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Haploid embryonic stem cell lines derived from androgenetic and parthenogenetic rat blastocysts

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Abstract. The present study was conducted to establish haploid embryonic stem (ES) cell lines using fluorescent marker-carrying rats. In the first series, 7 ES cell lines were established from 26 androgenetic haploid blastocysts. However, only 1 ES cell line (ahES-2) was found to contain haploid cells ($1n = 20 + X$) by fluorescence-activated cell sorting (FACS) and karyotypic analyses. No chimeras were detected among the 10 fetuses and 41 offspring derived from blastocyst injection with the FACS-purified haploid cells. In the second series, 2 ES cell lines containing haploid cells (13% in phES-1 and 1% in phES-2) were established from 2 parthenogenetic haploid blastocysts. Only the phES-2 cell population was purified by repeated FACS to obtain 33% haploid cells. Following blastocyst injection with the FACS-purified haploid cells, no chimera was observed among the 11 fetuses; however, 1 chimeric male was found among the 47 offspring. Although haploid rat ES cell lines can be established from both blastocyst sources, FACS purification may be necessary for maintenance and chimera production.

Key words: Blastocyst injection, FACS purification, Haploid ES cells, Rat blastocysts

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Diploid rat embryonic stem (ES) cell lines have been established using several inhibitors of fibroblast growth factor receptor, mitogen-activated protein kinase (MEK), and glycogen synthase kinase 3 (GSK3) in differentiation-related pathways [1–3] and successfully used for rat transgenesis [4–6]. Haploid cells contain a single copy of each gene, facilitating the generation of loss-of-function mutations in a single step if haploid ES cell lines are available. Androgenetic haploid mouse ES cells can contribute to not only chimeric mouse production via conventional blastocyst injection but also semi-cloned mouse production via ooplasmic microinsemination [7]. A combination of the altered expression of two imprinted genes and CRISPR/Cas9-based genome editing would allow the stable production of gene-modified semi-cloned mice with androgenetic haploid ES cells [8]. Furthermore, in comparison with diploid ES cells, haploid ES cells can contribute more efficiently

to the generation of mutant mice with multiple knockouts or large deletions [9]. Different strategies for establishing pluripotent and germline-competent ES cell lines have also been applied to parthenogenetic haploid mouse blastocysts [10]. Moreover, Li *et al.* [11] have reported that haploid ES cells established from androgenetic red fluorescent protein (RFP)-positive rat blastocysts are suitable for producing transgenic rats by ooplasmic microinsemination with the RFP-labeled haploid ES cells. Although the production efficiency of transgenic rats by this approach is extremely low, haploid ES cell lines are an attractive tool for rat mutagenesis and screening. Therefore, the present study was conducted to establish haploid ES cell lines using knock-in (male) or transgenic (female) rats carrying a fluorescent marker (tdTomato or Venus).

In the first series of the experiment, 702 androgenetic haploids were prepared by removing the female pronucleus from Slc:SD × WDB-Rosa26^{em1(RT2)Nips} rat zygotes and transferred to allow blastocyst development for 4 days in the oviducts of pseudopregnant female rats. Among 495 harvests, 26 blastocysts (5%) were detected and seeded on mouse embryonic fibroblast feeder cells in the 2iF medium. Outgrowth was observed in 8 blastocysts, and tdTomato-positive ES cell colonies were established from 7 blastocysts (Table 1). Fluorescence-activated cell sorting (FACS) analysis of the 7 ES cell lines indicated that only 1 ES cell line (ahES-2) contained a haploid

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Table 1. Establishment of haploid rat ES cell lines from androgenetic and parthenogenetic blastocysts

Blastocyst source	Blastocysts seeded*	ES cell lines (%)		
		established	contained haploids	purified by FACS
Androgenetic	26	7 (27)	1 (14)	1 (100)
Parthenogenetic	2	2 (100)	2 (100)	1 (50)

* The 26 androgenetic blastocysts and 2 parthenogenetic blastocysts were produced by *in vivo* culture of 702 and 138 haploid zygotes, respectively.

