Production of Offspring from Azoospermic Mice with Meiotic

Failure: Precise Biparental Meiosis within Halved Oocytes

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While the large volume of mammalian oocytes is necessary for embryo

development, it can lead to error-prone chromosomal segregation during

meiosis. Conversely, we hypothesized that smaller oocytes would have a

great unidentified potential to stabilize unstable meiosis and improve the

development of the resultant embryos. Here, we show that reducing

ooplasmic volume can rescue highly error-prone fertilization using

primary spermatocytes by preventing segregation errors of chromosomes

during biparental meiosis. High-resolution live-imaging analysis revealed

that erroneous chromosome segregation occurred in most (90%)

spermatocyte-injected oocytes of normal size, but could be ameliorated to

40% in halved oocytes. The birth rate improved remarkably from 1% to 19%

($P < 0.0001$). Importantly, this technique enabled the production of

offspring from azoospermic mice with spermatocyte arrest caused by

STX2 deficiency, an azoospermia factor also found in humans. Thus,

contrary to popular opinion, oocytes inherently have a strong potential for

precise meiotic divisions, which can be evoked by reduction of the

ooplasmic volume. Their potential might help rescue cases of untreatable

human azoospermia with spermatocyte arrest. (170 words)

Keywords: azoospermia; fertilization; meiosis; oocyte; spermatocyte

INTRODUCTION

Fertilization is the process whereby female and male gametes (oocytes and

spermatozoa) unite to form a zygote. From the standpoint of their genomes, the

oocyte and spermatozoon are equivalent, but their history and cell type are quite

different. Oocytes acquire their large cytoplasm (the ooplasm) during oogenesis

to store all the components necessary for embryogenesis, including organelles,

proteins, metabolites, mRNAs, and other molecules. By contrast, the

contribution of spermatozoa to zygote formation and embryonic development is

largely limited to deposition of the paternal genome and oocyte activation.

Consequently, simple injection of a spermatozoon or even the sperm head

(nucleus) into a mature oocyte results in normal fertilization, leading to embryo

development and birth of offspring (Ogura et al., 2005; Palermo et al., 1992).

Fertilization of oocytes does not even require mature sperm nuclei, because

injection of nuclei from immature spermatozoa (spermatids) is sufficient for

normal fertilization and embryo development to term (Ogura et al., 2005).

Indeed, in one clinical study, 90 babies were born following round spermatid

injection, without any significant adverse effects (Tanaka et al., 2018).

Therefore, a large ooplasm helps determine the embryo's

developmental potential. However, it is known that this feature does not always

provide benefits for development. We and others have shown that a large

ooplasm is linked to error-prone chromosomal segregation, by analyzing

high-resolution images of meiotic chromosomes in oocytes with artificially

increased or decreased ooplasmic volume (Kyogoku and Kitajima, 2017; Lane

and Jones, 2017). Thus, the evolution of a particular ooplasmic mass in a

species might have arisen as a delicate trade-off between meiotic fidelity and

post-fertilization developmental competence. In our analysis, it was clear that a

large ooplasm was detrimental, because the meiotic chromosomes showed

frequent abnormal behavior, which could have led to aneuploidy and embryonic

death. Conversely, one can postulate that a smaller ooplasm might be more

beneficial than a larger one in terms of chromosomal behavior, but there is no

evidence for this because intact oocytes undergo meiotic divisions normally

during oogenesis and fertilization in experimentally tractable animal models such

as mice.

As mentioned above, normal diploid embryos can be obtained using

spermatids because they are already haploid, as are mature spermatozoa.

However, the use of primary spermatocytes for fertilization is considered to be

ineffective because they are premeiotic germ cells. Theoretically, the

chromosomes of primary spermatocytes might be able to contribute to the

construction of diploid embryos after two meiotic divisions within oocytes.

Indeed, we and another group have reported the birth of mice following

spermatocyte injection into oocytes, but the success rates were low at 1% to 3%

per embryo transferred (Kimura et al., 1998; Ogura et al., 1998). This was mostly

caused by the death of embryos shortly after implantation. When we observed

the reconstructed oocytes at metaphase II (MII), there was a high incidence of

chromosomal aberrations (Miki et al., 2006; Ogura et al., 1998). Since then,

there have been no technical improvements in spermatocyte injection. However,

the use of primary spermatocytes for conception should be explored, given that

many cases of nonobstructive azoospermia in humans are associated with

spermatogenic arrest at the primary spermatocyte stage (Enguita-Marruedo et

al., 2019).

