Human Reproduction (2013) 28:531-537.

Production of offspring after sperm chromosome screening: an experiment using the mouse model

Watanabe H, Kusakabe H, Mori H, Yanagimachi R, Tateno H

Production of offspring after sperm chromosome screening: an experiment using the mouse model

Running title: Sperm chromosome screening

H. Watanabe[1], H. Kusakabe[1], H. Mori[2], R. Yanagimachi[3] and H. Tateno[1]

[1]Department of Biological Sciences, Asahikawa Medical University, Asahikawa 078-8510, Japan.
[2]Department of Animal Science, School of Agriculture, Ibaraki University, Ibaraki 300-0332, Japan.
[3]Institute for Biogenesis Research, University of Hawaii, Honolulu, Hawaii 96822, USA.

Correspondence to: Hiroyuki Watanabe, Ph.D.
Department of Biological Sciences,
Asahikawa Medical University,
Asahikawa 078-8510, Japan
e-mail: h-wata@asahikawa-med.ac.jp
Tel: +81-166-68-2731
Fax: +81-166-68-2783

Abstract

BACKGROUND: Chromosomal aberrations in gametes are a major cause of pregnancy loss in women treated with assisted reproductive technology. To prevent the transmission of chromosomal aberrations from fathers to offspring, we performed sperm chromosome screening (SCS) prior to fertilization using the mouse as a model.

METHOD: A single spermatozoon of a male mouse with or without a Robertsonian translocation was injected into an enucleated oocyte to allow the replication of sperm chromosomes. One of the sister blastomeres of a haploid androgenic 2-cell embryo was used for chromosome analysis. The other blastomere was fused with an unfertilized oocyte, activated, and allowed to develop to a blastocyst before transfer to a surrogate mother.

RESULTS: We could analyze sperm chromosomes in the blastomere of an androgenic 2-cell embryo and were able to culture zygotes with and without aberrant chromosomes before embryo transfer. The karyotypes of the offspring faithfully reflected those of the blastomeres used for SCS.

CONCLUSION: This study showed that it is possible to produce zygotes without any paternally inherited aberrations by examining the genome of individual spermatozoa prior to embryo production.

Key Words: assisted reproductive technology/chromosome analysis/diagnosis/sperm

Introduction

Some women who became pregnant after the application of assisted reproductive technology (ART) repeat abortions. A major cause of pregnancy loss after ART, similar to that of spontaneous abortion after natural conception, is the presence of chromosomal aberrations in embryos that are inherited from the paternal and/or maternal gametes (Bettio et al., 2008; Martinez et al., 2010). The incidence of chromosomal aberrations in embryos may be higher when the fathers have abnormal karyotypes, such as 47,XXY and 47,XYY. Men with these karyotypes have a high risk of producing spermatozoa with sex chromosome aneuploidies. Some infertile men without any chromosomal aberrations may produce aneuploid and/or diploid spermatozoa because of the occurrence of aberrant meiosis during spermatogenesis (Egozcue et al., 2000). In addition to numerical anomalies, structural chromosomal aberrations in spermatozoa are responsible for pregnancy loss (Zini et al., 2008).

Although preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) have been used (Franssen et al., 2011) to avoid aneuploidy-related abortions, the clinical benefits of PGD/PGS remain uncertain (Franssen et al., 2011; Harper et al., 2010). PGD/PGS is an invasive method in which cleaving embryos or blastocysts receive microsurgical operation. Blastocyst biopsy may not allow sufficient time for a detailed genetic diagnosis (unless the blastocysts are frozen), as the blastocysts must be transferred to mothers by day 6 of development. Furthermore, there are legal restrictions regarding the use of PGD/PGS in some countries. Thus, genetic screening of spermatozoa and oocytes prior to fertilization is a method that should be considered (Yanagimachi, 2011). The production of offspring after sperm chromosome screening (SCS) reported in this study consisted of four steps: (1) replication of the sperm chromosomes, (2) analysis of one copy of the replicated sperm chromosomes, (3) construction of a zygote using

another set of chromosomes, and (4) production of a transferable embryo. To our knowledge, there are no reports on the successful genomic screening of spermatozoa, although some attempts have been made using the mouse as a model (Takeuchi et al., 2007, 2008).

Materials and methods

Experimental design

The procedure used for SCS in this study is summarized in Figure 1. Briefly, a spermatozoon was injected into an enucleated MII oocyte (Figure 1A) to produce a haploid androgenic 2-cell embryo (Figure 1B). One of the two sister blastomeres was fused with a zona-free MII oocyte to induce premature chromosome condensation (PCC) for the analysis of sperm chromosomes (Figure 1C). The other sister blastomere was fused with another zona-free MII oocyte to construct a diploid zygote (Figure 1D). This zygote was cultured until the blastocyst stage. Based on chromosome analysis of the first sister blastomere, embryos with known sperm chromosome constitutions were selected (Figure 1E) and transferred to the surrogate mothers. Some androgenic blastomeres were cryopreserved in liquid nitrogen before fusion with zona-free MII oocytes for embryo production (Figure 1F). We used the spermatozoa of male mice with a Robertsonian translocation to determine whether it is possible to preselect embryos with known chromosome constitutions prior to the embryo transfer.