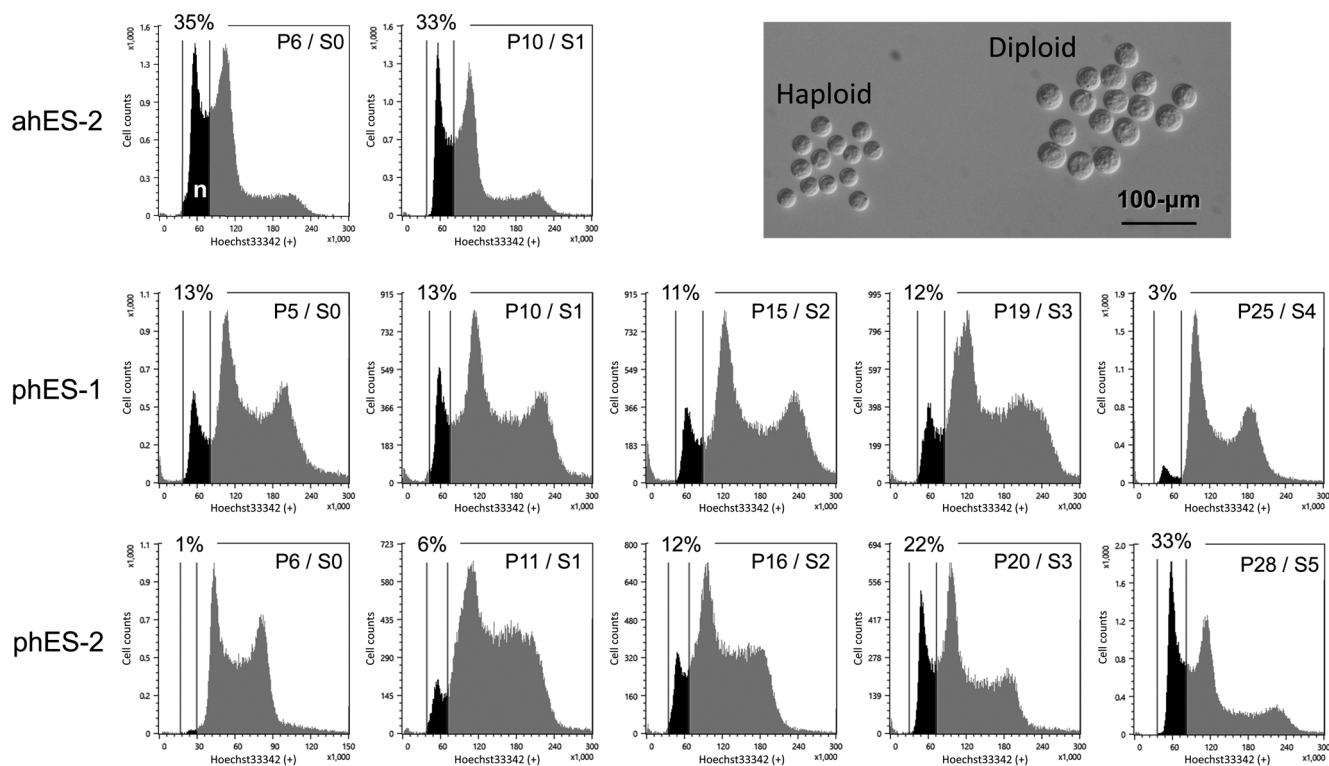


Fig. 1. FACS histograms of ahES-2, phES-1, and phES-2 cell populations. The phES-2 cell line was successfully maintained from passages 6 to 28 with 5 rounds of FACS purification. However, the haploid cells of the phES-1 cell population were lost during FACS passaging. P: passage number, S: sorting number.