Based on these findings, we expected that reducing the ooplasmic volume

might improve the chromosomal integrity of spermatocyte-injected oocytes and

increase the survival rate of the resultant embryos. In this study, by employing

high-resolution live-imaging techniques, we analyzed the segregation patterns of

the maternal and paternal chromosomes within spermatocyte-injected oocytes

with or without reduction of the ooplasm. Furthermore, we examined whether

such reduction could improve the birth rate following spermatocyte injection and

whether this technology could be applied to azoospermic mice having a mutation

causing spermatocyte arrest.

RESULTS

Reduction of the Recipient Ooplasmic Volume Increases the Rate of
Normal Diploidy in Spermatocyte-injected Oocytes

Fertilization with primary spermatocytes was achieved by injecting a

spermatocyte nucleus into immature oocytes at the germinal vesicle (GV) stage

followed by arrest at the metaphase of meiosis I (MI) induced by cytochalasin D

treatment (**Figure 1A**). Here, the maternal and paternal (spermatocyte-derived)

chromosomes were synchronized, forming a single chromosomal mass. After

removal of cytochalasin D, they underwent meiotic division with protrusion of the

first polar body to reach the MII stage (**Figure 1A**). This reconstructed MII

"zygote" could be activated artificially to resume meiosis and form a one-cell

embryo having one zygotic nucleus (**Figure 1A**). To test the developmental

ability of reconstructed embryos, we transferred these MII chromosomes to

freshly prepared enucleated MII oocytes (Ogura et al., 1998).

Recipient oocytes with half the normal ooplasmic volume were prepared by

aspiration using a large glass pipette (**Movie S1**). Using these halved oocytes,

we first analyzed the chromosomal integrity of spermatocyte-injected oocytes. In

control oocytes without spermatocyte injection (i.e., oocyte chromosomes only),

the proportion of oocytes with normal chromosomes was 97% at MII (**Figure 1B**

and Table S1). In spermatocyte-injected oocytes with intact ooplasm, the

proportion of normal chromosomes was decreased significantly to 2% (1/59, $P <$

0.0001) (**Figure 1B and Table S1**). The most frequent abnormality (86%, 51/58)

was the presence of prematurely separated sister chromatids (**Figure 1B and**

Table S1). When spermatocytes were injected into half-sized oocytes, the

proportion of MII oocytes with normal chromosomes improved significantly to

13/62 (21%; $P <$ 0.005, vs the intact cytoplasm group) because of the decreased

number of separated chromatids (**Figure 1B and Table S1**). Thus, while

chromosomal normality was largely lost during meiosis I in

spermatocyte-injected oocytes, chromosomal aberrations could be prevented in

a significant proportion of oocytes by reduction of the ooplasmic mass.

Reduction of the Recipient Ooplasm Corrects the Behavior of
Spermatocyte-derived Chromosomes During Meiosis

Next, we sought to study how chromosomal behavior was influenced by the

ooplasmic volume and which of the two parental (maternal or paternal)

chromosomes was more vulnerable to the stress of biparental meiosis. The

high-resolution three-dimensional (3D) live imaging system reported in our

previous studies was employed for analyzing the chromosomal behavior during

meiosis I (Kitajima et al., 2011; Kyogoku and Kitajima, 2017). To this end, it was

essential to discriminate the origins of the chromosomes via fluorescence

microscopy. Interestingly, the paternal (spermatocyte-derived) chromosomes

could be distinguished from the maternal chromosomes by the relatively lower

fluorescent intensities of the histone H2B-mCherry marker (**Figure 2A**). Our 3D

visualization of individual chromosomal positions showed that biparental meiosis

exhibited more frequent misalignment of paternal chromosomes at late MI,

compared with the maternal chromosomes (**Figures 2B, C and Movie S2**).

Halving ooplasmic volume significantly reduced the number of misaligned

paternal chromosomes (**Figures 2B, C and Movie S3**), an effect that we

expected based on our previous observations (Kyogoku and Kitajima, 2017).

Thus, paternal chromosomes are susceptible to errors in ooplasm-hosted

biparental meiosis, which can be tuned by reducing the ooplasmic volume.

We then analyzed how biparental meiosis in normal-sized ooplasms results

in chromosomal abnormality. Our technique of complete centromere tracking

using 3D imaging (Kitajima et al., 2011; Sakakibara et al., 2015) enabled us to

demonstrate that 89% of biparental meiotic divisions showed errors in

chromosomal segregation at anaphase I (**Figures 2B, 3A and Movie S2**).

Almost all of the errors were of spermatocyte origin (**Figure 3A**). Categorization

of anaphase trajectories showed that predominant error patterns were balanced

and unbalanced predivisions (premature segregation of sister chromatids at MI)

(**Figure 3B**), consistent with our observation of separated chromatids in MII

spreads (**Figures 1B and Table S1**).