Reagents and media

All chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated. Oocytes/zygotes were cultured in Chatot–Ziomek–Bavister (CZB) medium (Chatot et al.,

1989) supplemented with 5.56 mM D-glucose and 5 mg/mL of bovine serum albumin (BSA, AlbuMax; GibcoBRL, 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 0.1 mg/mL of polyvinyl alcohol (cold-water soluble; Sigma-Aldrich, St. Louis, MO, USA) instead of BSA (H-CZB). Spermatozoa were collected in Toyoda–Yokoyama–Hosi (TYH) medium (Toyoda et al., 1971) supplemented with 20 mM HEPES-Na, 5 mM $NaHCO_3$, and 0.1 mg/mL of polyvinyl alcohol instead of BSA (H-TYH). The pH of both H-CZB and H-TYH media was adjusted to approximately 7.4. Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10,000 IU of penicillin G potassium (Meiji Seika Pharmaceutical, Tokyo, Japan) and 10% fetal bovine serum (FBS; Cell Culture Technologies, Lugano, Switzerland) was used to culture skin cells of offspring. CZB and D-MEM were used under 5% CO_2 in air, and H-CZB and H-TYH were used under pure air.

Animals

Hybrid (C57BL/6 × DBA/2) F_1 mice (BDF_1; Japan SLC Inc., Shizuoka, Japan) were used to collect oocytes and spermatozoa. ICR mice (Charles River Laboratories Japan Inc., Yokohama, Japan) were used as surrogate mothers. Heterozygous carriers (ICR × C57BL/10) of a Robertsonian translocation between chromosomes 6 and 15 (rob (6;15)) were also used as other sperm donors because they innately provide spermatozoa with normal, balanced, and unbalanced chromosome constitutions (Mori et al., 1995). All experiments were performed according to the Guideline for Animal Experiments of the Asahikawa Medical University.

Preparation of oocytes and spermatozoa for intracytoplasmic sperm injection (ICSI)

Superovulation was induced in BDF_1 females aged 7–11 weeks via intraperitoneal (i.p.)

injection of 10 IU of eCG (Aska Pharmaceutical, Tokyo, Japan), followed by i.p. injection of 10 IU of hCG (Aska Pharmaceutical) 48 h later. Oocytes were recovered from the oviducts of treated mice between 14 and 16 h after hCG injection and immediately denuded of their cumulus cells in H-CZB containing 0.1% (w/v) hyaluronidase (Sigma-Aldrich). The cumulus-free oocytes were thoroughly washed with CZB and maintained in fresh CZB at 37°C until ICSI. Spermatozoa from the cauda epididymis of males aged 7–14 weeks 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% polyvinyl pyrrolidone under paraffin oil (Merck Japan, Tokyo, Japan) in a Petri dish, for ICSI.

Production of haploid androgenic embryos

Oocytes were enucleated in H-CZB containing 5 μg/mL of cytochalasin B (Sigma-Aldrich), as described elsewhere (Wakayama et al., 1998). Before sperm injection, a group of 15 oocytes was transferred to a droplet (5 μL) of H-CZB under paraffin oil, which had been placed next to a sperm-containing droplet in the same dish. A spermatozoon was aspirated into the injection pipette tail first, followed by separation of the tail from the head by application of a few piezo pulses. The heads were individually injected into enucleated oocytes according to the method of Kuretake et al. (1996). Each series of ICSI experiments was completed within 1 h. ICSI eggs were cultured in a droplet (100 μL) of fresh CZB under paraffin oil at 37°C until they reached the 2-cell stage.

Fusion of blastomeres and oocytes

At 24 h after ICSI, the androgenic 2-cell embryos were treated with 0.5% (w/v) protease (Kaken Pharmaceuticals, Tokyo, Japan) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline to digest the zona pellucida and separate the sister blastomeres. Concomitantly,

fresh MII oocytes were successively treated with 0.1% hyaluronidase and 0.5% protease to remove the cumulus cells and the zona pellucida. One androgenic blastomere and one zona-free MII oocyte were then fused using the Hemagglutinating Virus of Japan (HVJ) Envelope Cell Fusion Kit (GenomeOne-CF; Ishihara Sangyo, Osaka, Japan). Briefly, the MII spindle of the recipient oocyte was located at the 9 o'clock position on a holding pipette attached to the micromanipulator and the HVJ envelope was sprayed on the surface of the oocyte at the 3 o'clock position using a fine glass pipette (20–25 μm in diameter). Subsequently, the zona-free oocyte was paired with a blastomere (Supplementary Movie 1). The paired oocytes/blastomeres were individually maintained for 30 min in CZB until complete fusion (Supplementary Movie 2). MII oocytes to be used for induction of PCC in blastomeres were maintained in medium containing the calcium-chelating agent BAPTA-AM (10 μM) (Sigma-Aldrich) to prevent their activation.

Culture of fused zygotes

The fused zona-free zygotes were maintained in CZB for 1.5 to 2 h at 37°C, and then transferred to calcium-free CZB containing 10 mM strontium chloride to allow their activation. Thirty minutes later, the zygotes were washed with CZB and individually cultured for 96 h in the same medium by placing them in a small depression in the surface of a culture dish, which was created using an aggregation needle (BLS, Budapest, Hungary). The development of each embryo was examined every 24 h.

Vitrification of blastomeres

Some androgenic blastomeres were cryopreserved in liquid nitrogen for 1–2 weeks using Cryotop (Kitazato BioPharma, Shizuoka, Japan) and a Vitrification Kit (VT101 and VT102, for vitrifying and warming, respectively; Kitazato BioPharma). Vitrifying and warming were

performed according to the manufacturer's instructions. After warming, blastomeres were cultured in CZB for 1.5 to 2 h and then used to construct fused zygotes, as described above.

