/BSA), blastomeres were

incubated with anti-ß-tubulin monoclonal antibody (1:100 dilution; Sigma-

Aldrich) in D-PBS for 1–2 h at 37°C. The cells were stored at 4°C in D-

PBS/BSA overnight. The cells were then incubated in D-PBS containing an

anti-mouse IgG-FITC-conjugated antibody (1:400 dilution; Sigma-Aldrich)

for 1 h at 37°C in dark. Finally, the cells were mounted on a glass slide and

covered with a mounting medium containing 4′-6-diamidino-2-phenylindole

(DAPI) (Vectashield; Vector Laboratories, Burlingame, CA, USA) for

fluorescent microscopy.

1

2 *Interphase fluorescence in situ hybridization (FISH) analysis*

3 *Preimplantation embryos*

4   Morulae and blastocysts were transferred into hypotonic solution

5 consisting of 1:1 mixture of 1% sodium citrate and 30% fetal bovine serum

6 (FBS; PAA laboratories, Etobicoke, Canada). Morulae and blastocysts were

7 kept in the solution for 1−3 min and 30 min, respectively, at room

8 temperature. The cytological slides of embryos were prepared using the

9 gradual-fixation/air-drying method (Mikamo and Kamiguchi, 1983). The

10 slides were treated at 50°C for 2 h in air. Subsequently, in situ hybridization

11 was carried out as follows. The slides were denatured in 2×SSC/70%

12 formamide (pH 7.0) at 72°C for 2 min, dehydrated in a series of ethanol (70%,

13 85%, and 100%), and air-dried. Mouse FISH probes (Kreatech, Amsterdam,

14 Netherlands) specific to *Aurka* and *Tlk2* genes on chromosomes 2 and 11,

15 respectively, were applied to the denatured slides. The slides were covered

16 with a glass coverslip, and incubated in a humidified chamber at 37°C

17 overnight. Finally, the slides were covered with *SlowFade*®Gold antifade

18 reagents containing DAPI (Invitrogen, Carlsbad, CA, USA) for fluorescent

19 microscopy.

20

21 *Conceptuses at 11.5 and 18.5 days of pregnancy*

22   When 2-cell embryos grew morulae, they were transferred into the

23 uteri of pseudopregnant CD1 female mice that had been mated with

vasectomized male mice 3 days ago. Sixteen to 21 morulae were transferred

into each recipient. Nine or 16 days after the transfer (embryonic stage of

day 11.5 and day 18.5; abbreviated as E11.5 and E18.5, respectively), the

females were sacrificed, and the conceptuses were retrieved from the uteri.

Embryos/fetuses and placentas were separately minced thoroughly in D-

PBS (−). In the fetuses at E18.5, the liver and the spleen were selectively

screened. The pieces of tissues in D-PBS (−) were further broken up to

collect cells by forcing them to pass through a mesh filter (150 µm). Cell

suspensions were centrifuged for 5 min at 550 ×g, supernatants were

removed, and 3 mL of 75 mM potassium chloride were added to the tubes for

hypotonic treatment. Twenty min after the treatment, 7 mL of fixative

(methanol:acetic acid=3:1) were added gently and mixed with the cell

suspensions. After washing by two centrifugations for 5 min at 550 ×g, the

cells were resuspended in 50 µL of the fixative. Twenty µL of the cell

suspensions was placed on a slide in a temperature- and humidity-

controlled chamber (HANABI; ADSTEC, Funabashi, Japan) and dried. The

slides were then treated at 50 ºC for 2 h in air. FISH was performed as

described above.

Analysis of green fluorescent protein (GFP) expression

Preimplantation embryos

PB2s from 2-cell embryos of GFP-transgenic mice were individually

fused with one of sister blastomeres of 2-cell embryos of BDF1 mice. The

successfully fused embryos were cultured in mCZB medium at 37°C under

1   5% CO_2 in air. When the fused embryos reached the 4-cell (48h after

2   fertilization), morula (72h after fertilization), or blastocyst (96h after

3   fertilization) stages, they were fixed in 4 % paraformaldehyde (PFA) for 10

4   min. The embryos were mounted on a glass slide and covered with a

5   mounting medium containing DAPI. Images of embryos with GFP-positive

6   cells were taken under a fluorescent microscope.

