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—Original Article—

Experimental approach to prezygotic chromosome screening using only a single pair of gametes in mice

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Abstract. During *in vitro* embryo production, chromosome screening is essential to prevent pregnancy losses caused by embryonic chromosome aberrations. When the chromosome screening is completed before fertilization, gametes are effectively utilized as genetic resources. The aim of this study was to investigate whether chromosome screening of gametes accompanied by fertilization would be feasible using a single mouse spermatozoon and oocyte. Metaphase II oocytes were divided into a cytoplast and a karyoplast. For genome cloning of the gametes, androgenic and gynogenic embryos were produced by microinjection of sperm into cytoplasts and parthenogenetic activation of karyoplasts, respectively. Pairs of blastomeres from androgenic and gynogenic embryos were fused electrically to produce diploid embryos, which were transferred into pseudopregnant surrogate mothers to examine fetal development. Blastomeres from androgenic and gynogenic embryos were individually treated with calyculin A—a specific inhibitor of type 1 and 2A protein phosphatases—for 2 h to induce premature chromosome condensation. Thereafter, chromosome analysis of blastomeres, reflecting the genetic constitution of individual spermatozoa and oocytes, was performed, and we confirmed that most of the androgenic and gynogenic 2-cell embryos had a haploid set of chromosomes in their sister blastomeres. The reconstructed embryos from blastomeres of androgenic and gynogenic 2-cell embryos could be implanted and develop into live fetuses, albeit at low efficiency. This study indicates that prezygotic chromosome screening and embryo production using a single pair of gametes may be practicable.

Key words: Assisted reproductive technology, Chromosome screening, Gamete, Genetic diagnosis, Premature chromosome condensation

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In *in vitro* embryo production (IVP), the production of genetically normal embryos is greatly desired. IVP procedures potentially carry a risk of producing embryos with chromosome aberrations [1, 2], which leads to early embryonic loss. Additionally, it is well known that single-nucleotide polymorphisms are associated with phenotypic characteristics. For example, single-nucleotide polymorphism in tumor necrosis factor-α affects reproductive performance in dairy cows [3]. Thus, examination of the genetic constitution of each gamete used for insemination offers a great advantage in animal breeding. The genetic constitution of embryos developed *in vitro* can be examined with preimplantation genetic diagnosis/screening (PGD/PGS) using their first and second polar bodies and blastomeres. A new approach to PGD and PGS, array comparative genomic hybridization, has been developed and has had a significant effect on the detection of imbalanced chromosome aberrations in embryos [4, 5]. Alternatively, classical chromosome karyotyping combined with florescence *in situ* hybridization (FISH) remains a valid and feasible way to detect balanced chromosome aberrations. Usually, these genetic analyses are conducted using postzygotic materials. Clearly, the ideal method is to produce embryos with known genetic conditions from gametes analyzed prezygotically.

Some studies have reported successful genome cloning of spermatozoa [6–9] and oocytes [10] using androgenic and gynogenic embryos, which can be used for genetic screening prior to fertilization. Moreover, these haploid blastomeres from androgenic or gynogenic embryos could be used for the production of biparental diploid embryos [6, 9–13]. We recently reported a method for detecting chromosomal aberrations in spermatozoa before fertilization using a mouse model [14]. Briefly, a single spermatozoon was injected into an enucleated oocyte, and the sperm genome was duplicated in the ooplasm and divided equally into the sister blastomeres of a 2-cell embryo. The blastomeres were fused individually with fresh metaphase (M)II oocytes to induce premature chromosome condensation (PCC), which enabled us to examine the sperm chromosomes and produce diploid zygotes. Normal embryos derived from the reconstructed zygotes were selected based on the chromosome screening and then transferred into pseudopregnant surrogate mothers to obtain offspring. In this way, it would be possible to avert any chromosomal aberrations arising from the spermatozoa. However, three oocytes are needed to accomplish this chromosome screening: one for duplication of the sperm DNA, one for the induction of PCC and one for producing diploid zygotes. If chromosome analysis of a spermatozoon could be implemented concurrently with that of the oocyte by use of a single oocyte, the technology of prezygotic chromosome screening would be greatly advanced.

Wakayama and Yanagimachi [15] demonstrated that live offspring

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could be produced successfully from an oocyte in which about half of the cytoplasm had been removed. This suggested that half of the cytoplasm in an oocyte could be used for chromosome analysis. Calyculin A (Caly A), a specific inhibitor of type 1 and 2A protein phosphatases, can easily induce PCC during cell culture, even if the cells are in interphase [16, 17]. Practically, Caly A-induced PCC can be used for cytogenetic analysis of fetal cells from amniotic fluid [18]. Thus, Caly A-induced PCC may be useful for chromosome screening of spermatozoa and oocytes prior to fertilization.