Predivisions are error patterns observed following premature separation of

bivalent chromosomes into univalents during the prometaphase and metaphase

in naturally aged oocytes (Sakakibara et al., 2015). Therefore, we carefully

analyzed the prometaphase–metaphase trajectories of the chromosomes that

underwent segregation errors in biparental meiosis. This analysis revealed that

most of the errors were preceded by premature separation of bivalent

chromosomes into univalent-like structures (**Figures 2B, 3C and Movies S2,**

S3). Importantly, decreasing (halving) the recipient ooplasm mass significantly

suppressed the premature bivalent separation of chromosomes (75% in controls

vs 31% in halved oocytes) (**Figure 3D**) and chromosome segregation errors

(89% in control vs 46% in halved oocytes) (**Figures 3A, B, and D**). Thus, the

chromosomal aberrations found in spermatocyte-injected oocytes were largely

attributable to the premature separation of spermatocyte-derived chromosomes,

and about half of such aberrations could be prevented by reducing the size of

the recipient ooplasm (**Figure 3E**).

Reduction in the Recipient Ooplasm Improved the Birth Rates Following
Spermatocyte Injection

Next, we examined whether reduction of the ooplasm volume could improve the

developmental ability of spermatocyte-derived embryos. When we reconstructed

embryos using normal-sized oocytes and transferred them into recipient

females, only 1% (1/96) developed into offspring (**Figure 4A and Table S2**),

consistent with our previous reports (Miki et al., 2006; Ogura et al., 1998). By

contrast, when we used halved oocytes, 19% (17/90) of the reconstructed

embryos developed into live offspring, achieving a nearly 20-fold improvement

(P < 0.0001, **Figure 4A and Table S2**). The pups born by this improved method

had body and placental weights within the normal ranges (**Figure S1**). We

allowed three male pups to grow into adults and confirmed that they were all

fertile by mating them with normal female mice.

Spermatocyte Injection Rescued Azoospermia Caused by Meiotic Arrest

Finally, we applied this improved spermatocyte injection method to mouse

strains with azoospermia caused by spermatogenic arrest at the primary

spermatocyte stage. If the chromosomes of spermatocytes are functionally and

structurally intact, we surmised that their normal meiotic divisions might be

induced by the meiotic machinery of recipient oocytes. We performed our

studies on *Stx2^{repro34}* mice (hereafter, *repro34* mice) that carry a mutation in the

Stx2 (syntaxin 2) gene induced by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis

(Fujiwara et al., 2013). Its human homologue, *STX2*, has been identified as a

causal factor of nonobstructive azoospermia (Nakamura et al., 2018). Both

mouse *Stx2* and human *STX2* mutations are characterized by the formation of

large syncytial spermatocytes because of their inability to maintain intercellular

bridges (Fujiwara et al., 2013; Nakamura et al., 2018) (**Figure 4B**). We

confirmed that the nuclei within these syncytial cells of *repro34* mice were

derived from spermatocytes by examining their prophase I chromosomes

following injection into MII oocytes (**Figure 4C**). We reconstructed embryos

using the nuclei collected from these syncytial spermatocytes (**Figure 4D and**

Movie S4). After 41 embryos were transferred into recipient females, five pups

(four female and one male) were born (**Figure 4E**). All of these pups carried the

point mutation in the *Stx2* gene (**Figure 4F**). We also applied this technique to

spermatocytes from *Exoc1* (exocyst complex component 1)-deficient mice that

also show syncytial spermatocytes (Osawa et al., 2021). Three pups (two female

and one male) carrying the mutation were born at term (**Figure S2**). All the eight

pups derived from *Stx2*- or *Exoc1*-deficient spermatocytes looked normal in

appearance and their body and placental weights were within normal ranges,

except for the body weight of pups from *Stx2*-deficient spermatocytes (**Figure

S1**). They grew into normal-looking adults and were proven to be fertile.

Chromosomal Analysis of Offspring Born Following Spermatocyte
Injection

As described above, all the pups born following the injection of wild-type

spermatocytes or mutant spermatocytes grew into fertile adults. We then

analyzed their chromosomal constitution in detail by multicolor fluorescence *in*

situ hybridization (FISH). Among the three male mice derived from wild-type

spermatocytes, two had a normal karyotype, but one had XYY sex

chromosomes (**Figure S3**). Among the five mice (four female and one male)

derived from *Stx2*-deficient spermatocytes, two female mice and one male

mouse were normal, but one female had an XO chromosome and another had a

shortened X chromosome (**Figure S4**). Among the three female mice derived

from *Exoc1*-deficient spermatocytes, one had an XO chromosomal

configuration. No abnormalities were found in the autosomes of the mice

examined.