7   To determine the location of GFP-positive cells in blastocysts, inner

8   cell masses (ICM) and trophectoderm (TE) cells were identified by the

9   expressions of their respective markers, Oct4 (Nichols et al., 1998) and Cdx2

10   (Beck et al., 1995). Blastocysts were fixed with 4% PFA in D-PBS overnight

11   at 4°C, and then permeabilized with 0.1% Triton X-100 in D-PBS for 10 min

12   at room temperature. They were incubated in D-PBS containing mouse anti-

13   Oct4 IgG (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) for 1

14   h at 37°C in dark and incubated in D-PBS containing Alexa-Fluor-546-

15   labeled anti-rabbit IgG (1:100 dilution; Molecular probes, Eugene, OR, USA)

16   for 1 h at 37°C in dark. Subsequently, the blastocysts were incubated in D-

17   PBS containing mouse anti-Cdx2 IgG (1:1 dilution; BioGenex, San Ramon,

18   CA, USA) for 1 h at 37 °C in dark, and continued to be incubated in D-PBS

19   containing Alexa-Fluor-350-labeled anti-mouse IgG (1:100 dilution;

20   Molecular probes) for 1 h at 37°C in dark. Finally, the blastocysts were

21   placed on a glass slide and covered with a mounting medium (Vectashield).

22   The slides were observed under a confocal laser-scanning microscope

23   (FV1000-D; Olympus, Tokyo, Japan).

1

2 *Conceptuses at E11.5*

3   Morulae were transferred to the pseudopregnant females as described

4 previously. Females were sacrificed at E11.5, and conceptuses were

5 retrieved from the uteri. The conceptuses were fixed with 4% PFA in 0.1 M

6 phosphate buffer solution (pH 7.2; abbreviated as 0.1 M solution) at 4°C

7 overnight in dark. After being washed with 0.1 M solution, the conceptuses

8 were immersed in 10% sucrose in 0.1 M solution for 3 h, followed by

9 treatment with 20% sucrose in 0.1 M solution for 3 h, and 30% sucrose in 0.1

10 M solution overnight. The conceptuses were embedded in Optimal Cutting

11 Temperature (O.C.T.) compound (Sakura Finetechnical Co., Ltd., Tokyo,

12 Japan) and rapidly frozen by use of the liquid nitrogen. The samples were

13 stored at −80°C until use. Cryostat sagittal sections (6 μm in thickness)

14 were prepared using a cryostat (CM3050; Leica Biosystems, Nussloch,

15 Germany). The sections were mounted with a mounting medium containing

16 DAPI, and GFP fluorescence was screened with a fluorescent microscope.

17 The observations were made at least every 5 sections per conceptus.

18

19 *Statistical analysis*

20   Percentile data for preimplantation development were transformed

21 into arcsine values for statistical analysis. When data were compared

22 between two different groups, one-way analysis of variance (ANOVA)

1 followed by Student's *t*-test was used. Statistical significance was accepted

2 at $p < 0.05$.

3

4 **Results**

5 <u>Experiment 1:</u> *Preimplantation development of mixoploid embryos*

6   First, we observed spindle and chromosomes at the first metaphase after

7 cell fusion to examine whether two nuclei of blastomere and PB2 origin can

8 unite. Twenty-three blastomeres were fused with PB2s and

9 immunocytologically prepared after the disappearance of nuclear membrane.

10 Based on the features of spindle and chromosome alignment, 18 blastomeres

11 were determined to be at metaphase, and others were at prophase or

12 telophase. All cells at metaphase formed a bipolar spindle. In 16 of them,

13 condensed chromosomes of PB2 origin aligned on the equatorial plane of the

14 spindle along with chromosomes of the host blastomere (Figure 1). The

15 others had less condensed chromosomes of PB2 origin on the equatorial

16 plane.

17   Subsequently, $2n/3n$ mixoploid 2-cell embryos, the blastomeres of which

18 were fused with BP2s, were followed up to the blastocyst stage. Among the

19 45 2-cell embryos cultured, all grew morphologically normal 4-cell embryos

20 and morulae, and finally 41 (91.1%) achieved blastocyst formation on

21 schedule. These percentages were similar to those of sham-operated controls

22 (Table 1).

