Sex Identification and Development of Single Blastomeres from 2-cell Mouse Embryos

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Abstract. Single blastomeres were isolated from 2-cell mouse embryos and analysed for their sex by using partially deleted Y-chromosome as a marker. Sex identification of 83% of the embryos was achieved with conventionally Giemsa-stained chromosome preparations. The other half-embryos were cultured individually or recombined with another half-embryo of the same sex, and were transferred to pseudopregnant recipient females. Six percent of the half and 18% of the recombined embryos developed into live fetuses. The sex of these fetuses was identical with that determined prior to embryo transfer. Sex analysis in this way was accurate and early enough to allow utilization of sex-known embryos for development and sex differentiation studies at the cleavage stage.

Key words: Partially deleted Y-chromosome, Single blastomere, Karyotyping, Development of sex-known embryos.

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Numerous attempts have been made to study the genetic traits of preimplantation embryos. One trait that has received much attention is sex. The mammalian Y chromosome plays a crucial part in sex determination: an embryo that inherits a Y chromosome develops as a male whereas an embryo lacking a Y chromosome develops as a female [1]. In eutherian mammals, evidence suggests that sex determination is equivalent to testis determination [2]. The sex determining gene(s) on the Y chromosome induces testicular development, and subsequent male sexual differentiation is a consequence of the hormonal products of the testis [3]. The Y-encoded testis-determining gene has been designated *TDF* (testis-determining factor) in humans and *Tdy* (testis-determining-Y chromosome) in mouse [3, 4]. It is likely that many different genes are required for both male and female sex differentiation, and it is

assumed the *Tdy* must act within the context of the other regulatory molecules. There is no doubt that sexing preimplantation embryos facilitates the utilization of sex-known embryos for further study and understanding of the mechanism of sex determination and differentiation.

In the present study, we attempted to sex single blastomeres separated from 2-cell mouse embryos with a partially deleted Y chromosome as a marker, and the observation on development of the sex-known single blastomeres was conducted.

Materials and Methods

Mice

The mice used in the experiments were 8–22-week-old mature B10·BR-Y^{del} males, 4-week-old C57BL/6N (B6) and mature ICR females. B10·BR-Y^{del} (Y^{del}) mouse was first established as a mutant of B10·BR mouse at the National Institute of

Genetics of Japan [5]. This mouse was introduced to the Department of Animal Pathology, Institute of Medical Science, University of Tokyo in 1988 and maintained by brother-sister mating. Female B6 and ICR mice were used as the oocyte donor and recipient mouse respectively. Both of these mice were purchased from Clea Japan Inc.

Treatment of spermatozoa with Percoll solution

Sperm suspensions were prepared by releasing the contents of each pair of cauda epididymidis into 0.8 ml of TYH medium [6] overlaid by mineral oil, and incubated at 37°C with 5% CO₂ in air for 30 min to allow dispersal of the spermatozoa. 0.5 ml of the sperm suspension was loaded onto the gradient of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in TYH and centrifuged at $250 \times g$ for 10 min at room temperature. The sperm-rich bottom fraction was transferred to another tube and overlaid with 0.5 ml of fresh TYH medium to allow the spermatozoa to swim up to the added TYH medium during 30 min incubation. The spermatozoa, which swam up and will be referred to as Percoll-treated spermatozoa hereinafter, were used for *in vitro* fertilization.

In vitro fertilization

Four-week-old C57BL/6N female mice were induced to superovulate with i.p. injections of 5 i.u. pregnant mares' serum gonadotropin (PMSG) (Sankyo Zoki Co. Japan) and 48 h later 5 i.u. human chorionic gonadotropin (hCG) (Sankyo Zoki Co. Japan). Unfertilized, cumulus-intact eggs were recovered 14–16 h after the hCG injection and released directly to 0.2 ml droplets of TYH medium, which contained spermatozoa in concentrations of $1\text{--}5 \times 10^5$ cells/ml. All droplets of the medium were overlaid with mineral oil and the eggs and the spermatozoa were incubated at 37°C in an atmosphere of 5% CO₂ in air for 5–6 h. The eggs possessing a second polar body and both male and female pronuclei were considered fertilized and were cultured in Whitten's medium (WM) [7] containing 0.1 mM EDTA [8] after washing several times.