Embryo transfer

Blastocysts were transferred into each oviduct of ICR females aged 8–14 weeks on the first day of pseudopregnancy to investigate fetal development. Recipients were sacrificed on day 16 of pregnancy and the number of implantation sites and live fetuses was recorded. The fetuses were morphologically examined. For offspring delivery, blastocysts were also transferred into pregnant females. Offspring from transferred embryos were identified using hair color.

Preparation and analysis of chromosome slides

The fused zygotes were maintained in CZB for 2 h at 37°C to induce PCC in paternal chromosomes. The zygotes were then placed in hypotonic CZB (diluted 2-fold with pure water) for 10 min at room temperature. Chromosome spreads were prepared using the gradual-fixation/air-drying method (Mikamo and Kamiguchi, 1983). The slides were stained with 2% Giemsa (Merck) in buffered saline (pH 6.8) for 10 min. Some slides were subjected to conventional C-banding stain to identify centromeric heterochromatin. For the analysis of paternal chromosomes derived from spermatozoa of mice carrying a Robertsonian translocation, fluorescence *in situ* hybridization (FISH) was performed according to the manufacturer's instructions to detect rob(6;15) using DNA probes specific for chromosomes 6 and 15 (Applied Spectral Imaging, Migdal Haemek, Israel). After hybridization, slides were sealed with VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) before examination under a fluorescence microscope.

For chromosome analysis of offspring derived from the spermatozoa of carriers of rob(6;15), skin cells from the tail tip were cultured in D-MEM for 3–5 days. The cells were then

treated with 0.05 μg/mL of colcemid (GibcoBRL) for 2–3 h and then recovered by treating with 0.25% trypsin/1mM EDTA solution. The cells were placed in a hypotonic 0.075 M KCl solution for 30 min at room temperature and fixed with a methanol/acetic acid (3:1) mixture. Chromosome spreads were air dried and subjected to FISH, as described above.

Statistical analysis

All experiments were performed at least three times. Statistical analyses were performed using logistic regression analysis using the JMP software (SAS Institute, Cary, NC, USA). Differences were considered significant at $P < 0.05$.

Results

Chromosome distribution in blastomeres of androgenones

The injection of spermatozoa into enucleated oocytes led to the successful development of 93.1% (162/174) to the 2-cell stage. Among them, 123 androgenones at the 2-cell stage (246 blastomeres) were used to determine whether two blastomeres of each androgenone had identical chromosome constitutions. Chromosomes in two blastomeres of androgenones were examined after fusion of blastomeres with MII oocytes, with care being taken not to mix up blastomeres of two different androgenones. When blastomeres were fused with MII oocytes, 98.8% (243/246) of the zygotes were successfully fused. Two hundred and thirty fused zygotes (115 pairs) were fixed on glass slides and 218 zygotes had analyzable chromosomes originating from blastomeres at the G2 phase of the cell cycle (Figure 2B–D). Among them, it was possible to analyze concurrently the chromosomes of 104 pairs in both sister blastomeres. Chromosomes originating from blastomeres

and MII oocytes were easily distinguished based on their morphology (Figure 2A); chromosome spreads originating from blastomeres were analyzed. As shown in Table I, 85 pairs (81.7%) had normal chromosomes in each blastomere; nine pairs (8.7%) had chromosomes with identical abnormalities in two blastomeres; and 10 pairs (9.6%) had normal chromosomes in one blastomere and abnormal chromosomes in the other blastomere. Together, ~90% of sister blastomeres in androgenones had identical chromosome constitutions.

Development of embryos that received haploid nuclei of androgenones

The developmental competence of MII oocytes fused with fresh or cryopreserved blastomeres from androgenones was investigated (Table II). In the case of fresh blastomeres, the fusion of 372 zona-free MII oocytes with a blastomere of a 2-cell androgenone and subsequent activation resulted in 284 (76.3%) zygotes that had two pronuclei and two polar bodies (Figure 2E). They were considered as being "fertilized normally." There were 52 zygotes with incomplete extrusion of polar bodies and 36 zygotes with unusually large polar bodies. These were considered as zygotes with abnormal ploidy and were discarded. Among the reconstructed zygotes, 53.8–63.6% developed to blastocysts and 22.6% of blastocysts that were transferred developed to live fetuses. Among 115 cryopreserved blastomeres, 110 (95.6%) were successfully defrosted. One hundred and eight blastomeres were each fused with an MII oocyte to produce 77 diploid zygotes. Among these zygotes, 34 (44.2%) developed into blastocysts. This value was significantly lower ($P < 0.05$) than that of zygotes produced using nonfrozen blastomeres (63.6%). However, the rates of implantation (47.1%) and fetal development (14.7%) after embryo transfer were similar to those of the zygotes produced using nonfrozen blastomeres (42.9 and 22.6%, respectively).

Production of offspring after SCS

Haploid androgenic 2-cell embryos were prepared using the spermatozoa of males carrying a Robertsonian translocation. One blastomere of each embryo was used for chromosome analysis. FISH identified chromosome constitution: normal, balanced (rob(6;15)), or unbalanced (Figure 3). The other blastomere was used without cryopreservation for the production of offspring. Table III summarizes the results of experiments in which blastomeres with a normal chromosome constitution and those with a rob(6;15) were separately fused with zona-free MII oocytes and allowed to develop to blastocysts before transfer to surrogate mothers. The karyotypes of the offspring faithfully reflected those of the blastomeres used for SCS (Supplementary Figure 1). Delivered offspring were all phenotypically normal and developed into fertile adults.