In this study, we devised an effective method for prezygotic chromosome screening of both gametes in parallel with embryo production and performed two experiments: one was to test the validity of Caly A for chromosome analysis of blastomeres, and the other was to demonstrate the technical feasibility of embryo production in the process of prezygotic examination of gamete chromosomes.

Materials and Methods

Experimental design

A strategy for achieving prezygotic examination of gamete chromosomes in parallel with embryo production is summarized in Fig. 1. Briefly, the zona pellucida of single MII oocytes was partially dissected to separate the karyoplasts from cytoplasts (Fig. 1A). The karyoplasts were activated with strontium chloride to produce haploid gynogenic embryos (Fig. 1B), while cytoplasts were injected with single spermatozoa to produce haploid androgenic embryos (Fig. 1C). At 24 h after sperm injection or parthenogenetic activation, single androgenic and gynogenic sister blastomeres of 2-cell embryos were cultured in Caly A for 2 h to induce PCC (Fig. 1D), and the chromosomes were analyzed for embryo selection (Fig. 1E). The remaining blastomeres were placed back into the original zona pellucida and fused with each other electrically (Fig. 1F) to construct diploid embryos for transfer (Fig. 1G).

To demonstrate the viability of this method, two experiments were designed as follows. Experiment 1 was performed to investigate the efficiency of Caly A-induced PCC for chromosome analysis of blastomeres from androgenic, gynogenic and normally fertilized embryos. Haploid and diploid karyotypes were prepared in androgenic and gynogenic embryos. Briefly, microinjecting one or two spermatozoa into enucleated oocytes produced androgenic embryos. Gynogenic embryos were produced from oocytes activated with strontium chloride for 1 h and cultured with or without 2.5 μg/ml cytochalasin B. Fertilized embryos were produced by microinjection of sperm into MII oocytes. These embryos were cultured for 24 h to the 2-cell stage, and each blastomere was treated with Caly A at different concentrations (10 or 20 nM) for 2 h. Thereafter, chromosome spreads were prepared and examined for the incidence of PCC and for chromosome morphology.

In Experiment 2, the viability of the reconstructed embryos produced by the fusion of blastomeres of androgenic and gynogenic embryos was investigated (see Fig. 1A, B, C, E and G). First, it was ascertained that chromosomes were equally divided into the sister blastomeres in androgenic, gynogenic and reconstructed embryos. Second, developmental competence of the reconstructed embryos with half the volume of cytoplasm was observed. The developmental assay was conducted independent of prezygotic examination of gamete chromosomes. As a control, 2-cell embryos were produced by microinjection of sperm into MII oocytes, followed by removal of one of the blastomeres to reduce the cytoplasm.

Reagents and media

All chemicals were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise stated. Oocytes/zygotes were cultured in Chatot–Ziomek–Bavister (CZB) medium [19] supplemented with 5.56 mM D-glucose and 5 mg/ml bovine serum albumin (BSA, AlbuMAX; Gibco, Auckland, New Zealand). Collection and micromanipulation of oocytes were performed in modified CZB medium supplemented with 20 mM HEPES-Na, 5 mM $NaHCO_3$ and 3 mg/ml of polyvinyl alcohol (PVA, cold-water soluble; Sigma-Aldrich, St. Louis, MO, USA) instead of BSA (H-CZB). Spermatozoa were collected in Toyoda–Yokoyama–Hosi (TYH) medium [20] supplemented with 20 mM HEPES-Na, 5 mM $NaHCO_3$ and 3 mg/ml of polyvinyl alcohol instead of BSA (H-TYH). CZB medium was used under an atmosphere of 5% CO_2 in air, and H-CZB and H-TYH media were used under an atmosphere of pure air.

Animals

All animals were purchased from Charles River Laboratories (Yokohama, Japan). The oocytes and spermatozoa were collected from hybrid (C57BL/6 × DBA/2) F_1 mice (BDF_1). ICR mice were used as surrogate mothers. All experiments were performed according to the Guidelines for Animal Experiments of Asahikawa Medical University.

Preparation of oocytes and spermatozoa

Female BDF_1 mice, 7–12 weeks of age, were superovulated by intraperitoneal injection of 10 IU equine chorionic gonadotropin (Aska Pharmaceutical, Tokyo, Japan) followed by injection of 10 IU human chorionic gonadotropin (Aska Pharmaceutical) 48 h later. The oocytes were recovered from the oviducts between 14 and 16 h after human chorionic gonadotropin injection and denuded of their cumulus cells by treatment with 0.1% (w/v) bovine testicular hyaluronidase (Sigma-Aldrich) in H-CZB. The cumulus-free oocytes were thoroughly washed with CZB and maintained in fresh CZB at 37 C.