DISCUSSION

DISCUSSION

Here, we addressed whether reducing the recipient ooplasm could ameliorate

the embryonic death rate caused by the meiotic errors that can occur in

mammalian oocytes. To this end, we employed an assisted fertilization system

using primary spermatocytes, which need simultaneous biparental meiosis

within oocytes: namely, meiosis with doubled chromosomes. Following

spermatocyte injection into halved oocytes, the proportion of normal

chromosomes at MII increased from 2% to 21% and the birth rate increased from

1% to 19%. These results demonstrate unequivocally that reducing the mass of

the ooplasm indeed helps to normalize chromosomal behavior, leading to better

survival of embryos to term. It would be interesting to test whether this strategy

could also correct the meiotic errors that are frequently found in oocytes from

aged female mammals (Mihajlović and FitzHarris, 2018). In humans, these

meiotic errors in oocytes are known to increase with advanced age and to

reduce conception rates significantly (El Yakoubi and Wassmann 2017; Mikwar

et al., 2020). In these errors, diverse mechanistic defects are involved, such as

defects in chromosomal cross-over formation, cohesin loss and spindle

deformation (Ma et al., 2020; Mihajlović and FitzHarris, 2018). We suspect that

reducing the mass of the ooplasm might help rescue or prevent at least some of

these defects.

The nearly 20-fold improvement in the birth rate following spermatocyte

injection into halved oocytes was much better than we expected. It is known that

meiosis in female and male mammals differs largely with respect to the

underlying molecular mechanisms and cell cycle progression patterns, such as

absence of the interphase between two meiotic divisions in female germ cells.

Consistent with this, many strains of gene knockout mice carrying mutations of

meiosis-related factors show male or female infertility (Biswas et al., 2021;

Jamsai and O'Bryan, 2011). Our findings imply that the meiotic chromosomes of

female and male germ cells have structural commonalities that allow

mechanistic interchangeability between them. Nevertheless, most chromosomal

aberrations were identified as of spermatocyte origin with a high incidence of

premature sister chromatid segregation during meiosis I. Most of these errors

were preceded by premature separation of bivalent chromosomes into

univalent-like structures. Our results reveal a novel effect of ooplasmic reduction

that can suppress premature separation of chromosomes, at least in the context

of biparental meiosis. These suggest that spermatocyte-derived chromosomes

are more vulnerable to physical or biochemical properties associated with a

large ooplasm, such as spindle size, and ooplasmic dilution of nuclear factors

(Kyogoku and Kitajima, 2017). The delayed alignment of spermatocyte-derived

chromosomes to the MI spindle (**Figures 2B and C**) might also reflect these

differences. In other words, maternal meiotic chromosomes might have evolved

special mechanisms that efficiently avoid segregation errors in a large ooplasm.

Chromosomal analysis by multicolor FISH revealed that four of the 11

spermatocyte-derived offspring carried chromosomal abnormalities that were

restricted to the sex chromosomes. There were no abnormalities in autosomes

in any of the mice analyzed. This sex-chromosome-biased chromosomal

aberration may be explained by the high embryonic lethality of autosomal

aneuploidy, which might have selected embryos with normal autosomes for

survival to term. It might also have resulted from yet undiscovered special

characteristics of sex chromosomes, especially those derived from

spermatocytes. All the abnormal patterns found in the sex chromosomes—XO,

XX with a shortened X, and XYY—can be explained by segregation errors of

spermatocyte XY chromosomes during meiosis I (see **Figures 3B and E**). It is

known that sex chromosomes are prepared to undergo meiosis later than

autosomes as they require the formation of the XY body (Kauppi et al., 2011).

Therefore, it is possible that spermatocytes that had not completed this stage

might have been selected for injection accidentally.

This study has practical implications for treating spermatogenic arrest

caused by meiotic arrest. Given the complexity of meiosis, many genes are

involved in its regulation, as revealed by mouse gene knockout models (Jamsai

and O'Bryan, 2011). Defects in some of these genes might cause failure of

meiosis and spermatogenic arrest at the primary spermatocyte stage. The

results of this study will help identify the types of meiotic arrest that can be

rescued or prevented by the spermatocyte injection technique we developed

here. Such information would provide invaluable clues for human clinical

research aiming to develop treatments for meiosis-related male infertility. In

addition, we propose another important implication of this study: at present,

complete *in vitro* gametogenesis is possible for female germ cells (Hikabe et al.,

2016), but not for male germ cells. One of the major causes of this sex-specific

difference in *in vitro* gametogenesis is the inability of male germ cells to undergo

meiosis *in vitro*. If male primordial germ cell-like cells derived from induced

pluripotent stem cells (Hayashi et al., 2011) could be cultured to form pachytene

spermatocytes, injecting them into immature oocytes as substitute gametes

might produce offspring by skipping *in vivo* male gametogenesis completely.