cell population. The percentage of FACS-purified haploid ES cells in the ahES-2 cell line was 35% (Fig. 1). The ahES-2 cells, assessed by karyotyping at passage-17/sorting-2, were a heterogeneous population of cells including haploid (20 + X; 24%), diploid (40 + XX; 74%), and aneuploid (39 + XX; 2%) cells (Fig. 2). The stem cell marker genes (*Oct4*, *Rex1*, and *rNanog*) and trophectoderm-specific marker gene (*Cdx2*) in the haploid cells of the ahES-2 cell line were examined by reverse transcription (RT)-PCR analysis, which confirmed the expression of these genes (Fig. 3). To produce haploid ES cell-derived chimeras, blastocysts were injected with 10–20 ES cells that were identified as haploids (or diploids as controls) following FACS. No chimeras were obtained from the 10 fetuses and 41 offspring of foster mothers transferred with 17 and 65 haploid ES-injected blastocysts, respectively (Table 2). In contrast, 11 and 5 chimeras were obtained from the 30 fetuses and 10 offspring of foster mothers transferred

with 35 and 16 diploid ES-injected blastocysts, respectively. Germline transmission of the tdTomato gene was confirmed in 4 out of the 14 G1 offspring of 1 chimeric female.

In the second series of the experiment, 138 parthenogenetic haploids were prepared by activating the oocytes of WI-Tg(CAG/Venus)^{Nips} female rats with ionomycin and cycloheximide. After *in vivo* culture for 4 days, 2 blastocysts (2%) were detected among 117 harvests. Both blastocysts contributed to outgrowth and the subsequent establishment of Venus-positive ES cell lines (Table 1). FACS analysis indicated that both cell lines contained haploid cells (13% in phES-1 and 1% in phES-2) (Fig. 1). Repeated FACS during passaging was performed to purify the haploid ES cell population. Only the phES-2 cell population was successfully passaged through 5 rounds of FACS purification with an increased haploid cell percentage of 33%. The phES-2 cells, assessed by karyotyping at passage-36/

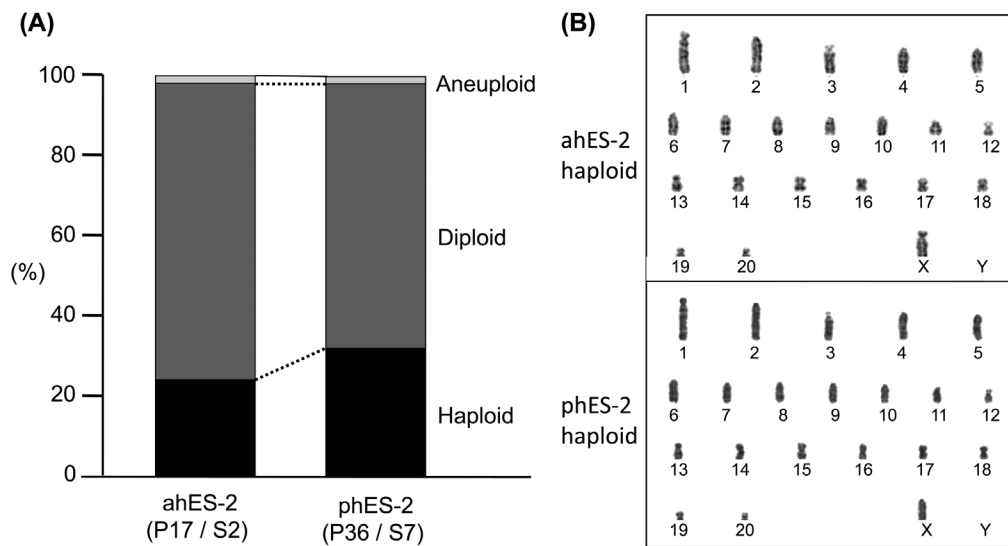


Fig. 2. (A) Haploid/diploid/aneuploid distribution based on karyotypic analysis of the ahES-2 and phES-2 cell lines. (B) Haploid cells in both cell lines had a set of 20 + X chromosomes. P: passage number, S: sorting number.