Spermatozoa from the cauda epididymis of male mice, 7–14 weeks of age, were allowed to disperse in H-TYH. A small amount of sperm suspension was immediately transferred into a droplet (5 μl) of H-TYH containing 10% polyvinylpyrrolidone under sterile paraffin oil in a Petri dish for sperm microinjection.

Preparation of cytoplasts and karyoplasts

Zonae pellucidae of oocytes were partially dissected as reported previously [21] with slight modifications (Supplementary Movie S1). Briefly, the zona pellucida opposite the MII spindle was incised halfway around using a blunt-end micropipette (15–20 μm in diameter) by applying piezo pulses. The zona-cut oocytes were transferred into H-CZB containing 2.5 μg/ml cytochalasin B (Sigma-Aldrich). Approximately half of the cytoplasm volume was aspirated into another micropipette (30–35 μm in diameter) with care being taken not to aspirate the MII spindle, and the cell was then pinched off to produce a cytoplast [15]. Then, the MII-karyoplast was removed

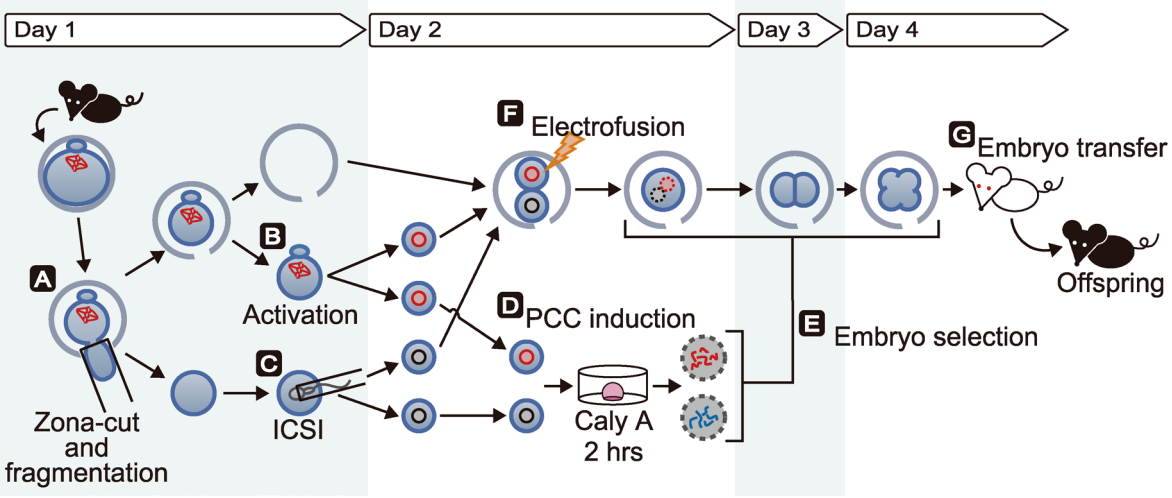

Fig. 1. Flowchart of chromosome screening of gametes. (A) Preparation of the karyoplast and cytoplast. (B and C) Production of gynogenic (B) and androgenic (C) embryos. (D) Induction of premature chromosome condensation (PCC) by calyculin A (Caly A). (E) Chromosome analysis and selection of transferable embryos. (F) Production of biparental diploid embryos by electrofusion. (G) Embryo transfer to obtain offspring (fetuses). Details of the procedure are provided in the Materials and Methods.

from the zona pellucida using the same micropipette and placed in H-CZB. The first polar body was removed from the zona pellucida and discarded. Cytoplasts, karyoplasts and zonae pellucidae were stored in CZB at 37 C until use.

Intracytoplasmic sperm injection (ICSI) and parthenogenetic activation

Before ICSI, a group of 15 oocytes was transferred to a droplet (5 µl) of H-CZB under sterile paraffin oil, which had been placed next to a sperm-containing droplet in the same dish. A spermatozoon was aspirated tail first into the injection pipette, followed by separation of the tail from the head using a few piezo pulses. The heads were individually injected into MII oocytes, enucleated oocytes [22] or cytoplasts according to the method described by Kuretake *et al*. [23]. ICSI-generated zygotes were cultured in a droplet (100 µl) of fresh CZB under sterile paraffin oil at 37 C until they reached the 2-cell stage. When androgenic embryos were produced by microinjection of sperm into cytoplasts, the osmolality of H-CZB was increased further to 30 mOsm by the addition of 5.5 mg/ml D-sorbitol (Sigma-Aldrich) because cytoplasts are vulnerable to damage from the microinjection procedure.