This could be the ultimate strategy to enable conception in cases of male

patients with germ cell loss. These scenarios could open up new methods for

treating human male infertility, although there are a number of ethical and

technical issues—for example, the high incidence of sex chromosome

abnormalities—that need to be resolved before these strategies could be used

by clinics offering assisted reproductive technology.

MATERIALS AND METHODS

Mice

All animal experiments were approved by and performed according to the

principles of the Institutional Animal Care and Use Committees at RIKEN,

Tsukuba and Kobe Branches. B6D2F1 and C57BL/6NCrSlc mice were

purchased from Japan SLC. ICR mice were purchased from CLEA, Japan.

Repro 34 mice carrying the $Stx^{repro34}$ mutation were introduced from Okayama

University. The *repro34* mutation was induced in a C57BL/6J male and

subsequently a congenic line with the C3HeB/FeJ background was created

(Akiyama et al., 2008). Germline-specific *Exoc1* conditional knockout mice were

generated by breeding $Exoc1^{tm1c(EUCOMM)Hmgu}$ mice (Skarnes et al., 2011) with

Nanos3-Cre driver mice (kindly gifted by Dr Y. Saga, RIKEN BRC RBRC02568),

which express Cre in spermatogonia (Osawa et al., 2021; Suzuki et al., 2008).

Collection of oocytes

Female B6D2F1 mice (9–12 weeks old) were injected with 7.5 IU of equine

chorionic gonadotropin (eCG, ASKA Pharmaceutical, Tokyo,Japan). Forty-four

to forty-eight hours after injection, fully grown oocytes at the GV stage were

collected from large antral follicles and released into M2 medium supplemented

with 150 μg/ml dibutyryl cyclic (dbc) AMP (Merck KGaA). After being freed from

cumulus cells by pipetting, oocytes were cultured for at least 1 hours in MEM)

Merck KGaA) supplemented with 50 μg/mL gentamicin, 0.22 mM Na-pyruvate, 1

μg/ml epidermal growth factor (EGF), 150 μg/ml dbc AMP, and 4 mg/ml bovine

serum albumin (BSA), (mMEM) (Fulka and Langerova, 2014) at 37°C in an

atmosphere of 5% CO_2 in humidified air, until micromanipulation.

Collection of primary spermatocytes

Spermatogenic cells were collected from the testes of male B6D2F1, C57BL/6N,

and ICR mice (12–16 weeks old) by a mechanical method as reported in a

previous study (Ogura and Yanagimachi, 1993). Briefly, the testes were placed

in erythrocyte-lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM EDTA; pH

7.2). After the tunica albuginea had been removed, the testes were transferred

into a cold (4°C) Dulbecco's phosphate-buffered saline (PBS) supplemented

with 5.6 mM glucose, 5.4 mM sodium lactate, and 3 mg/ml BSA (GL-PBS)

(Ogura et al., 1996). The seminiferous tubules were cut into small pieces using a

pair of fine scissors and pipetted gently to allow spermatogenic cells to be

released into the medium. The cell suspension was filtered through a 38-μm

nylon mesh and washed twice by centrifugation (200g for 4 min). After gentle

washing, the cells were resuspended in GL-PBS and stored at 4°C until

microinjection.

Micromanipulation

To make half-sized oocytes, oocytes at the GV stage were transferred to M2

medium (Merck Millipore) containing 7.5 μg/ml cytochalasin D (Merck KGaA)

and 60 mM NaCl for 10 min at 37°C. All manipulations were performed under an

inverted microscope with a Piezo-driven micromanipulator (PrimeTech). The

zona pellucida was opened by piezo drilling and one-third to half of the

ooplasmic volume was aspirated with an injection pipette (inner diameter 25 μm)

at 37°C (**Movie S1**). After manipulation, oocytes were cultured in mMEM

containing 7.5 μg/ml cytochalasin D and 40 mM NaCl at 37°C in an atmosphere

of 5% CO_2 in air. About 1–1.5 hours later, primary spermatocytes (pachytene to

diplotene stages) were injected into oocytes that were induced to arrest at the MI