Discussion

In this study, we replicated chromosomes of a single spermatozoon, quickly analyzed one replicated chromosome set, constructed a zygote using the remaining set of sperm chromosomes, and produced offspring with known male chromosome constitutions. We visualized sperm chromosomes by inducing PCC within a fresh MII oocyte. Prior to fixation of oocytes, oocytes were treated with a hypotonic (1/2 diluted) CZB medium. This medium yielded better and more consistent chromosome spreads than did conventional hypotonic solutions, such as 1% sodium citrate (Araki et al., 2005) and a 1:1 mixture of 1% sodium citrate and 30% FBS (Tateno and Kamiguchi, 2007). Our chromosome analyses of androgenic 2-cell embryos revealed that 9.6% of the embryos had different chromosome constitutions in the two sister blastomeres. This may represent structural chromosomal aberrations generated artificially by the ICSI procedure. According to previous studies (Tateno and Kamiguchi, 2007; Watanabe et al., 2010a,b), 6–13% of

ICSI mouse zygotes have unstable structural chromosomal aberrations. Unstable structural chromosomal aberrations occurring during cleavage after sperm irradiation have also been reported (Tateno et al., 2011). Nevertheless, the high homology (90.4%) in chromosome constitution between the two sister blastomeres of androgenic 2-cell embryos used here for chromosome analysis indicates that this embryonic stage is suitable for SCS. Prior to this study, we attempted to use haploid androgenic 4-cell embryos for SCS, because they provide four sister blastomeres instead of two, which would increase the reliability of the chromosome analysis. To our disappointment, sister blastomeres of haploid androgenic 4-cell embryos did not enter the G2 phase synchronously.

In this study, whole androgenic blastomeres at the G2 phase were fused with MII oocytes using an HVJ envelope to produce diploid zygotes, whereas in previous studies karyoplasts isolated from androgenic blastomeres were electrofused with parthenogenetically activated oocytes (Takeuchi et al., 2007, 2008). The delivery rate in this study was slightly higher than that observed in previous studies. Therefore, it seems unlikely that a mixture of cytoplasm between different cell stages affects the subsequent development of fused zygotes.

The results of SCS using spermatozoa of carriers of rob(6;15) demonstrated that offspring could be obtained from sperm with preanalyzed chromosomes. This is the first report of successful SCS at the chromosomal level. Our success using a mouse model suggests that SCS may also be applicable to the spermatozoa of human translocation carriers. It may also be possible to apply advanced DNA technologies, such as array-comparative genomic hybridization and single-nucleotide polymorphism arrays, to genome-wide sperm genetic screening (SGS) (Harper and SenGupta, 2012). In addition, the technique of SCS can be used for sperm sexing in animal reproduction.

In this study, the two sister blastomeres of a haploid androgenic 2-cell embryo were

concurrently fused with MII oocytes. One fused pair was used for chromosome analysis and the other was used for embryo production. Although SCS/SGS will become simpler and quicker with the advancement of technologies, it would be ideal to freeze blastomeres of androgenones until fully mature unfertilized oocytes become available. We found that blastomeres of androgenic 2-cell embryos from normal BDF_1 male mice could be cryopreserved in liquid nitrogen using Cryotop (Kuwayama et al., 2005) and that zygotes constructed using cryopreserved blastomeres developed to live fetuses as well as did zygotes constructed using nonfrozen blastomeres (Table II). Based on this result, we tried to perform SCS and embryo production using cryopreserved blastomeres from a carrier of rob(6;15). However, the attempt was abortive because the resultant zygotes did not develop beyond the 2-cell stage. The genome of blastomeres from a carrier of rob(6;15) may be vulnerable to freezing compared with that of blastomeres from normal BDF_1 mice. In any case, the cryopreservation of blastomeres certainly facilitates the SCS approach proposed here.

Authors' role

H.W. performed all experiments and drafted the paper. H.K. performed the FISH analysis. H.M. managed rob(6;15) mice. R.Y. and H.T. designed the study and drafted the paper.

Acknowledgements

The authors thank Dr. Y. Fukui (Obihiro University of Agriculture and Veterinary Medicine) for his invaluable technical advice.

Funding

This study was supported by a Grant-in-Aid for Scientific Research (22·8495 and 23890013 to H.W.) from the Japanese Society for the Promotion of Science (JSPS).

Conflict of interest

None declared.

References

Araki Y, Yoshizawa M, Araki Y. A novel method for chromosome analysis of human sperm using enucleated mouse oocytes. Hum Reprod 2005;20:1244-1247.

Bettio D, Venci A, Levi Setti PE. Chromosomal abnormalities in miscarriages after different assisted reproduction procedures. Placenta 2008;29 Suppl B:126-128.

Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 1989;86:679-688.

Egozcue S, Blanco J, Vendrell JM, Garcia F, Veiga A, Aran B, Barri PN, Vidal F, Egozcue J. Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. Hum Reprod Update 2000;6:93-105.

Franssen MT, Musters AM, van der Veen F, Repping S, Leschot NJ, Bossuyt PM, Goddijn M, Korevaar JC. Reproductive outcome after PGD in couples with recurrent miscarriage carrying a structural chromosome abnormality: a systematic review. Hum Reprod Update 2011;17:467-475.