When gynogenic embryos were produced by parthenogenetic activation, oocytes or karyoplasts were cultured separately in Ca^{2+}-free CZB containing 10 mM strontium chloride ($SrCl_2$) for 1 h at 37 C. Subsequently, they were washed three times in fresh CZB and cultured in the same medium until they reached the 2-cell stage.

Reconstruction of diploid embryos from blastomeres of androgenic and gynogenic embryos

At 24 h after ICSI or parthenogenetic activation, androgenic or gynogenic 2-cell embryos were transferred individually into Ca^{2+}-free CZB to separate their blastomeres. One of the blastomeres from each of the two sources was transferred into an empty zona pellucida in H-CZB using a micropipette (about 50 µm in diameter; Supplementary Movie S2). Then, the blastomeres were electrically fused according to the method described by Wakayama *et al*. [24] with a slight modification. Briefly, a pair of the blastomeres within a zona pellucida was transferred into fusion medium consisting of 0.3 M mannitol, 0.1 mM $MgSO_4$ and 0.1% polyvinylpyrrolidone in a fusion chamber with electrodes set at 1 mm gaps. Fusion of the cell membrane was induced by applying alternating current (1 MHz for 5 sec) followed by direct current (150 V, 20 µsec) using a cell fusion generator (LF101; Nepa Gene, Chiba, Japan). Then, the paired blastomeres were immediately placed into fresh CZB to allow cell fusion.

We investigated spindle formation and chromosome constitution in the reconstructed embryos. For observation of the spindle, some of the reconstructed embryos at the first mitotic stage (approximately 1 h after the nuclear envelope had broken down) were fixed and permeabilized in 1% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.1% PVA and 0.2% Triton X-100 (Sigma-Aldrich) for 30 min, and then washed in PBS containing 1 mg/ml BSA. The embryos were incubated in mouse monoclonal anti-β-tubulin antibody (1:100; Sigma-Aldrich) for 1 h at 37 C, washed in PBS containing 1 mg/ml BSA and incubated in FITC-labeled goat anti-mouse IgG (1:100; KPL, Gaithersburg, MD, USA) for 1 h at 37 C. The embryos were placed on glass slides, sealed with VectaShield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and examined under an epifluorescence microscope. To investigate the chromosome constitution of the reconstructed embryos, 2-cell embryos were placed in CZB containing 0.02 µg/ml vinblastine sulfate at approximately 24–26 h after fusion and cultured until the nuclear envelopes of both sister blastomeres disappeared. Chromosome slides were prepared as described below.

Embryo transfer

At 48 h after blastomere fusion, the embryos were transferred into oviducts of ICR females, 8–16 weeks of age, on the first day of pseudopregnancy. Recipients were euthanized by cervical dislocation on day 16 of pregnancy to investigate the numbers of implantation sites and live fetuses.

PCC induced by Caly A

At 24 h after ICSI or parthenogenetic activation, blastomeres of the 2-cell embryos were transferred into CZB containing 2.5 μg/ml cytochalasin B, 0.02 μg/ml vinblastine sulfate and 10 or 20 nM Caly A (Wako Pure Chemical Industries, Osaka, Japan) and incubated for 2 h to induce PCC.

Chromosome preparation and multicolor fluorescence in situ hybridization (mFISH)

Blastomeres and embryos were kept in a hypotonic solution consisting of 0.24% (w/v) sodium citrate and 48% (v/v) fetal bovine serum in pure water for 10 min at room temperature. Chromosome spreads were prepared using a gradual-fixation/air-drying method [25] and stained with 2% Giemsa solution (Merck Japan, Tokyo, Japan) in buffered saline (pH 6.8) for 10 min.

In some slides, individual chromosomes were identified using mouse mFISH probes, 21XMouse (MetaSystems, Altlussheim, Germany), according to the manufacturer's instructions. After hybridization, the slides were sealed with DAPI/Antifade (MetaSystems) solution and examined under an epifluorescence microscope fitted with the appropriate filter sets. Fluorescent images were captured, and individual chromosomes, shown in pseudocolor, were karyotyped using Isis software (MetaSystems).

Statistical analysis

Statistical analyses were performed using the JMP software (SAS Institute, Cary, NC, USA). Data were analyzed by logistic regression using the following model: $\ln(\alpha/1-\alpha) = \beta$ + main factor (the dose of Caly A and type of embryos for Experiments 1 and 2, respectively), where α = frequency of positive outcomes and β = the intercept. The odds ratio (OR) with 95% confidence interval (CI) was calculated. When the values were small, Fisher's exact probability test was performed.