stage by cytochalasin D. Oocytes were cultured in mMEM containing 7.5 μg/ml

cytochalasin D and 40 mM NaCl for 2 hours at 37°C in an atmosphere of 5%

CO_2 in humidified air. After washing in mMEM, the oocytes were cultured for 14–

17 hours until they reached the MII stage. The karyoplasts containing

chromosomes were removed and were then fused with fresh enucleated oocytes

using Sendai virus (HVJ; Ishihara Sangyo Co., Ltd.) in Hepes-buffered CZB

medium (Chatot et al., 1990) containing 7.5 μg/mL cytochalasin B. After

manipulation, the oocytes were cultured in CZB medium containing 7.5 μg/mL

cytochalasin B for 1 hours at 37°C in an atmosphere of 5% CO_2 in humidified air

until complete fusion occurred. Reconstructed oocytes were activated by

culturing them in Ca^{2+}-free CZB medium containing 8 mM $SrCl_2$ for 20 min. After

washing, the oocytes were cultured in CZB medium for 24 hours under 5% CO_2

in humidified air at 37°C.

Embryo Transfer

Embryos that reached the 2-cell stage by 24 hours were transferred into the

oviducts of Day 1 pseudopregnant ICR strain female mice (9–12 weeks old). On

day 19.5, recipient females were euthanized and their uteri were examined for

live fetuses. In some experiments, live fetuses were nursed by lactating foster

ICR strain mothers. After weaning, they were checked for fertility by mating with

ICR mice of the opposite sex.

Chromosome preparation of oocytes

The MII oocytes were treated with 0.5% actinase E (Kaken Pharmaceutical Co.)

for 5 min at room temperature to loosen the zona pellucida and then treated with

a hypotonic solution (1:1 mixture of 1.2% sodium citrate and 60% fetal bovine

serum, FBS; Merck KGaA) for 10 min at room temperature. Chromosome slides

were prepared using a gradual-fixation/air-drying method (Mikamo and

Kamiguchi, 1983). Briefly, oocytes were treated with Fixative I (methanol:acetic

acid:distilled water = 5:1:4) for 6–8 min and put onto a glass slide with a small

amount of Fixative I. Then, the oocytes were treated with Fixative II

(methanol:acetic acid = 3:1) for 2 min, followed by treatment with Fixative III

(methanol:acetic acid:distilled water = 3:3:1) for 1 min. The slides were air-dried

under conditions of 50%–60% humidity at 22–24°C. For conventional

chromosome analysis, the slides were stained with 2% Giemsa (Merck KGaA)

for 8 min. C-band staining was used to distinguish between structural

chromosome aberrations and aneuploidy (Tateno and Kamiguchi, 2007).

Chromosome analysis by multicolor fluorescence *in situ* hybridization

(FISH)

Spleens were removed under sterile conditions from mice produced by

spermatocyte injection. Lymphocytes were isolated from the spleen and

incubated in a tissue culture tube at a cell concentration of 1×10^6/ml in

RPMI1640 (Nacalai Tesque) containing lipopolysaccharide (10 μg/ml, Merck

KGaA), concanavalin A (3 μg/ml, Nacalai Tesque), 2-mercaptoethanol (50 μM,

Nacalai Tesque), and FBS (6%) at 37°C under 5% CO_2 in humidified air for 48

hours. Colcemid (KaryoMAX, Gibco) at a concentration of 0.02 μg/ml was added

to the cell suspension for the last 2 hours of culture to arrest the cell cycle at

metaphase. The cells were centrifuged at 420*g* for 5 min and resuspended in 3

ml of a hypotonic solution (0.075 M KCl). Twenty minutes later, 2 ml of Carnoy's

fixative (methanol:acetic acid = 3:1) was added to the cell suspension. Cells

were centrifuged at 420*g* for 5 min and resuspended in 5 ml of fresh Carnoy's

fixative. Centrifugations and fixations were repeated three times. Chromosome

preparations were made using a Hanabi metaphase spreader (ADSTEC). For

multicolor FISH analysis, the chromosome slides were hybridized with

21XMouse (MetaSystems) according to the manufacturer's protocol. For

denaturation of chromosomal DNA, the slides were incubated in 2 × saline

sodium buffer (SSC) at 70°C for 30 min and then treated with 0.07 M NaOH at

room temperature for 1 min. The denatured slides were washed in 0.1 × SSC

and 2 × SSC at 4 °C for 1 min each and dehydrated with a series of 70%, 95%,

and 100% ethanol. Multicolor FISH probes were denatured at 75°C for 5 min and

applied to the chromosome slides. After hybridization at 37°C for 48 hours in a

humidified chamber, the chromosome slides were treated with 0.4 × SSC at

72°C for 2 min, washed in 2 × SSC with 0.05% Tween20 (Merck KGaA) at room

temperature for 30 seconds, and rinsed in distilled water. For counterstaining,

the slides were covered by a coverslip with DAPI/Antifade (MetaSystems). The

chromosome slides were observed using fluorescent microscopy. Fluorescence

images were captured using a high-sensitive digital camera (α7s, SONY). The

images were imported into the ChromaWizard software (Auer et al., 2018) to

assign fluorescence colors to each chromosome. Based on these fluorescence

colors, the chromosome numbering was determined. Ten metaphase cells per

mouse were analyzed for karyotyping.