1   Two-color FISH analysis was applied to interphase cells of morulae and

2   blastocysts to confirm the presence of $3n$ cells at these embryonic stages.

3   Out of the 36 morulae and 40 blastocysts analyzed, $3n$ cells were observed in

4   34 (94.4%) morulae and 33 (82.5%) blastocysts (Figure 2). Among the $2n/3n$

5   morulae found, there was great variation in the proportion of $3n$ cells to

6   total cells of embryos. However, the percentages of $3n$ cells did not exceed

7   50% except in one case (Supplemental Figure 1). The numbers of $3n$ cells

8   were not counted in blastocysts because of the overlapping of cells, which

9   made accurate counting difficult.

10

11   <u>Experiment 2</u>: *Postimplantation development of mixoploid embryos*

12   A total of 166 morulae were transferred to 9 recipient females. Among

13   these females, 5 were sacrificed at E11.5 and 4 were sacrificed at E18.5. Ten

14   and 9 live conceptuses were obtained at E11.5 and E18.5, respectively (Table

15   2). Two-color FISH analysis was applied to more than 150 interphase cells of

16   embryonic/fetal and placental cells. At E11.5, $3n$ cells were found in 4

17   conceptuses: all embryos and 3 placentas. Four conceptuses at E18.5 were

18   mixoploidy; $3n$ cells were found in 3 fetuses and all placentas. There was a

19   great variation in the percentage of $3n$ cells among the conceptuses at E11.5,

20   while the percentage was considerably low in conceptuses at E18.5.

21   Majority of $2n/3n$ conceptuses were normal in appearance, however, one

22   of embryos at E11.5 showed slight growth retardation, and a large placenta

23   was observed in one case at E18.5.

1

2 <u>**Experiment 3**</u> : Activities of PB2 genome in mixoploid embryos

3 *1) GFP expression in preimplantation embryos*

4   The $2n/3n$ mixoploid embryos were produced using PB2s originated from

5 GFP-transgenic mice to visualize the activities of PB2 genome in the $3n$ cell

6 line. There were no GFP signals at 2-cell and 4-cell stages, while 46.2%

7 (18/39) of morulae and 50.0% (18/36) of blastocysts emitted GFP

8 fluorescence (Figure 3).

9   The distributions of $3n$ cells with GFP signals were determined in

10 additional 19 blastocysts. Among them, 2 cases (10.5%) had GFP signals in

11 ICM, 15 cases (78.9%) had the signals in TE, and 2 cases (10.5%) had the

12 signals in both ICM and TE (Figure 4).

13   Incidentally, the preimplantation development of the $2n/3n$ mixoploid

14 embryos that originated from PB2s of GFP-transgenic mice was comparable

15 to that of $2n/3n$ mixoploid embryos that originated from PB2s of BDF1 mice

16 (Supplemental Table 1).

17

18 *2) GFP expression in conceptuses at E11.5*

19   A total of 116 morulae were transferred to recipient females. Among

20 them, 38 (32.8%) were implanted and 12 (10.3%) survived at E11.5. In

21 cryosection of the live consceptuses, no GFP-positive $3n$ cells were found in

22 any embryonic tissue, but it was noteworthy that one placenta had GFP-

23 positive $3n$ cells (Figure 5).

1

2 **Discussion**

3   The developmental potential of $2n/3n$ mixoploid mouse embryos may

4 be dependent on the timing of fusion of PB2 with blastomere, because mouse

5 PB2 of 2-cell embryo arrests the cell cycle at S-phase. Our previous study

6 found that when a PB2 was fused with a blastomere of a 2-cell embryo in

7 G1- and early S-phase 17–19 h after fertilization, more than 80% of the

8 daughter cells had morphologically normal nuclei. On the contrary, when a

9 PB2 was fused with a blastomere in G2-phase 27–28 h after fertilization,

10 more than 70% of the daughter blastomeres had abnormal nuclei (Hino et

11 al., 2013). In this study, we fused individual PB2s with individual

12 blastomeres of 2-cell embryos 17–19 h after fertilization. It seems likely that

13 this timing of cell fusion achieved a successful outcome in the formation of

14 bipolar spindle and preimplantation development.