Results

Experiment 1: Caly A-induced PCC

Blastomere nuclear morphologies following Caly A treatment are shown in Fig. 2A–E. In this study, breakdown of the nuclear envelope was used as a criterion for the induction of PCC. Good chromosome spread, as shown in Fig. 2E, was recorded as an "analyzable karyoplate." Analysis using logistic regression revealed that the dose of Caly A affected the induction of PCC (OR, 90.78; 95% CI, 45.86–214.33). The rates of induction of PCC were significantly higher in 20 nM Caly A ($P < 0.001$) than in 10 nM Caly A (Fig. 2F). As shown in Fig. 2G, the rates of analyzable karyoplates in androgenic and gynogenic embryos—calculated as a proportion of the numbers of blastomeres with PCC—were also affected by the dose of Caly A (OR, 3.19; 95% CI, 2.42–4.21), being significantly increased ($P < 0.001$) in 20 nM compared with 10 nM regardless of the embryo's ploidy. However, analyzable karyoplates were observed in many blastomeres of ICSI embryos regardless of the dose of Caly A, and there was no difference in the PCC rates between the groups treated with 10 and 20 nM. Treatment with 20 nM Caly A tended to shorten the chromosomes more markedly than treatment with 10 nM Caly A.

Experiment 2: Gamete chromosome screening, embryo production and developmental assay

In this experiment, 99.2% (607/612) of oocytes were successfully divided into karyoplasts and cytoplasts following incision of the zona pellucida. Almost all cytoplasts injected with a spermatozoon formed a pronucleus (98.8%: 423/428), and the resultant androgenic embryos developed to the 2-cell stage with high frequency (90.5%: 383/423). By contrast, 95.7% (440/460) of MII karyoplasts extruded a second polar body following treatment with $SrCl_2$, and 439 of 440 gynogenic embryos (99.8%) developed to the 2-cell stage. Using these reliable techniques, the feasibilities of chromosome analysis by Caly A-induced PCC and embryo production by blastomere fusion were examined.

The chromosome constitutions of the androgenic and gynogenic 2-cell embryos were analyzed by inducing PCC with 20 nM Caly A (Table 1). More than 77.4% of them had haploid blastomeres (n = 20) without chromosome aberrations. Additionally, mFISH analysis revealed that these haploid blastomeres had a normal chromosome complement (Supplementary Fig. 1A and B: online only). Thus, most of the androgenic and gynogenic 2-cell embryos had a normal haploid set of chromosomes in both blastomeres, even though the amount of cytoplasm was halved. Therefore, blastomeres of androgenic and gynogenic embryos (without performing chromosome examination) were randomly fused with each other to construct diploid embryos. The fused embryos at the first mitotic division had a single metaphase spindle on which all chromosomes were aligned in the equatorial plane (Fig. 3A), demonstrating that the nuclei of androgenic and gynogenic origins had undergone syngamy. The fused embryos were fixed at the metaphase of the 2-cell stage to examine their chromosomal constitutions (Fig. 3B). Of 87 embryos examined (Table 1), 77.0% were diploid, with both of the sister blastomeres having a normal chromosomal complement (n = 40; Fig. 3C). Based on this result of cytogenetic analysis, the developmental competence of these fused embryos was monitored to verify the technical feasibility of embryo production using the present protocol.

To produce diploid embryos, blastomeres of androgenic and gynogenic embryos were randomly fused as mentioned above. The rate of fusion was 96.8% (240/248 pairs). The developmental competence of the 240 reconstructed zygotes is summarized in Table 2. The fused embryos developed successfully to the 2-cell stage (95.5–97.4%) by 24 h after fusion and to the 4-cell stage (90.9–94.9%) by 48 h after fusion. These 4-cell embryos reached the 8-cell stage according to the normal schedule for mouse embryo development and underwent "compaction" as seen in normal 8-cell embryos (Fig. 3D). At 72 h after fusion, blastocyst formation was observed in 79.5% of the embryos. Embryonic development of the fused embryos was comparable to that of the control. As shown in Table 2, transfer of the compacted 4-cell embryos supported normal fetal development (Fig. 3E), although the rates of implantation (15.6%) and live fetus