sorting-7, were a heterogeneous population of cells including haploid (20 + X; 32%), diploid (40 + XX; 66%), and aneuploid (41 + XX; 2%) cells (Fig. 2). RT-PCR analysis of 1n cells of the phES-2 cell line confirmed the expression of *Oct4*, *rNanog*, and *Cdx2* genes (Fig. 3). Only 1 (2%) chimeric rat offspring was obtained from the 11 fetuses and 47 offspring developed from the 20 and 62 injected blastocysts, respectively (Table 2, Fig. 4). The germline transmission of the *Venus* gene from this chimeric rat to G1 progeny was not examined because the chimeric rat was male. It is well known that the genetic characteristics of XX ES cells are not transmitted from chimeric males to their offspring. Nagashima *et al.* [12] reported that when XX inner cell mass donor cells were injected into XY porcine blastocysts, the phenotypic sex of the resulting chimeric pig was male with no germline cells from the donor cell lineage. Isotani *et al.* [13] reported that XX-XY chimeric mice whose X chromosomes were tagged with GFP had a few green XX germ cells within the seminiferous tubules of the testis; however, the germ cells developed as “eggs” and not “sperm”. A weak Venus-positive fluorescence was observed in the brain, heart, lung, stomach, pancreas, intestine, kidney, and muscle but not in the thymus and testis of the chimeric rat (7 weeks old). With diploid phES-2 cells, no chimeric rats were obtained from the 12 fetuses and 15 offspring developed from 26 and 16 injected blastocysts, respectively. Since the FACS purification causes cell damage, blastocyst injection without FACS will increase the efficacy of chimera production. This study was the first attempt at establishing rat ES cell lines from parthenogenetic haploid blastocysts.

Haploid ES cells can be spontaneously diploidized during *in vitro* culture [7–11] and differentiation [7, 11, 14]. Our results suggest that rat haploid ES cells need to be purified by FACS during passaging. Li *et al.* [11] reported that 4–5 rounds of FACS purification resulted in the enrichment of androgenetic haploid rat blastocyst-derived ES cells with approximately 90% of haploid cells, and the haploid-enriched

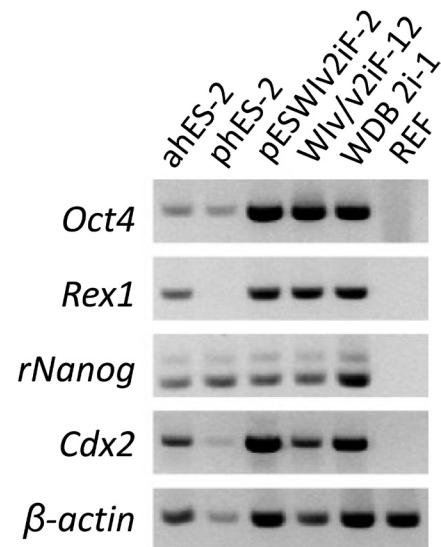


Fig. 3. Expression of the stem cell marker genes (*Oct4*, *Rex1*, and *rNanog*), trophectoderm-specific marker gene (*Cdx2*), and reference gene (*β-actin*) in the ahES-2 and phES-2 cell lines. Other lanes include the positive controls (pESWiv2iF-2, Wiv/v2iF-12, and WDB2i-1) and negative control (REF: rat embryonic fibroblast).