Separation of blastomeres at the 2-cell stage

About 30 h post-insemination, 2-cell embryos were treated with 0.5% pronase (Kaken Seiyaku Co., Tokyo) in Hepes-WM for 3–5 min to remove zona pellucida and washed by passage through

several drops of WM. Blastomere pairs were obtained by gentle pipetting the zona-free 2-cell embryos with a micropipette with an internal diameter of 70–100 μm . One of the blastomeres was subjected to karyotypic analysis, and the other was transferred to a WM droplet for embryo culture.

Karyotyping and sexing of single blastomeres isolated from 2-cell embryos

Each isolated blastomere was transferred to a 10 μl drop of Colcemid solution (0.06 $\mu\text{g/ml}$ in WM, Life Technologies Inc. Grand Island, U.S.A.) and incubated overnight to stop mitosis. The blastomere was then transferred to a hypotonic solution of 40% fetal calf serum (FCS, GIBCO, Grand Island, NY, U.S.A.) and treated for 10–15 min. One blastomere at a time was placed on a clean slide, and 1–2 drops of a freshly prepared fixative mixture of 3:1 methanol:acetic acid were dropped onto the blastomere. The slides obtained were conventionally stained with 2% Giemsa solution (E. Merck) in phosphate buffer at pH 6.8 [9, 10].

Sex analysis of the blastomeres was performed by examining the chromosome preparations under a microscope. The blastomeres bearing 40 chromosomes including a partially deleted Y-chromosome, which appeared to be only about half the size of the normal one, were classified as males and those without the deleted Y-chromosome were considered to be females.

Aggregation of two sexed half-embryos

Each of 2 male or female half-embryos developed from single blastomeres of 2-cell embryos was placed into a drop of 10 μl phytohemagglutinin P solution (PHA, 50 $\mu\text{g/ml}$ in WM, Difco Laboratories, Detroit, Michigan, U.S.A.), and aggregated together with 2 well polished, micro glass needles, just before compaction occurred. The recombined embryos were incubated at 37°C for 10–15 min and washed several times prior to embryo culture.

Embryo culture and transfer

All treated embryos were cultured individually in drops of 10 μl WM containing 0.1 mM EDTA, in an 60 mm plastic culture dish (Corning Inc. N.Y., U.S.A.). Since in preliminary experiments the highest viability of zona-free embryos was obtained when day 4 recipient mice were used, the

blastocysts developed from single blastomeres and aggregated embryos were transferred into the uteri of ICR recipient mice (Clea Japan Inc.) on day 4 of pseudopregnancy under anesthesia with an i.p. injection of 2.5% Avertin [11]. Pseudopregnancy was induced by mating the females with vasectomized males, and the day on which female mice were found with vaginal plugs was counted as Day 1. The recipient mice were killed by cervical dislocation on Day 18 of gestation and examined for live fetuses and implantation sites. The sex of the fetuses was diagnosed by examining their gonads under a dissecting microscope.

Determination of number of component blastomeres

At 72 and 96 h post-insemination, some of the half embryos were placed on clean slides, fixed with a fixative of 3:1 methanol:acetic acid and stained with 2% Giemsa solution after 15–30 min hypotonic treatment in 0.8% trisodium citrate solution. The number of component blastomeres per embryo was determined by counting the nuclei

under a microscope.

Statistical analysis

The results of the embryo transfer were analyzed by X^2 test to determine the significance of differences between the developmental rate of the half and recombined embryos, and the male and female half-embryos. Student's *t*-test was used for testing the differences between average number of cells in the morulae and blastocyst stages in male and female half embryos.

Results

In vitro fertilization

All of the embryos were obtained from *in vitro* fertilization for exact evaluation of embryonic development and to facilitate the sex diagnosis. Among the 764 of the oocytes examined, 675 were fertilized, giving a 88% fertilization rate.

Fig. 1. The metaphase plates from single blastomeres of 2-cell embryos. (M) Male blastomere possessing a partially deleted Y chromosome (arrowed). (F) Blastomere without Y chromosome and classified as female. Reproduced at 1000 $\times$.

Table 1. Sexing of single blastomeres from 2-cell stage embryos by karyotyping analysis

No scored	No and (%) at metaphase	Polyploid	Analysable no. /diploid (%)	Sex ratio		
				Male	Female	% of male
659	653(99)	18(3n), 1(4n)	528/634(83)	281	247	53

Sex determination was performed only with diploid blastomeres. The embryos possessing 40 chromosomes including a deleted Y-chromosome were classified as male. The others were considered to be female. Values are for 7 replicates.