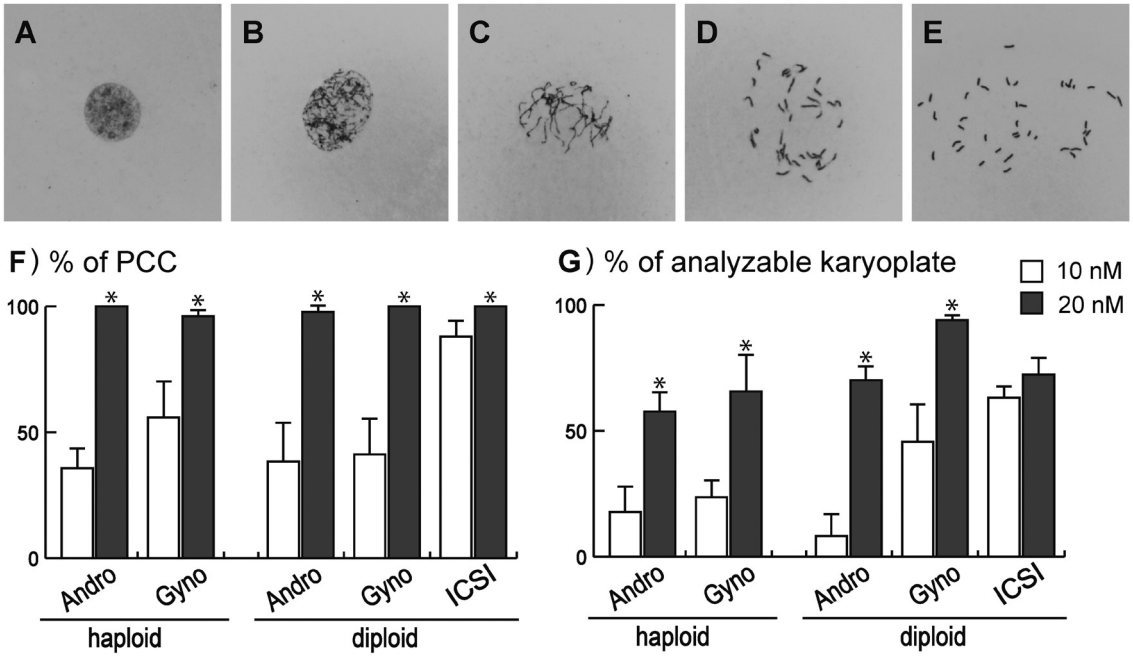

Fig. 2. Calyculin A (Caly A) treatment of the 2-cell stage blastomeres. (A–E) Morphology of blastomere nuclei in ICSI-generated embryos after Caly A treatment (10 nM for 2 h). Nuclei with breakdown of the nuclear envelope (C–E) were judged as having undergone premature chromosome condensation (PCC). Nuclei with good chromosome spreads such as that shown in E were classified as having analyzable karyoplates. (F and G) Comparison of the percentages of blastomeres with PCC (F) and analyzable karyoplates (G) after Caly A treatment at two different concentrations. The percentages of analyzable karyoplates were calculated based on the numbers of blastomeres with PCC. Data are shown as the mean ± SEM calculated from each replicate. Asterisks indicate values significantly different from their counterparts treated at 10 nM ($P < 0.05$).

Table 1. Type and incidence of chromosome aberrations in androgenic, gynogenic and reconstructed 2-cell embryos

Type of embryos	No. of embryos examined	Normal (%)*	Numerical aberration (%)			Structural aberration (%)†	Combined (%)‡
			Mosaicism	Hypoploidy	Hyperploidy		
Androgenic	53	41 (77.4)	3 (5.7)	0 (0)	2 (3.8)	6 (11.3)	1 (1.9)
Gynogenic	60	57 (95.0)	0 (0)	0 (0)	0 (0)	3 (5.0)	0 (0)
Reconstructed§	87	67 (77.0)	10 (11.5)	4 (4.6)	0 (0)	3 (3.4)	3 (3.4)

* Both blastomeres of 2-cell embryos had 20 or 40 normal chromosomes each. † Structural aberrations consisted of chromosome and chromatid breaks and chromosome exchange. Even in cases where these aberrations were found in one blastomere, the embryos were recorded as having structural aberrations. ‡ Embryos had numerical and structural aberrations in a blastomere. § Reconstructed embryos were produced from blastomeres without performing chromosome analysis.

formation (3.2%) were extremely low ($P < 0.05$) compared with those of the control (63.9% and 26.4%, respectively).

Discussion

Chromosome screening of gametes before fertilization is an effective method for preventing the transfer of embryos with chromosome damage in IVP. This study sought to establish an innovative method in which only a single pair of gametes is used for both screening and embryo production. Consequently, we achieved three technical improvements: (i) the use of oocyte fragments (karyoplasts and cytoplasts), (ii) haploid genome cloning using androgenic and gynogenic embryos and (iii) induction of PCC by Caly A. This method of chromosome screening of gametes might prove to be a breakthrough for producing genetically normal embryos during IVP.