Harper J, Coonen E, De Rycke M, Fiorentino F, Geraedts J, Goossens V, Harton G, Moutou C, Pehlivan Budak T, Renwick P et al. What next for preimplantation genetic screening (PGS)? A position statement from the ESHRE PGD Consortium Steering Committee. Hum Reprod 2010;25:821-823.

Harper JC, SenGupta SB. Preimplantation genetic diagnosis: State of the ART 2011. Hum Genet 2012;131:175-186.

Kuretake S, Kimura Y, Hoshi K, Yanagimachi R. Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol Reprod 1996;55:789-795.

Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online 2005;11:300-308.

Martinez MC, Mendez C, Ferro J, Nicolas M, Serra V, Landeras J. Cytogenetic analysis of early nonviable pregnancies after assisted reproduction treatment. Fertil Steril 2010;93:289-292.

Mikamo K, Kamiguchi Y. A new assessment system for chromosomal mutagenicity using oocytes and early zygotes of the Chinese hamster. In Ishihara T and Sasaki MS (eds) Radiation-Induced Chromosome Damage in Man. 1983. Alan R. Liss, New York, pp.411-432.

Mori H, Yoshida H, Omori K, Ishio S. Chromosome segregation patterns in heterozygous mice for Robertsonian translocation. J Mamm Ova Res 1995;12:S35.

Takeuchi T, Neri QV, Cheng M, Palermo GD. Cloning the male genome. Hum Reprod 2007;22(Suppl. 1):i62.

Takeuchi T, Neri QV, Rosenwaks Z, Palermo GD. Offspring generated from androgenic 'octets'. Hum Reprod 2008;23(Suppl. 1):i103.

Tateno H, Kamiguchi Y. Evaluation of chromosomal risk following intracytoplasmic sperm injection in the mouse. Biol Reprod 2007;77:336-342.

Tateno H, Kusakabe H, Kamiguchi Y. Structural chromosomal aberrations, aneuploidy, and mosaicism in early cleavage mouse embryos derived from spermatozoa exposed to gamma-rays. Int J Radiat Biol 2011;87:320-329.

Toyoda Y, Yokoyama M, Hosi T. Studies on the fertilization of mouse eggs in vitro: I. In vitro fertilization of eggs by fresh epididymal sperm (in Japanese). Jpn J Anim Reprod 1971;16:147-151.

Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998;394:369-374.

Watanabe H, Suzuki H, Fukui Y. Fertilizability, developmental competence, and chromosomal integrity of oocytes microinjected with pre-treated spermatozoa in mice. Reproduction 2010a;139:513-521.

Watanabe H, Suzuki H, Tateno H, Fukui Y. A novel method for detection of chromosomal integrity in cryopreserved livestock spermatozoa using artificially fused mouse oocytes. J Assist Reprod Genet 2010b;27:581-588.

Yanagimachi R. Problems of sperm fertility: a reproductive biologist's view. Syst Biol Reprod Med 2011;57:102-114.

Zini A, Boman JM, Belzile E, Ciampi A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. Hum Reprod 2008;23:2663-2668.

Figure legends

Fig. 1: Flowchart of SCS. Details of the procedure are provided in the "Materials and methods" section. Polar bodies were omitted in the illustration.

Fig. 2: Chromosome spreads and morphological features of zygotes produced by fusion of androgenic haploid blastomeres with MII oocytes. (A) Chromosomal preparation of two fused zygotes derived from the two sister blastomeres of an androgenic haploid 2-cell embryo. Each zygote had a G2 prematurely condensed chromosome (G2-PCC) spread of blastomere origin (arrows) and an MII chromosome spread of oocyte origin (arrowheads). The broken line indicates the outline of fused zygotes. (B) Normal haploid (n = 20) chromosome spread of blastomere origin. (C) C-band staining of (B). (D) Structural chromosomal aberration of blastomere origin (arrow). (E) Diploid zygote with two pronuclei and two polar bodies. (F) Blastocysts derived from diploid zygotes after cultivation for 96 h. Bar = 100 μm.

Fig. 3: Fluorescence *in situ* hybridization (FISH) analysis of androgenic haploid G2 blastomeres derived from the spermatozoa of rob(6;15) carriers. Chromosomes 6 and 15 were visualized using red and green colors, respectively. (A) Normal karyotype. (B) Balanced karyotype with rob(6;15) (arrow). (C) Disomy of chromosome 15, including rob(6;15) (arrowhead) and an extra chromosome 15 (arrow). (D) *De novo* reciprocal translocation involving chromosome 6 (arrows).

Supplementary Fig. 1: Offspring derived from preanalyzed paternal chromosomes. (A) The

offspring delivered (arrows) were morphologically normal and fertile. (B) Karyotype of normal skin cells. (C) Karyotype of skin cells with a balanced translocation. The karyotypes of the offspring were reflected in the results of SCS. Chromosomes 6 and 15 were visualized using red and green colors, respectively. The arrowhead indicates rob(6;15).

Table I. Homology of chromosome constitution in blastomeres of 2-cell androgenones

No. of 2-cell androgenones examined	Chromosome constitution (%)		
	Normal/Normal	Normal/Abnormal[a]	Abnormal/Abnormal[b]
104	85 (81.7)	10 (9.6)	9 (8.7)

[a] Chromosomal aberrations consisted of chromosome and chromatid breaks (eight and two cases, respectively).

[b] Chromosomal aberrations consisted of spermatozoan hypohaploidy, structural anomaly, and mosaicism (three cases each).