15   In this study, interphase FISH analysis revealed that the percentage

16 of $3n$ cells in morulae was usually less than 50%. Comparable data have

17 been reported in a study using chimeric mouse embryos. Chromosome

18 analysis of $2n \leftrightarrow 3n$ chimeric mouse embryos at the blastocyst stage, in which

19 $3n$ cells were produced by suppressing extrusion of a PB2 by treatment with

20 cytochalasin B, found that the proportions of $3n$ cells and $2n$ cells among

21 analyzable cells were approximately 40% and 60%, respectively (Azuma et

22 al., 1991a). In addition, the study showed that the total cell number of the

23 digynic $3n$ embryos was about half of that of normal $2n$ embryos after *in*

vitro culture for 96 h. These findings suggest that the cell cycle of $3n$ cells is slower than that of $2n$ cells in the mixoploid embryos.

The present study found that $3n$ cells of PB2 origin survived, and organized into the embryonic/fetal tissues and the placenta at E11.5 and E18.5, though the percentage of $3n$ cells was low at E18.5, compared to that at E11.5. Previous mouse studies have reported that $3n$ cells distributed poorly to $2n \leftrightarrow 3n$ chimeric embryos or fetuses at E9.5 and E12.5 (Everett et al., 2007) and at E8–19 (Suwińska et al, 2005). Nonetheless, $3n$ cells were found in the organs of adult mice when the $2n \leftrightarrow 3n$ chimeric embryos were produced by aggregation of $2n$ embryos and $3n$ embryos by suppression of PB2s (Azuma et al., 1991b; Suwińska et al, 2005). When the $2n \leftrightarrow 3n$ chimeric mouse embryos were produced by fusion of individual karyoplasts from haploid 4-cell embryos with individual blastomeres of $2n$ 2-cell embryos, the chimerism was not expressed in the coat and the blood of adults (Suwińska et al, 2005). The latter method resembles our production method of $2n/3n$ mixoploid embryos, in which individual haploid PB2s were fused with individual $2n$ blastomeres (Hino et al., 2013). The survivability of the $3n$ cells in $2n/3n$ mixoploid embryos may be influenced by the mechanism underlying the mixoploidy formation.

As far as GFP signals in $2n/3n$ mixoploid embryos are concerned, PB2 genomes of $3n$ cells seemed to be incapable of working up to the 4-cell stage, and then reactivated by the morula stage. However, there was no GFP signal in about a half of molurae/blastocysts, although the developmental

assay showed that $2n/3n$ embryos frequently (88–100%) develop to

morulae/blastocysts (Table 1, Supplemental Table 1). The low incidence of

morulae/blastocysts with GFP signals suggests that quite a few of $2n/3n$

morulae/blastocysts might have failed to fulfill the reactivation of PB2

genomes. Because PB2 genomes of mouse embryos remain highly

methylated during the preimplantation period (Santos et al., 2002),

demethylation might be inadequate to fulfill reactivation of PB2 genomes in

the GFP-negative morulae/blastocysts.

When Oct4 and Cdx2 were used as markers for ICM and TE cells,

respectively, GFP signals were more frequently observed in TE than in ICM.

This suggests that $3n$ cells might be non-randomly distributed during

blastocyst formation in $2n/3n$ mixoploid embryos produced by fusion of

individual PB2s and blastomeres. The preferential allocation of polyploidy

in TE cells has been found in $2n \leftrightarrow 3n$ chimeric blastocysts produced by

aggregation of a $2n$ embryo and a $3n$ embryo at 8-cell and morula stages

(Azuma et al., 1991a), in which $3n$ embryos were produced by suppressing

PB2s using cytochalacin B. The same observation has been reported in

$2n \leftrightarrow 4n$ chimeric blastocysts produced by aggregation of a $2n$ embryo and a

$4n$ embryo during the 4-cell stage (Everett and West, 1996, 1998; Tang et al.,

2000; MacKay and West, 2005). In these studies, $4n$ embryos were produced

by inhibiting cytokinesis at second cleavage using cytochalasin B. Poor

distribution of $4n$ cells to embryonic ectoderm was also found in $2n \leftrightarrow 4n$

mouse chimeras at postimplantation stages (James et al., 1995; Goto et al.,

2002). The non-random allocation of polyploid cells may also be true in

$2n/4n$ mouse mosaic embryos (Tarkowski et al., 1977).