The ability to predict the genetic constitution of offspring from the results of prezygotic examination of gamete chromosomes should be confirmed. In our previous study of prezygotic sperm chromosome screening [14], we produced normal offspring by using a sperm sample of a carrier of a Robertsonian translocation to evaluate the application of screening. However, the frequency of embryonic loss in the present study was too high to perform such tests. Barra and Renard [12] reported that fusion of blastomeres of 2-cell haploid gynogenic mouse embryos, in which one of the two nuclei was replaced by a paternal one, could lead to full-term development. Although fusion of blastomeres of 2-cell androgenic and gynogenic embryos could produce viable fetuses in the present study (Table 2), the fetal development rates were far from satisfactory. It should be noted that

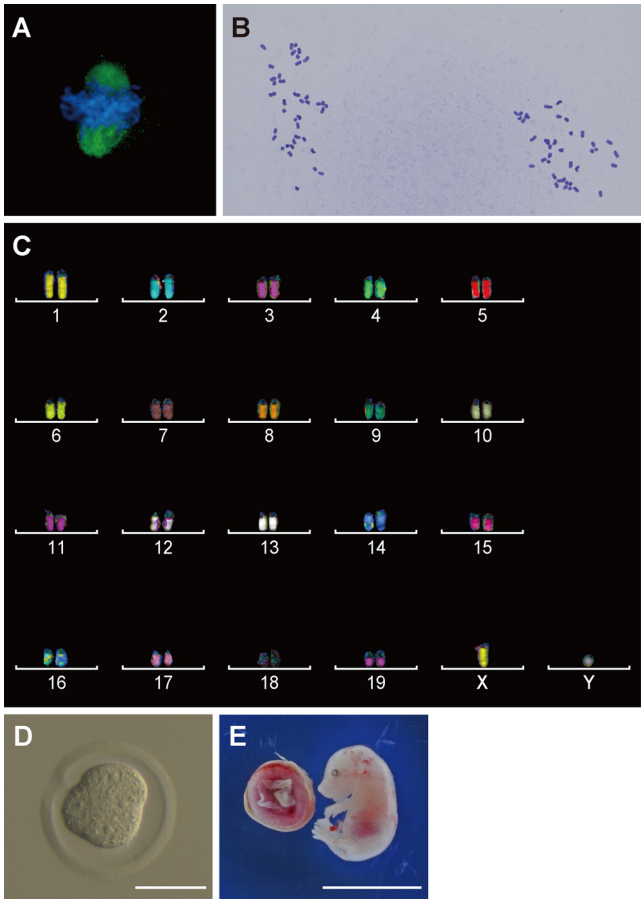

Fig. 3. Chromosome segregation and fetal development of reconstructed embryos fused with haploid blastomeres of androgenic and gynogenic embryos. (A) Mitotic spindle at the first mitosis of the reconstructed embryo. Nuclei from the blastomeres of androgenic and gynogenic embryos coordinately formed a single spindle in the reconstructed embryo. (B) Chromosome spreads of the reconstructed 2-cell embryo. Two sets of 40 chromosomes are seen. (C) mFISH analysis of a blastomere in the reconstructed embryo showing a full component of chromosomes. (D) A transferable embryo developed to the compacted 4-cell stage. Bar = 50 µm. (E) Normal live fetus obtained by embryo transfer of the reconstructed embryo. Bar = 10 mm.

the rates of implantation and live fetus formation ranged widely in Experiment 2 (0–38.9% and 0–11.1%, respectively). Although we cannot fully account for the embryonic losses, several explanations are plausible. For example, the biparental embryos used in this study were derived from fusion of pairs of blastomeres from androgenic and gynogenic embryos, which were formed using cytoplasts and karyoplasts, respectively. Thus, the cytoplasmic volume of the resulting embryos was reduced by almost half (Fig. 1F). This reduced volume might be a reason for insufficient developmental competence. However, the developmental competence of embryos with reduced cytoplasm was higher in the control (Table 2) and in a previous study [15]. A more reasonable explanation for the embryonic loss may be that the present procedures to produce biparental embryos by blastomere fusion are stressful and generate *de novo* chromosome aberrations during cleavage. Although a number of embryos (about 77%) had a normal chromosome complement, at least in the 2-cell stage (Table 1), we cannot exclude the possibility that the proportion of embryos with chromosome aberrations increases with progression of embryonic development, as reported previously [5, 26]. In addition, there is the possibility that the epigenetic states of the biparental embryos were inappropriate, leading to poor developmental competence. Indeed, fusion of somatic cells with embryonic germ cells [27] and embryonic stem cells [28] induces epigenetic modifications in the resulting hybrid cells.