Table II. Developmental competence of oocytes fused with androgenic blastomeres

Type of blastomere	No. of diploid zygotes constructed	No. (%) of zygotes developed to				No. of blastocysts transferred	No. (%) of	
		2-cell	4-cell	8-cell/ morula	blastocyst		implantations	live fetuses
Fresh	130[a]	111 (85.4)	103 (76.2)	97 (74.6)	70 (53.8)[c,d]	–	–	–
	154[b]	–	–	–	98 (63.6)[c]	84	36 (42.9)	19 (22.6)
Cryopreserved	77[b]	–	–	–	34 (44.2)[d]	34	16 (47.1)	5 (14.7)

[a] Zygotes were cultured to examine development up to the blastocyst stage.

[b] Zygotes were cultured to produce transferable blastocysts.

[c,d] Values without a common superscript were significantly different ($P < 0.05$).

Table III. Production of offspring after sperm chromosome screening

Karyotype of blastomeres used for production of offspring	No. of embryos transferred	No. (%) of offspring [sex]	No. (%) of offspring with		
			normal karyotype	rob(6;15)	unknown[a]
Normal	66	18 (27.3) [5F, 13M]	16 (88.9)	0 (0)	2 (11.1)
rob(6;15)	22	5 (22.7) [2F, 3M]	0 (0)	5 (100)	0 (0)

[a] Two offspring could not be karyotyped because they were stillborn and their cells failed to grow during cell culture.

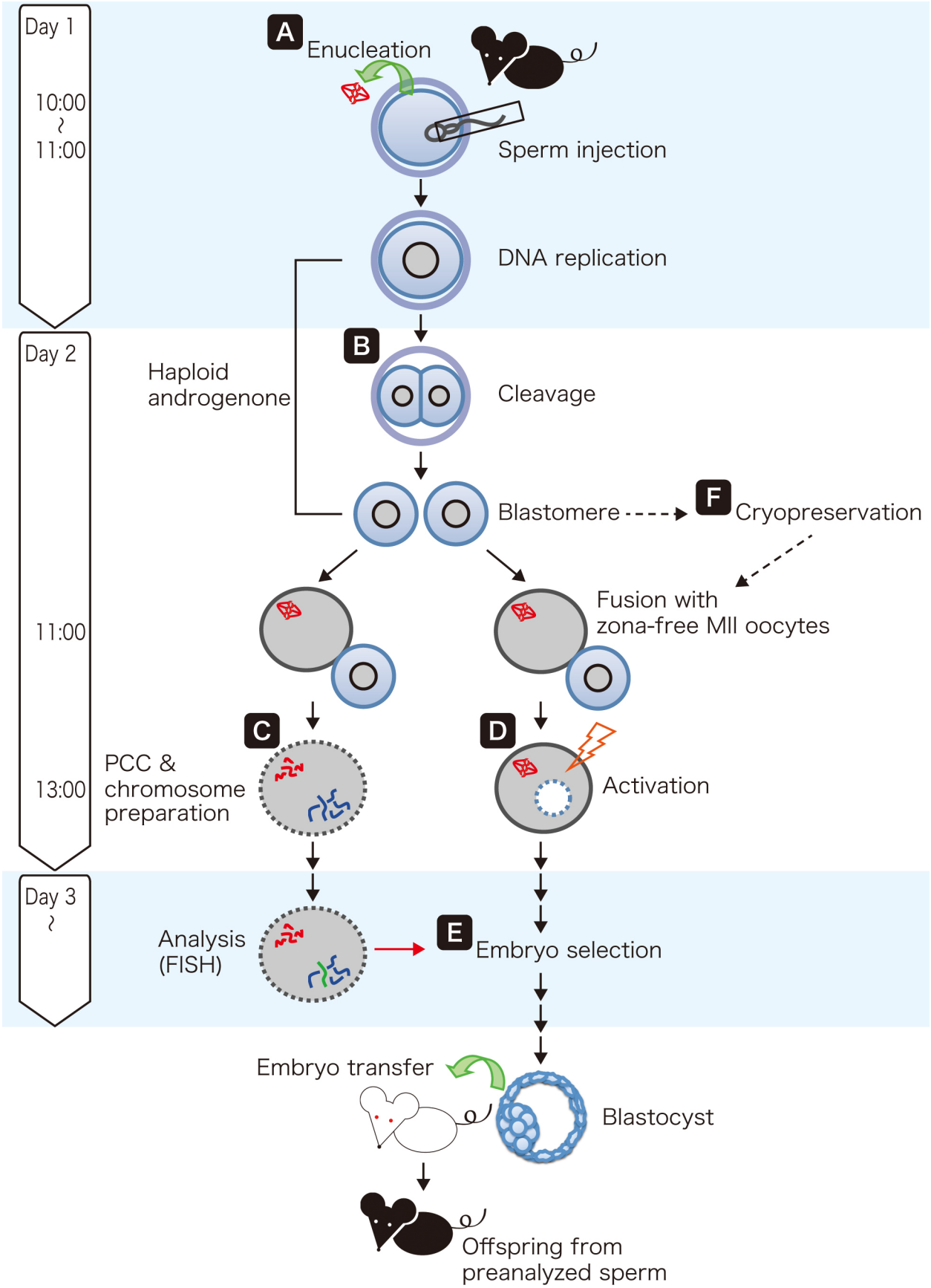

Day 1
10:00
~
11:00
A
Enucleation
Sperm injection
DNA replication
Day 2
Haploid
androgenone
B
Cleavage
Blastomere
F
Cryopreservation
Fusion with
zona-free MII oocytes
11:00
C
D
PCC &
chromosome
preparation
13:00
Activation
Day 3
~
Analysis
(FISH)
E
Embryo selection
Embryo transfer
Blastocyst
Offspring from
preanalyzed sperm