ES cell lines could be maintained for over 40 passages with FACS purification at every 5 passages. However, the FACS purification performed in our study was not as effective as that in the study by Li *et al.* Takahashi *et al.* [15] reported that the *Wee1* kinase inhibitor stabilizes mouse haploid ES cells without FACS purification by accelerating the G2/M transition. Nevertheless, we failed to confirm this

Table 2. Generation of chimeric rats by blastocyst injection with ahES-2 and phES-2 cells

Cell source *	Blastocysts transferred	E14.5 fetuses + full-term offspring (%)	Chimeric fetuses + offspring (%)
ahES-2 haploid	17 + 65	10 + 41 (62)	0 + 0 (0)
ahES-2 diploid	35 + 16	30 + 10 (78)	11 + 5 (40)
phES-2 haploid	20 + 62	11 + 47 (71)	0 + 1 (2)
phES-2 diploid	26 + 16	12 + 15 (64)	0 + 0 (0)

* Cells from the ahES-2 and phES-2 cell lines were FACS-purified prior to blastocyst injection.

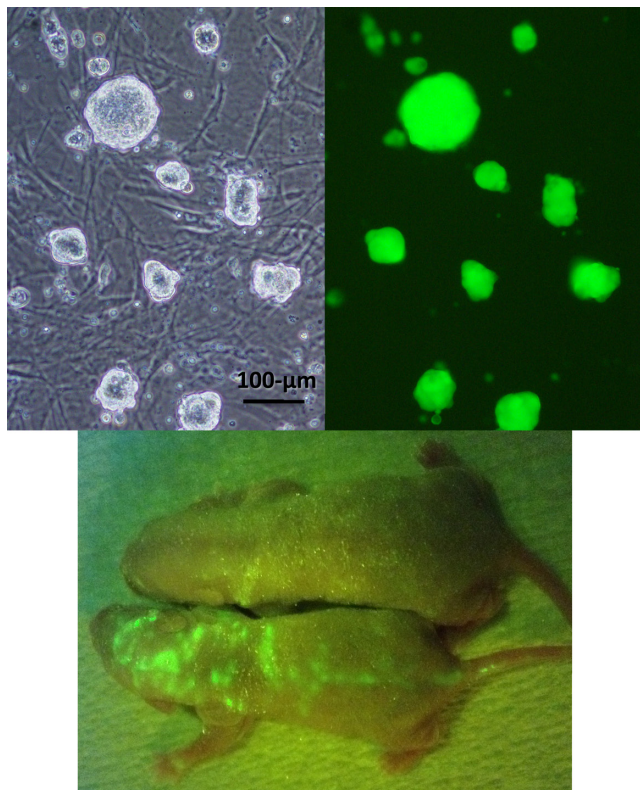


Fig. 4. Morphology of the phES-2 cell line (upper left), its Venus fluorescence (upper right), and chimeric male offspring produced by blastocyst injection with haploid phES-2 cells (bottom).

positive effect since the spontaneous diploidization of rat haploid ES cells was not prevented by the *Wee1* kinase inhibitor (data not shown). Two reagents for *Wee1* kinase inhibition (PD166285 and MK1775) have been reported to be effective in mice [15]; however, we used only one of the two inhibitors (PD166285). It remains unclear whether the origin of haploid blastocysts (embryo source and strain background) is associated with the frequency of FACS purification. It is also unknown whether the time-dependent reduction of haploid ES cells during passaging is caused by the rapid spontaneous diploidization of haploid cells or the faster proliferation of diploid cells present in the In cell population even after FACS. If the former possibility alone is true, as described by Leeb and Wutz [10], the edited mutation in a single allele of haploid ES cells can be spontaneously copied to the

paired allele, thus easily generating homogenous mutations in both alleles. The germline competency of presumptive diploid ES cells in the ahES-2 cell line was demonstrated. The microinsemination of 89 ovulated oocytes with ahES-2 haploid cells did not produce any viable rat offspring (data not shown). However, this study does not completely exclude the possibility of haploid rat ES cells as male gametes. In the phES-2 cell line derived from a parthenogenetic haploid blastocyst, a ubiquitous but weak fluorescence expression of the Venus gene was observed in a chimeric male rat (Fig. 4) despite the failure in detecting *Rex1* expression by RT-PCR (Fig. 3). The limited or nonexistent contribution of haploid ES cells to chimeric rat production in the present study may be explained by the delayed timing of diploidization after blastocyst injection, which depends on the characteristics of the cell line. Li *et al.* [11] reported that only very few haploid cells (1.5%) were detected in E7.5 chimeric rat embryos. Further studies using additional haploid ES cell lines will be helpful to understand the mechanism underlying the optimal diploidization of the ES cells.