In this study, we tried to histologically detect GFP-positive cells in conceptuses at E11.5 to understand the allocation and the genomic activity of $3n$ cells in the embryos and the placentas. None of the 12 conceptuses examined had GFP-positive cells in embryonic tissues, but one had GFP-positive cells in the placental tissue. Our FISH analysis at the same embryonic stage revealed that incidence of $2n/3n$ mixoploid conceptuses was less than 50%, and the percentage of $3n$ cells in the embryonic tissue was considerably low, except for one case. This may be the reason why GFP-positive cells could not be detected in histological sections of embryos.

In conclusion, when a PB2 was artificially fused with a blastomere of a 2-cell embryo, chromosomes of PB2 origin were synchronously organized as those of the host blastomere, and incorporated into the single mitotic spindle. The majority of $2n/3n$ mixoploid embryos developed to blastocysts, and $3n$ cells distributed to ICM and TE. Subsequently, $3n$ cells organized into the fetal and placental tissues. The GFP signal assay found that PB2 genomes of $3n$ cells were reactivated by the morula stage. Our findings suggest that mouse $2n/3n$ mixoploid embryos produced by fusion of individual PB2s with individual blastomeres may be available as an animal model to study the causative mechanisms underlying formation of human $2n/3n$ mixoploid embryos.

1

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5

Declaration of interest

7   The authors have no conflict of interest to declare.

8

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2 **Figure legends**

3

4 **Figure 1.** Bipolar spindle of a blastomere of a 2-cell embryo at the first

5   mitotic division after fusion with a PB2. Chromosomes of the PB2

6   were aligned along with chromosomes of the host blastomere on the

7   equatorial plane. Bar, 10 µm.

8

9 **Figure 2.** Two-color FISH analysis of interphase nuclei of $2n/3n$ mixoploid

10   embryos at the morula stage. (a) Cytological preparation of a whole

11   embryo. The nuclei were counterstained with DAPI. (b) Enlargement

12   of the frame in (a). The triploid cell nucleus (top) showed 3 green

13   (chromosome 2) and 3 red (chromosome 11) signals, while the diploid

14   cell nucleus (bottom) exhibited 2 green and 2 red signals. Bar, 10 µm.

15

16 **Figure 3.** Visualization of $3n$ cells in a $2n/3n$ mixoploid embryo by using a

17   PB2 of a GFP-transgenic mouse. The fusion was carried out at the

18   early 2-cell stage (17−19 h after fertilization). There were no GFP

19   signals in late 2-cell and 4-cell embryos. GFP-positive cells were

20   found in some morulae and blastocysts. Bar, 30 µm.

21

22 **Figure 4.** Distribution of GFP-positive $3n$ cells in inner cell masses (ICM)

23   and trophectoderm (TE) of $2n/3n$ mixoploid blastocysts. (a−d)

24   Distribution of GFP-positive $3n$ cells in ICM. Oct4 (red in color) and

25   Cdx2 (blue in color) were expressed in ICM (a) and TE (b),

26   respectively. The GFP signals were located on ICM (c). (d) is a

27   merged figure of (a−c). (e−h) Distribution of GFP-positive $3n$ cells in

28   TE. Expressions of Oct4 and Cdx2 are shown in (e) and (f),

29   respectively. The GFP-positive cells were located in TE (g). (h) is a

30   merged figure of (e−g). Bar, 30 µm.

31

1   **Figure 5.** Distribution of GFP-positive $3n$ cells in a conceptus at E11.5. (a)

2   Fetus and placenta. Bar, 2 mm. (b) Cryosection of placenta with

3   GFP-positive $3n$ cells (arrows). Bar, 1 mm. (c) High magnification of

4   a GFP-positive cell indicated by the right arrow in (b). Orange

5   emission is due to endogenous autofluorescence from placental

6   tissues. Bar, 100 µm.