Karyotyping and sexing single blastomeres separated from 2-cell embryos

Single blastomeres isolated from 2-cell stage embryos were subjected to karyotyping and sex analysis at 33–34 h after insemination. The blastomeres possessing 40 chromosomes including a deleted Y-chromosome were regarded as males and those without the deleted Y-chromosome were considered to be females (Fig. 1). The results are summarized in Table 1. Among the 659 preparations handled, 653 (99%) blastomeres were undergoing mitotic division and all were in the metaphase. Of these blastomeres, 19 were polyploids including both 3n and 4n ones. The remaining 634 were classified as diploids, of which 8 possessed 39 or 41 chromosomes and thus were treated as unanalyzable diploids. Finally 528 (83%)

Table 2. *In vitro* development of single blastomeres separated from 2-cell embryos

No cultured	No. and (%) developed to		
	2-cell (48 h)*	Morula (72 h)*	Blastocyst (96 h)*
413	389(94)	360(87)	339(82)

Separation of blastomeres was performed by pipetting the 2-cell embryos whose zonae pellucidae were removed with 0.5% Pronase solution. Each blastomere was cultured separately in a 10 μ l WM drop. *Time after insemination. Values are for 5 replicates.

Fig. 2. Microphotographs of *in vitro* cultured embryos developed from single blastomeres in the 2-cell stage. (a) in the morula stage 72 h post-insemination. (b) in the blastocyst stage 96 h post-insemination. Reproduced at 500 $\times$.

Table 3. The viability of sex-known blastocysts after embryo transfer

Sex of embryos	No. of embryos sexed and transferred	Females with implantation/recipients (%)	No. and % of embryos implanted	Live fetuses (%)	% of live fetuses in implanted embryos	Sex of fetuses	
						♂	♀
(a) Half-embryo							
♂	43	2/3(67)	19(44) ^a	5(12) ^e	26	5	0
♀	45	1/3(33)	5(11) ^b	0(0) ^f	0	0	0
Total	88	3/6(50)	24(27) ^c	5(6) ^g	21	5	0
(b) Recombined ¹⁾ embryo							
♂+♂	35	3/3(100)	23(66)	5(14)	22	5	0
♀+♀	31	3/3(100)	26(84)	7(23)	27	0	7
Total	66	6/6(100)	49(74) ^d	12(18) ^h	24	5	7

All blastocysts were transferred into the uteri of Day 4 pseudopregnant mice. Autopsy was made and fetuses were analyzed for gonadal sex on day 18 of gestation. 1) The recombined embryos were produced by aggregating 2 male or female half-embryos in the presence of PHA before compaction occurred. a vs. b, c vs. d $P < 0.01$; e vs. f, g vs. h $P < 0.025$. Values are for 3 replicates.

of the diploid blastomeres were able to be sexed, 281 males and 247 females being identified. Males accounted for 53%.

In vitro development of single blastomeres separated from 2-cell stage embryos

Single blastomeres separated from 2-cell embryos were cultured individually to avoid aggregation between blastomeres. A total of 413 single blastomeres of the 2-cell embryos were subjected to culture, and 389 (94%) developed to the 2-cell stage 48 h after insemination. At 72 and 96 hr, 360 (87%) and 339 (82%) of the single blastomeres developed into morulae and blastocysts, respectively (Table 2; Fig. 2).

The viability of sex-known blastocysts after transferring to recipient mice

Blastocysts developed from single blastomeres of 2-cell embryos were transferred into the uteri of Day 4 recipient mice 96 h after insemination. At the time of transfer, the sex of these embryos had been identified by karyotyping the other corresponding blastomeres at the 2-cell stage. The transfer results are shown in Table 3. Of 43 male and 45 female half-embryos, 19 (44%) and 5 (11%) were implanted and 5 male half-embryos developed into live fetuses. All of them proved to be male when their gonads were examined.