In conclusion, haploid ES cell lines can be established from both androgenetic and parthenogenetic rat blastocysts; however, FACS purification may be necessary for their maintenance and chimera production.

Methods

Animals

All animal experimental procedures were reviewed and approved by the Animal Care and Use Committee of the National Institute for Physiological Sciences, Okazaki, Aichi, Japan. Specific pathogen-free Wistar (CrIj:WI) and Sprague-Dawley (Slc:SD) rats were purchased from Charles River Japan (Kanagawa, Japan) and Japan SLC (Shizuoka, Japan), respectively. Rosa26^{em1(RT2)}Nips knock-in male rats (RGD ID: 853237) [16] and WI-Tg(CAG/Venus)^{Nips} female rats (RGD ID: 8552368) [3] were used for the production of androgenetic and parthenogenetic zygotes, respectively. These rats were housed under controlled lighting (14L:10D), temperature (25 ± 2°C), and humidity (65 ± 5%) with free access to laboratory diet and filtered water.

Chemicals and media

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The culture medium used to establish ES cell lines was the N2B27 medium [17] containing 1 μM MEK inhibitor PD0325901 (Axon Medchem, Groningen, The Netherlands), 3 μM GSK3 inhibitor CHIR99021 (Axon Medchem), 1,000 U/ml ESGRO®

(Merck Millipore, Darmstadt, Germany), and 10 μM forskolin (referred to as 2iF medium) [18]. For the enucleation of pronuclear zygotes, modified rat 1-cell embryo culture medium (mR1ECM) [19] supplemented with 22 mM HEPES and 5 mM NaHCO_3 (referred to as HEPES-mR1ECM) was used. The mR1ECM (300–310 mOsm/Kg) supplemented with 4 mg/ml bovine serum albumin was used for uterine flushing to harvest *in vivo*-cultured zygotes.

Preparation of haploid blastocysts

For androgenetic haploid zygotes, Slc:SD female rats (7–9 weeks old) were superovulated by intraperitoneal injections of 0.04 mg/kg luteinizing hormone-releasing hormone, 300 IU/kg equine chorionic gonadotropin (eCG; Aska Pharmacies, Tokyo, Japan), and 300 IU/kg human chorionic gonadotropin (hCG; Aska Pharmacies) at intervals of 48 h. Immediately after hCG injection, the female rats were mated overnight with WDB-Rosa26^{em1(RT2)Nips} male rats. After 21–23 h following hCG injection, zygotes were retrieved from the oviductal ampullae, and the surrounding cumulus cells were removed by short-term culture and pipetting in 0.1% hyaluronidase-containing HEPES-mR1ECM. The female pronucleus was removed in HEPES-mR1ECM supplemented with 5 $\mu\text{g}/\text{ml}$ cytochalasin B using a piezo-driven micromanipulator (PMAS-CT150; PrimeTech, Ibaraki, Japan).

For parthenogenetic haploid zygotes, juvenile homozygous WI-Tg(CAG/Venus)^{Nips} female rats (3 weeks old) were superovulated by intraperitoneal injections of 300 IU/kg eCG and 300 IU/kg hCG at intervals of 48–50 h. After 16 h following hCG injection, oocytes were retrieved from the oviductal ampullae, and the surrounding cumulus cells were removed by short-term culture and pipetting in 0.1% hyaluronidase-containing HEPES-mR1ECM. The oocytes were artificially activated with 5 μM ionomycin in mR1ECM for 5 min and subsequently treated with 5 $\mu\text{g}/\text{ml}$ cycloheximide in mR1ECM for 4 h at 37°C in 5% CO_2 . Then, single pronucleus formation and polar body extrusion were confirmed.