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11   **Supplemental Figure S1.**

12   Frequency distribution of $2n/3n$ mixoploid morulae (n = 34) in

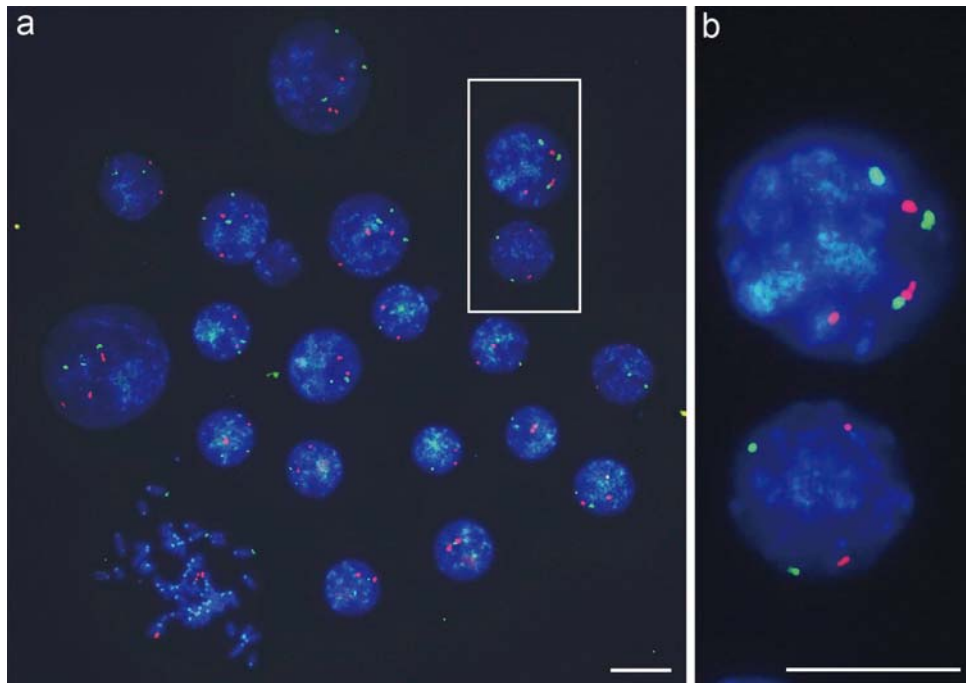13   relation to the percentage of $3n$ cells.
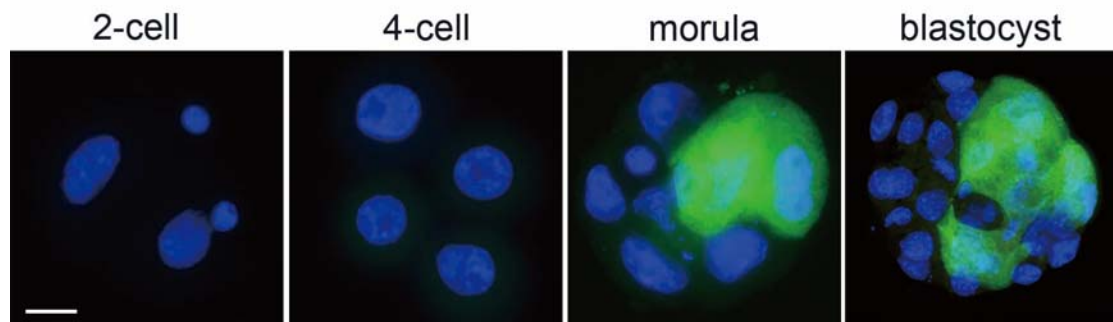
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Fig. 1