As the viability of the half-embryos was quite low, a control transfer experiment was conducted. Two sex-known male or female half-embryos were aggregated together and the recombined embryos were transferred into recipient mice 96 h post-

Table 4. Comparison of number of component blastomeres in male and female half-embryos at 72 and 96 h post-insemination

sex of embryos	Mean no. and SD of cells per embryo	
	Morula at 72 h	Blastocyst at 96 h
Male	7.73±1.92 (n=41)	22.69±5.28 ^{a)} (n=42)
Female	8.14±1.66 (n=35)	18.46±3.88 ^{b)} (n=26)

a vs. b $P < 0.01$. The total numbers of embryos determined in each item are shown in parentheses. Datas are for 4 replicates.

insemination (Table 3). Of 66 recombined embryos, 49 were implanted, and 5 male and 7 female live fetuses were obtained with sex identical to that diagnosed before embryo transfer. The implantation rate and the rate of development into live fetuses in recombined embryos were significantly higher than those in the half-embryos ($P < 0.01$, $P < 0.025$). But there was no significant difference in the proportion of live fetuses to implanted embryos in recombined (12/49) and half (5/24) embryos.

The difference between male and female embryos in vitro developmental potential

Since the implantation rate was significantly higher in male half-embryos ($P < 0.01$), and all of the 5 living fetuses from sexed half-embryos were male (Table 3), we examined whether male half-embryos would have a higher developmental

potential when male and female embryos were cultured *in vitro* under identical conditions. In the morula stage (72 h post-insemination), there was no significant difference between the 2 types of embryo in the number of component blastomeres. However, in the blastocyst stage (96 h post-insemination), the male half-embryos contained significantly more blastomeres per embryo than the female half ones (22.69:18.46, $P < 0.01$; Table 4).

Discussion

Experiments on sexing of mouse pre-implantation embryos and subsequent transfer of the sex-known embryos have been made with numerous approaches. Detection of the H-Y antigen [12, 13], determination of X-chromosome-linked enzymes [14] and enzymatic amplification of male specific repeated DNA [15] have been used to sex embryos at least in the 8-cell stage. However, evidence has shown that in the mouse the activation of the embryonic genome started in the 2-cell stage of the embryo [16] and male specific H-Y antigen was reported to be expressed in the 8-cell stage and after [17]. For the purpose of studies on embryonic development and sexual differentiation of the mammalian embryo, it is therefore considered to be necessary to sex embryos in an earlier stage.

In ordinary strains of the mouse, the Y-chromosome is similar in size to the 19th chromosome, i.e., the smallest autosome. The embryos bearing 3 smallest chromosomes were classified as males if the chromosome preparations were conventionally Giemsa-stained [9, 10]. But it is sometimes difficult to distinguish between males and females by this method, owing to the inappropriate constriction of chromosomes, if C-banding stain [10, 18] is not performed. In the case of Y^{del} mouse, however, the Y-chromosome is distinctly smaller than half the smallest autosome [19], thus facilitating the discrimination of male from female embryos without requiring time to produce the C-band.

Comparison after transfer of the viability of half and recombined embryos showed that although the implantation rate and the rate of development into live fetuses were significantly higher in the latter, there was no significant difference between

the two types of embryo in the proportion of live fetuses to implanted embryos. This implies that the reduction in embryo size or number of component blastomeres resulted in low viability in half-embryos by directly influencing the implantation of embryos. In addition, the low viability might be partly due to the use of *in vitro* fertilized eggs, since the rate of development of half-embryos in this study was lower than those reported in studies in which *in vivo* fertilized eggs were used [20, 21].

The results of half-embryo transfer reveal that all of the 5 living fetuses from sexed half-embryos were male, while none of the 45 female half-embryos developed into live fetuses, and the implantation rate was also significantly higher in the male half embryos. On the other hand, similar developmental rates were observed in both male and female recombined embryos, in which 2 half-embryos of the same sex were aggregated. These observations and the fact that the male half-embryos contained more blastomeres in the blastocyst stage than female ones suggest that the failure of the female half-embryos to develop into fetuses was not due to the damage caused by blastomere separation and culture, but due to difference(s) between male and female half-embryos in the developmental potential. Previous experiments have shown that after transfer of 2 groups of the mouse blastocyst embryos, fast cleavers included an excess of males, and slow cleavers more females [22]. From 67 mouse embryos from 10 litters collected on Day 9 of gestation, it was found that there was a sex difference in the stage attained [23]. Females were less developed on the whole and males were more developed, and on average at the time of examination males had 2 somites more than females. These investigations reveals that the sex effect is manifest before gonadal differentiation which was visible on day 12.5 post coitum [4], suggesting the possibility that sex-linked genes controlling the rate of cell division are expressed in an early stage embryo. It is therefore considered that further investigating this sex effect with sex-predicted embryos will have great significance in the study of embryonic development and the mechanisms of sex determination and differentiation of mammals.

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