Chromosome analysis by Giemsa banding (G-banding)

When multicolor FISH analysis revealed a possible chromosome deletion,

additional G-band staining was performed to identify the lost part of the

chromosomes. The chromosome slides were treated with 0.025% trypsin

(FUJIFILM Wako Pure Chemical Corporation) for 2 min at room temperature,

washed in PBS, and stained with 4% Giemsa for 8 min. Deletion sites were

determined according to the band pattern nomenclature of mouse chromosomes

(Nesbitt and Francke, 1973).

Live cell imaging

Live cell imaging

After linearization of the template plasmids, mRNA was synthesized using the

mMESSAGE mMACHINE KIT (Ambion). The synthesized RNAs were stored at

−80 °C until use. The *in vitro*-transcribed mRNAs (1.2 pl of 650 ng/μl major

satellite-mClover (Miyanari et al., 2013) and 0.6 pl of 350 ng/μl H2B-mCherry)

were microinjected into oocytes. These were cultured for 1 hours and then

subjected to micromanipulation. Live cell imaging was performed as described

(Kitajima et al., 2011; Sakakibara et al., 2015), with some modifications. Briefly,

a Zeiss LSM710 or LSM880 confocal microscope equipped with a 40 ×

C-Apochromat 1.2NA water immersion objective lens (Carl Zeiss) was controlled

by a multi-position autofocus macro (Politi et al., 2018). For centromere tracking

(**Figure 3**), 19 confocal z-sections (every 1.5 μm) of 512 × 512 pixel x/y images

covering a total volume of 35.4 × 35.4 × 28.5 μm were acquired at 200-second

intervals for at least 10 hours after spermatocyte injection into oocytes

expressing major satellite-mClover and H2B-mCherry. Centromere tracking was

performed as described (Kitajima et al., 2011; Sakakibara et al., 2015). The

parental origin of chromosomes was identified by the intensities of the

chromosomes and the centromeres (lower fluorescent intensity for the

spermatocyte chromosomes) (**Figure S2A**).

Statistical analysis

Live cell imaging

After linearization of the template plasmids, mRNA was synthesized using the

mMESSAGE mMACHINE KIT (Ambion). The synthesized RNAs were stored at

−80 °C until use. The *in vitro*-transcribed mRNAs (1.2 pl of 650 ng/μl major

satellite-mClover (Miyanari et al., 2013) and 0.6 pl of 350 ng/μl H2B-mCherry)

were microinjected into oocytes. These were cultured for 1 hours and then

subjected to micromanipulation. Live cell imaging was performed as described

(Kitajima et al., 2011; Sakakibara et al., 2015), with some modifications. Briefly,

a Zeiss LSM710 or LSM880 confocal microscope equipped with a 40 ×

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parental origin of chromosomes was identified by the intensities of the

chromosomes and the centromeres (lower fluorescent intensity for the

spermatocyte chromosomes) (**Figure S2A**).

Statistical analysis

The rates of chromosomal abnormalities and embryo development were

evaluated using Fisher's exact probability test. The body and placental weights

of pups were evaluated using Student's *t*-test.

SUPPLEMENTAL INFORMATION

482   Supplemental information can be found at ..

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AUTHOR CONTRIBUTIONS

487   N.O. and A.O. conceived the project. The project was developed jointly by A.O.,

488   T.K., S.M., H.T., T.S.K., and F.S. Experiments were carried out by N.O., T.H.,

489   H.K., Y.O., Y.F., and K.I. The paper was written by N.O., T.H., H.K., T.S.K., and

490   A.O.

DECLARATION OF INTERESTS

492   The authors declare no competing interests.

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Figure 1. Fertilization with Primary Spermatocytes

(A) The scheme of construction of a diploid fertilized oocyte using a primary

spermatocyte and a GV-stage oocyte. The chromosomes of the spermatocyte

and the oocyte are intermingled at MI to form a single chromosomal mass.

(B) Chromosomal analysis of MII oocytes that had been injected with primary

spermatocytes. In the spermatocyte-injected groups, normality was improved by

reducing the ooplasm mass (*$P < 0.005$ by Fisher's exact probability test).