The present results showed that genome cloning of spermatozoa and oocytes was essential for chromosome screening accompanied by fertilization and that mouse androgenic and gynogenic embryos could be produced at high efficiency, even though the volume of the oocytes (cytoplasts and karyoplasts) was reduced by half. Chromosome analysis of androgenic and gynogenic 2-cell embryos (Table 1) suggested that the genomes of spermatozoa and oocytes replicated normally and divided into sister blastomeres. Thus, genome cloning of gametes can be accomplished using only a single spermatozoon and an oocyte—at least in the mouse. On the other hand, the chromosome analysis results shown in Table 1 indicate that some blastomeres of androgenic 2-cell embryos had chromosome aberrations. Some of the aberrations might have been generated by the ICSI procedure, as shown in our previous study [14]. Although successful genome cloning of spermatozoa or oocytes using a similar method has been reported in bovine [9, 10] and human [6] models, the production of androgenic embryos occasionally leads to some problems. For

Table 2. Developmental competence of the reconstructed embryos derived from blastomeres of androgenic and gynogenic 2-cell embryos

Type of embryos	No. of diploid embryos cultured	No. (%) of embryos developed to			No. of embryos transferred	No. of recipients	No. (%) [range, %] of	
		2-cell stage	Compacted 4-cell stage	Blastocyst stage			Implantations	Live fetuses
Control*	89	79 (88.8)[a]	76 (85.4)[a]	–	72	4	46 (63.9)[a] [26.7–93.3]	19 (26.4)[a] [6.7–36.4]
Reconstructed	196	191 (97.4)[b]	186 (94.9)[b]	–	186	11	29 (15.6)[b] [0–38.9]	6 (3.2)[b] [0–11.1]
Reconstructed[†]	44	42 (95.5)[ab]	40 (90.9)[ab]	35 (79.5)	–	–	–	–

[a,b] Values without a common superscript are significantly different ($P < 0.05$). * Embryos fertilized by ICSI, in which a blastomere was removed at 24 h after ICSI, were prepared as the control. These embryos developed to the compacted 4-cell stage in the same manner as the reconstructed embryos. [†] This group of reconstructed embryos was cultured further to examine development up to the blastocyst stage.

example, microinjection of sperm into enucleated bovine oocytes was an inappropriate method for the production of androgenic embryos because of the lower developmental competence of the resulting embryos compared with *in vitro* fertilized enucleated oocytes [29]. In human trials, the reduction of cytoplasmic volume associated with oocyte enucleation was one of the factors that affected male pronuclear formation [6]. Furthermore, there is a possibility that acrosomal contents are detrimental for male pronuclear formation in oocytes fertilized by ICSI [30–32]. Therefore, genome cloning of spermatozoa in species other than the mouse requires scrupulous attention to the safe production of androgenic embryos.

Usually, a substantial number of metaphase cells are available for karyotyping the somatic cell sample. By contrast, there is a constraint in that only a single blastomere can be allocated to chromosome analysis of gametes. To overcome this disadvantage, induction of PCC by Caly A was attempted in this study. Caly A induces phosphorylation of the cell division cycle protein cdc25, followed by dephosphorylation of the cyclin B/cdc2 complex [16], which leads to PCC. Previous studies reported that large numbers of cells with readable chromosome spreads were obtainable in the analysis of lymphocytes [17] and amniotic cells [18] . The present study demonstrated that Caly A induced PCC in blastomeres, because chromosome preparation was complete at 2 h after treatment. This was similar in timing to induction of PCC by fusion with MII oocytes [14]. The proportion of cells showing PCC and the numbers of analyzable karyoplates following treatment with Caly A were higher at a dose of 20 nM than at 10 nM (Fig. 2F and G), although the chromosomes were markedly shortened at the higher dose. The mFISH approach was applicable to such shortened PCC chromosomes and was useful in identifying individual chromosomes (Fig. 3 and Supplementary Fig. 1).

The present method for prezygotic examination of gamete chromosomes allows the production of embryos without balanced chromosome aberration, as well as imbalanced aberrations. The balanced chromosome aberration causes chromosome nondisjunction and formation of imbalanced aberrations in gametes of the following generation. Similarly, chromosome mutations can be detected in gametes of parents accidentally exposed to environmental chemical mutagens and radiation. The method can be applied to DNA diagnosis of gametes because DNA amplification using a single haploid androgenic blastomere has already been achieved [9], leading to genome-wide diagnosis including the detection of genetic diseases. Through the use of single-nucleotide polymorphism analysis, gametes with a desirable haplotype may become available to obtain offspring with a predicted phenotype, accelerating animal breeding.

In conclusion, this study verified three key steps for the chromosome screening of gametes: (i) genome cloning of gametes using androgenic and gynogenic embryos, (ii) Caly A-induced PCC of the resulting different blastomeres and (iii) production of biparental diploid embryos by fusion of the remaining blastomeres. Cryopreservation of blastomeres of androgenic and gynogenic embryos would provide sufficient time for detailed chromosome analysis. When these steps are coordinated, chromosome screening of gametes and embryo production can be achieved by the use of only a single spermatozoon and oocyte. Although the cause of the early embryonic losses observed in this study remains uncertain, the approach to chromosome screening of gametes provides a powerful method of producing embryos with known genetic constitutions during IVP.

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