Fig. 2

2-cell　4-cell　morula　blastocyst

Fig. 3

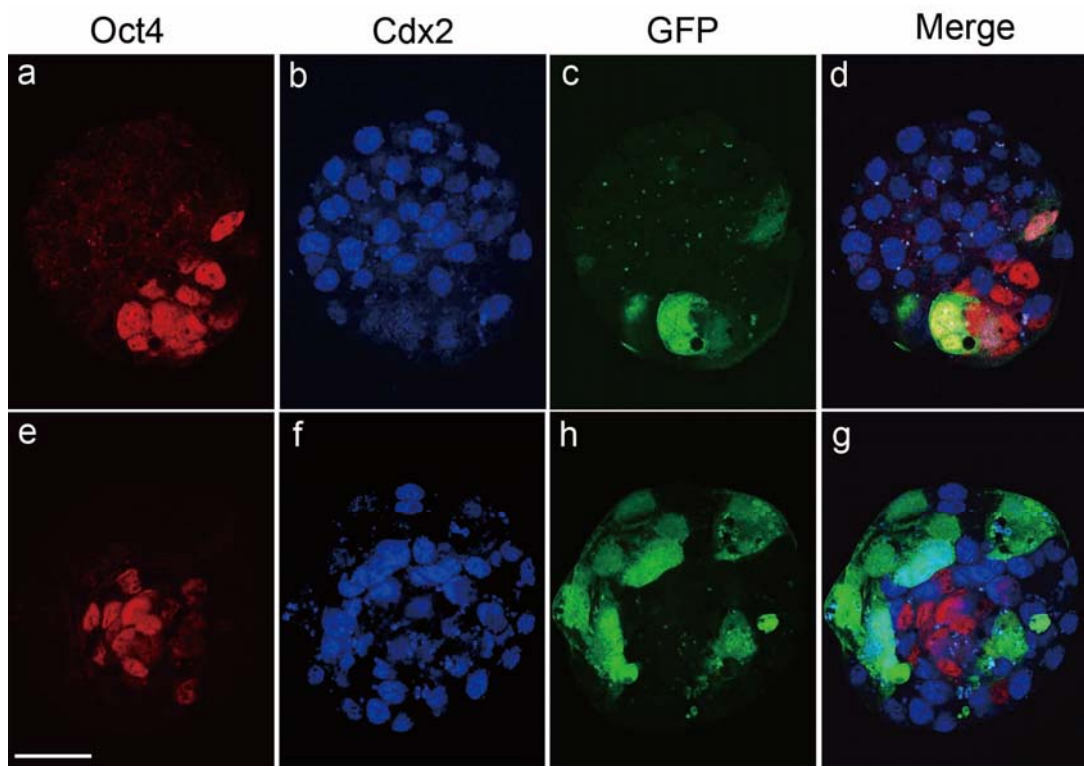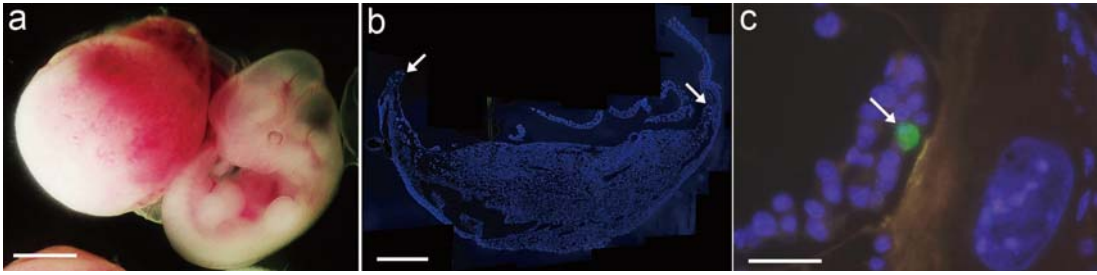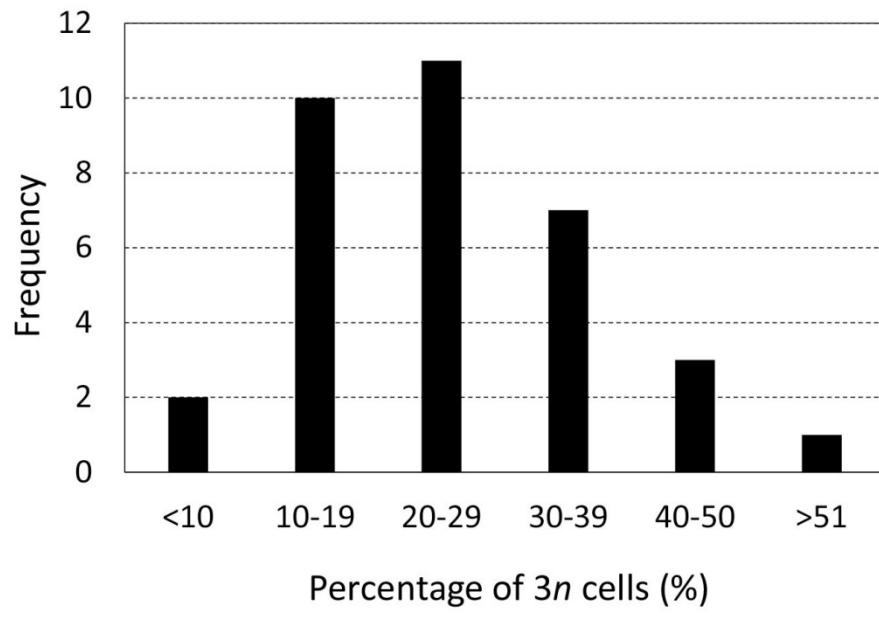| Oct4	Cdx2	GFP	Merge
a	b	c	d
e	f	h	g
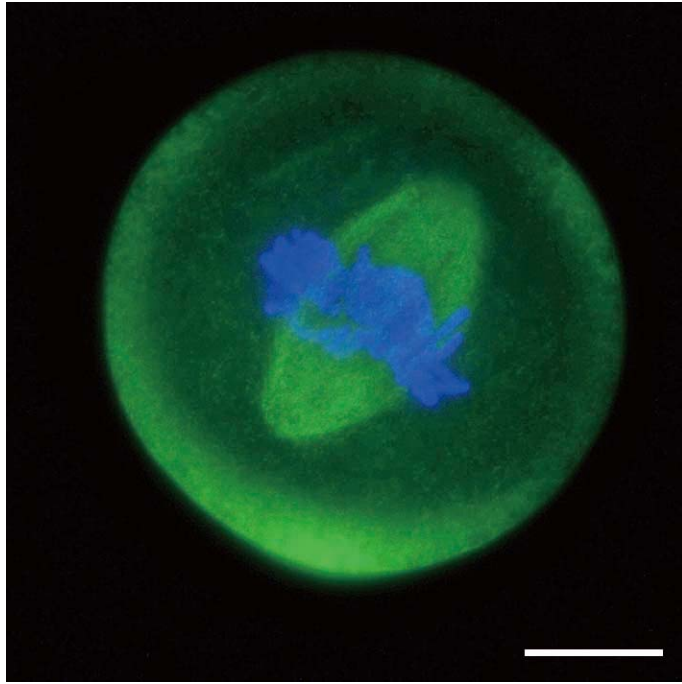
Fig. 4