Figure 1

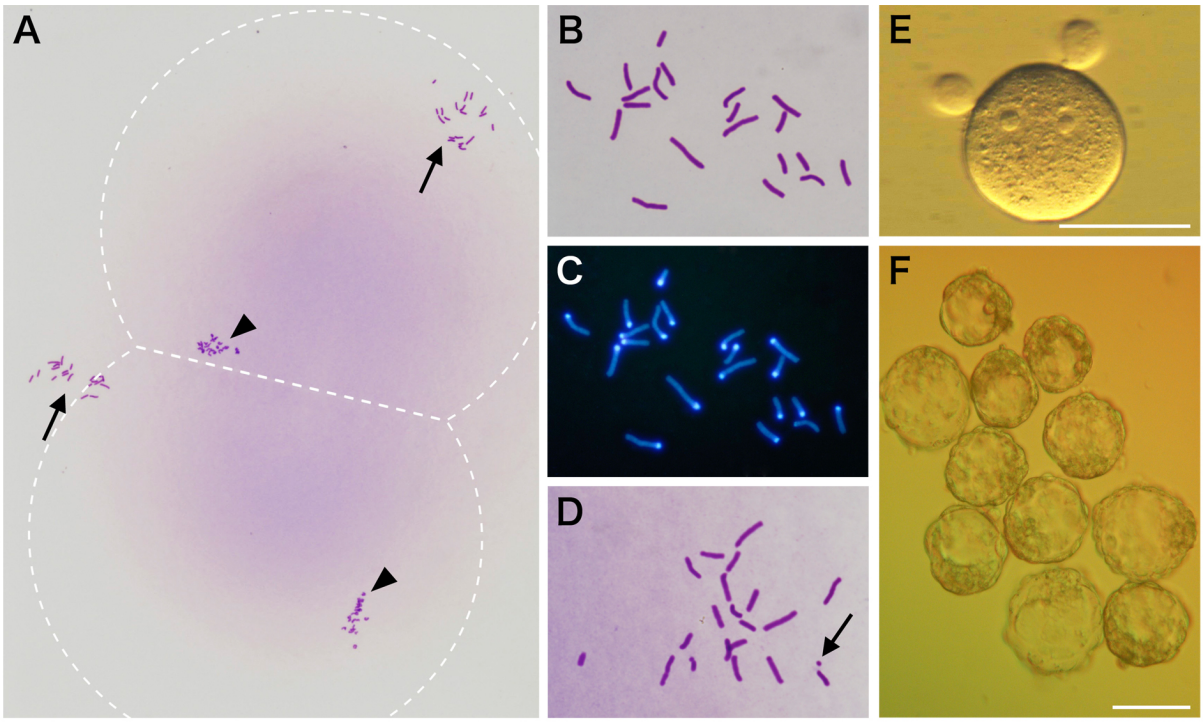


Figure 2

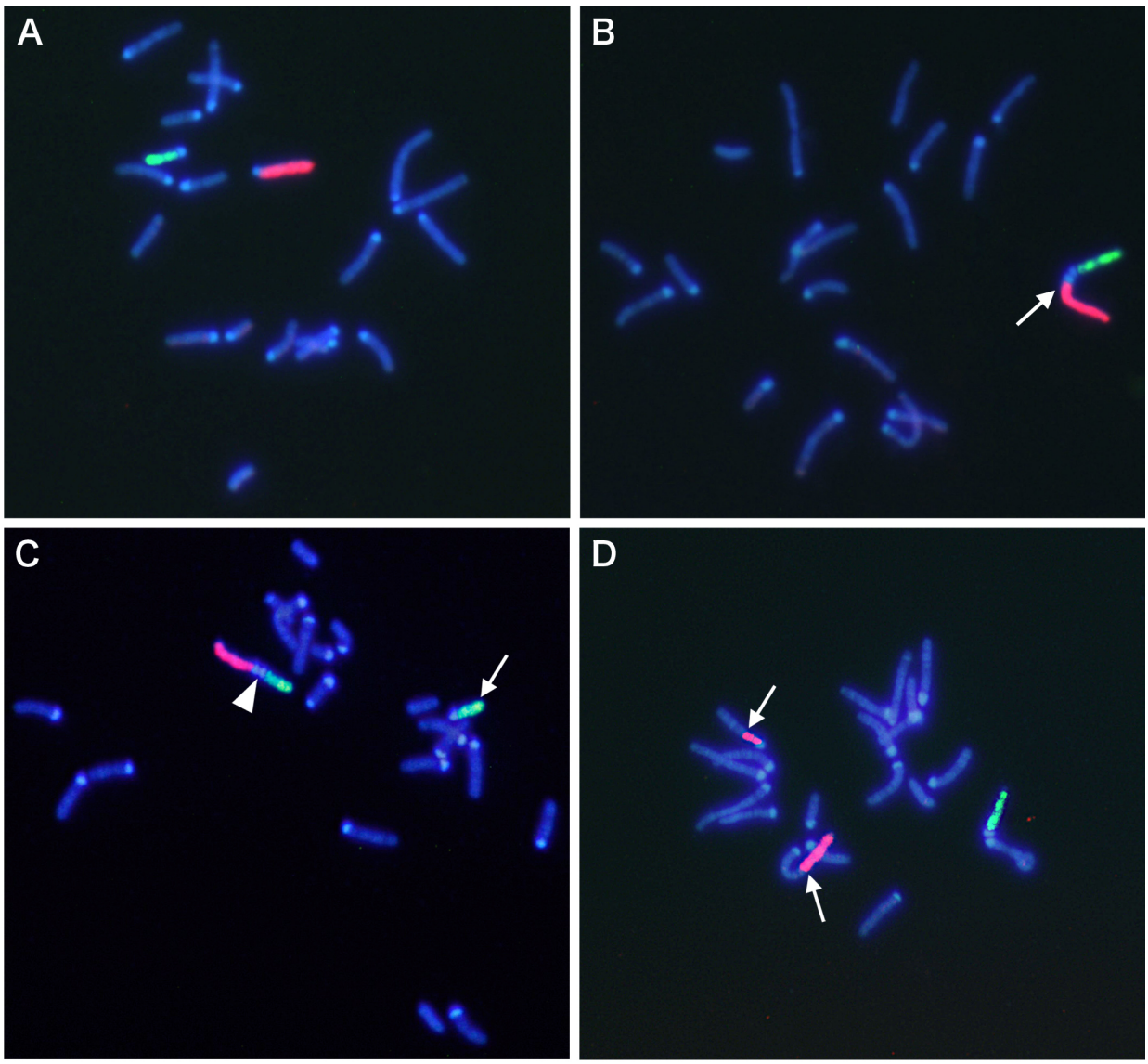