Arrows in the right figure indicate prematurely separated chromatids. For the

exact numbers in each case, see also **Table S1**. PB, polar body; GV, germinal

vesicle; MI, meiosis I; PN, pronuclear stage.

Figure 2. Live Imaging of Biparental Meiosis

(A) Identification of parental origin of the chromosomes was distinguishable based on H2B-mCherry fluorescent intensities (paternal chromosomes exhibit lower intensities). The z-projection image shows major satellite–mClover (centromeres, green) and H2B-mCherry (chromosomes, red). Time from anaphase onset is shown in h:min. Scale bar = 4 μm. The 3D-reconstructed image shows maternal (magenta) and paternal (cyan) chromosomes. Spots indicate centromeres.

(B) Chromosome tracking in 3D. The reconstructed images are viewed from the side of the metaphase plate. Signals are interpolated in the Z axis for visualization. White and red arrowheads, as well as red surfaces, indicate univalent-like chromosomes that underwent unbalanced predivision (premature segregation of sister chromatids). Scale bar = 4 μm.

(C) Halving the recipient ooplasmic mass rescued chromosome alignment. The numbers of misaligned chromosomes and their parental origin were determined in 3D (n = 39 and 17 oocytes). Error bars show the standard deviation. Student's t test was used to compare means. *P < 0.05. N.S., not significant.

Figure 3. Halving the Recipient Ooplasm Prevents Premature Separation of Paternal Chromosomes in Biparental Meiosis

(A) Halving the recipient ooplasm mass reduced chromosome segregation errors. Errors were determined by tracking all chromosomes at anaphase (n = 39 and 17 oocytes) (See also **Figure 2B**). The parental origin of errors is shown. Ooplasmic halving significantly reduced the rate of errors (**P < 0.01).

(B) Predivision was predominant in biparental meiosis. Chromosome segregation error patterns were categorized based on anaphase trajectories: nondisjunction (0:4 segregation), balanced predivision (2:2 sister chromatid segregation), unbalanced predivision (1:3 segregation including sister chromatid segregation), and complex patterns including predivision (multiple errors including sister chromatid segregation). Chromosome breakages (chromosomes lacking centromeres) were also observed.

(C) Univalent-like chromosomes. Images were 3D-reconstructed as in Fig. 2B and viewed from the top of the metaphase plate. Red surfaces with white arrowheads indicate univalent-like chromosomes. Scale bar = 3 μm.

(D) Halving the ooplasm volume suppressed the premature separation of paternal chromosomes. Oocytes were categorized based on whether the chromosomes exhibited premature separation into univalent-like structures prior to segregation errors.

(E) Summary of biparental meiosis. In normal-sized oocytes this frequently exhibits premature separation of paternal chromosomes into univalent-like structures. These chromosomes undergo predivision (premature segregation of sister chromatids), and thus result in separated chromatids in MII oocytes. Halving the ooplasmic volume reduced such errors.

A

B

C

D

E

Birth of mice from *Stx2*^{*repro34*} mutant spermatocytes

No. oocytes cultured	No. (%) 2-cells	No. transferred	No. (%) implanted	No. (%) offspring	With mutation
133	75 (56)	41	19 (46)	5 (12)	5

Results from two replicated experiments

Fig. 4

Figure 4. Birth of Spermatocyte-derived Offspring Following Embryo

Transfer

(A) Mouse pups born following spermatocyte injection (left) and the birth rates

following embryo transfer (right). For detailed results, see also **Table S2**.

(B) Histology of the testis of a *Stx2^{repro34}* mouse. Arrowheads indicate

multinucleated cells containing spermatocyte-like nuclei. There are no

spermatids or spermatozoa. Bar = 50 μm.

(C) An MII oocyte injected with a putative spermatocyte nucleus from a

multinucleated cell in a *Stx2^{repro34}* mouse testis, showing the typical paired

meiotic chromosomes. Bar = 20 μm.

(D) A multinucleated cell isolated from a *Stx2^{repro34}* mouse testis, showing four

nuclei. Differential interference contrast (left) and Hoechst-stained (right)

images. Bar = 10 μm.

(E) (left) mouse pups born following microinjection with putative primary

spermatocyte nuclei isolated from multinucleated cells; (right) birth rate of pups

following *Stx2^{repro34}* spermatocyte microinjection.

(F) Genomic sequencing confirming the origin of pups from *Stx2^{repro34}*

spermatocytes. Arrows indicate the expected point mutation of *Stx2^{repro34}*. Y

indicates a hybrid status with T and C bases.