Fig. 5

Supplemental Figure S1

Table 1. Preimplantation development of $2n/3n$ mixoploid embryos produced by fusion of individual second polar bodies and blastomeres of 2-cell embryos

Embryos	No. of embryos examined (No. of exp)	Embryonic stage		
		4-cell (%)	Morula (%)	Blastocyst (%)
$2n/3n$ embryo	45 (4)	45 (100)	45 (100)	41 (91.1)
$2n$ embryo (sham*)	45 (4)	45 (100)	45 (100)	44 (97.8)

*$2n$ embryos were manipulated in a similar manner as $2n/3n$ embryo except for the step of second polar body-blastomere fusion.

Table 2. Occurrence of 2n/3n mixoploidy in conceptuses at E11.5 and E18.5

Embryonic stages	No. of morulae		No. of $2n/3n$ conceptuses (%)	Distribution of $3n$ cells to tissues (%)
	transferred	developed to live conceptuses (%)		
E11.5	97	10 (10.3)	4 (4.1)	embryo (2.4) / placenta (0)
				embryo (19.5) / placenta (9.6)
				embryo (1.0) / placenta (3.4)
				embryo (3.5) / placenta (11.8)
E18.5	69	9 (13.0)	4 (5.8)	liver (0.3) / spleen (0) / placenta (0.5)
				liver (0) / spleen (0) / placenta (0.5)
				liver (1.0) / spleen (1.0) / placenta (1.5)
				liver (2.0) / spleen (0) / placenta (1.5)

Supplemental Table S1.

Development of $2n$/$3n$ mixoploid embryos produced by using PB2s of green mice

Embryonic stage		
4-cell	Morula	Blastocyst
97.2%	90.7%	87.8%
(n=36)	(n=43)	(n=41)