The androgenetic and parthenogenetic haploid zygotes were transferred into the oviducts of pseudopregnant Crj:WI rats at 0.5 days post coitum (dpc). Haploid blastocysts were harvested by flushing the uteri at 4.5 dpc and confirmed by tdTomato (510–560 nm) and Venus (460–500 nm) fluorescence under excitation light.

Establishment of haploid ES cells

Blastocysts were freed from their zonae pellucidae in acidified Tyrode's solution. The zona-free blastocysts were cultured for 7 days in the 2iF medium on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (37°C, 5% CO_2). Outgrowths from the blastocysts were disaggregated and transferred to new culture vessels containing the same culture medium (passage-1: P1). These tentative haploid ES cell lines were maintained by medium exchange every other day and trypsinization/expansion every 3 days (P2 plus). Each cell line was analyzed by FACS to confirm whether the cell population contained haploid cells. Briefly, ES cells were trypsinized, washed using DMEM (Gibco[®], Life Technologies[™], Grand Island, NJ) supplemented with 10% FBS (Gibco[®]), stained with 50 μM verapamil + 10 $\mu\text{g}/\text{ml}$ Hoechst33342 for 30 min at 37°C, and filtered with a nylon mesh (55 nm). The cell suspension was FACS-purified (sorting-0: S0) with SH800 (Sony, Tokyo, Japan). When the ES cell

population was found to contain less than 30% haploid cells, the ES cell line was purified by repeated FACS during passaging to enrich the cell line with the haploid cells (1 FACS at every 4–6 passages).

Karyotypic and RT-PCR analyses of haploid ES cells

The karyotype of ES cells derived from androgenetic blastocysts (ahES-2; P17/S2, n = 50) and parthenogenetic blastocysts (phES-2; P36/S7, n = 50) was determined by G-band staining (Nihon Gene Research Laboratories, Miyagi, Japan). Each cell was classified as haploid (n), diploid (2n), or aneuploid ($2n \pm 1$). The expression of the stem cell marker genes (*Oct4*, *rNanog*, and *Rex1*), trophectoderm-specific marker gene (*Cdx2*), and reference gene (*β -actin*) was examined by RT-PCR analysis. The primer sets used were same as those described previously [20]. Briefly, total RNA was extracted from each sample using a RNeasy[®] mini Kit (Qiagen, Germantown, MD). Then, cDNA was prepared using the Superscript[™] III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and amplified with TaKaRa LA Taq[®] (Takara Bio, Shiga, Japan) for 30 cycles at 95°C for 30 s, at 55°C (or 60°C for *rNanog*) for 30 sec, and at 72°C for 60 sec. A parthenogenetic diploid ES cell line (pESWIv2iF-2 [20]) and two diploid ES cell lines (WIv/v2iF-12 [21] and WDB2i-1 [18]) were used as positive controls, and rat embryonic fibroblast (REF) cells were used as a negative control.

Pluripotency and germline competency of haploid ES cells

Chimeric rats were generated by blastocyst injection of ES cells derived from the ahES-2 and phES-2 cell lines. Approximately 10 to 20 cells (G0/G1 phase), classified either as haploids or diploids following FACS (Fig. 1), were microinjected into each of the E4.5 Crj:WI blastocyst, and the injected blastocysts were transferred into the uteri of E3.5 pseudopregnant Crj:WI female rats to allow fetal development (autopsied E14.5) or full-term development. Chimeric rats were identified by coat color or tdTomato/Venus fluorescence. The germline competency of haploid ES cell lines was examined by a conventional approach using the G1 generation offspring of chimeric female rats.

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