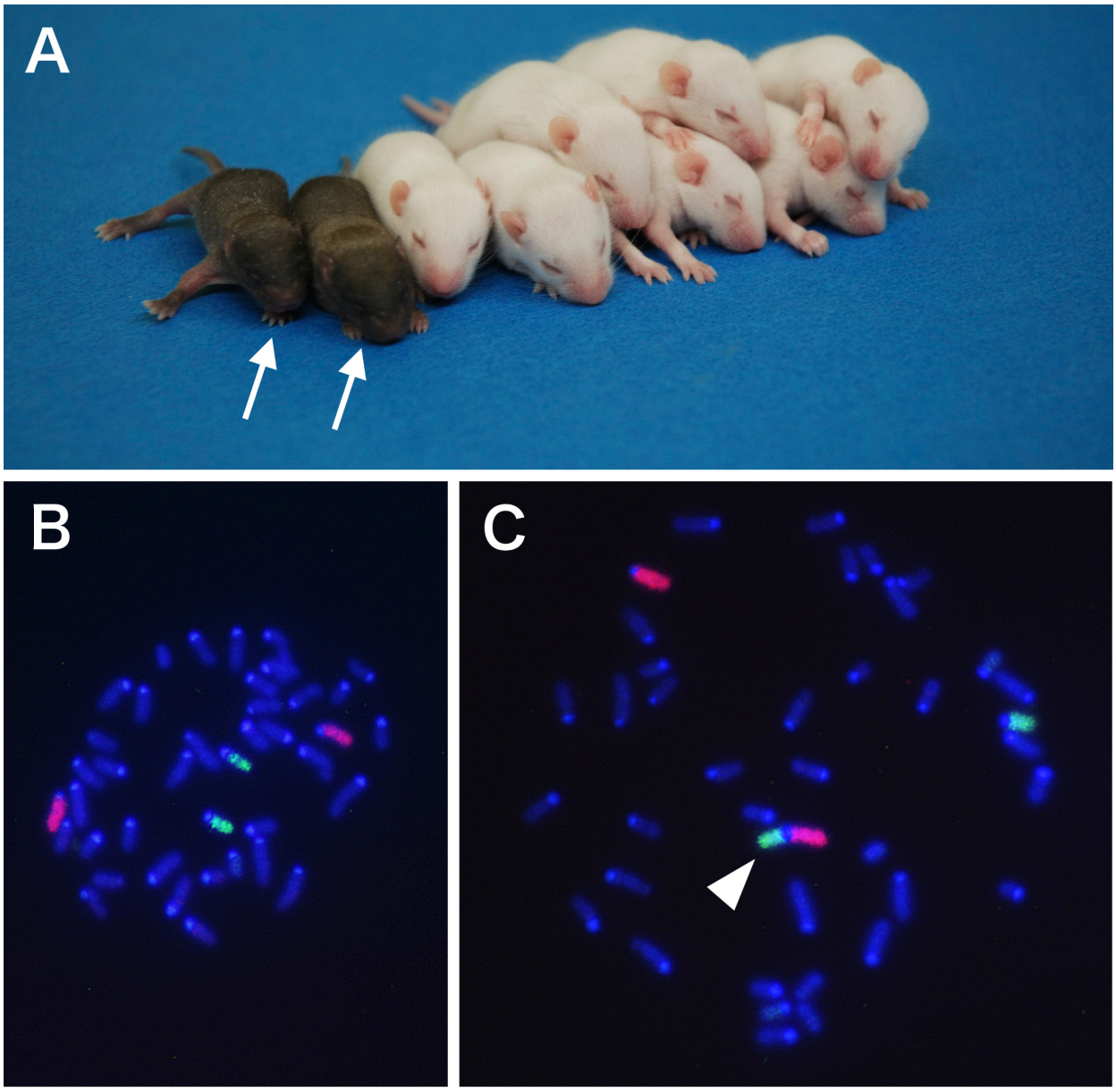


Figure 3



Supplementary Figure 1