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Transfer of a Mouse Artificial Chromosome into Spermatogonial Stem Cells Generates Transchromosomic Mice

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SUMMARY

The introduction of megabase-sized large DNA fragments into the germline has been a difficult task. Although microcell-mediated chromosome transfer into mouse embryonic stem cells (ESCs) allows the production of transchromosomic mice, ESCs have unstable karyotypes and germline transmission is unreliable by chimera formation. As spermatogonial stem cells (SSCs) are the only stem cells in the germline, they represent an attractive target for germline modification. Here, we report successful transfer of a mouse artificial chromosome (MAC) into mouse germline stem cells (GSCs), cultured spermatogonia enriched for SSCs. MAC-transferred GSCs maintained the host karyotype and MAC more stably than ESCs, which have significant variation in chromosome number. Moreover, MAC-transferred GSCs produced transchromosomic mice following microinjection into the seminiferous tubules of infertile recipients. Successful transfer of MACs to GSCs overcomes the problems associated with ESC-mediated germline transmission and provides new possibilities in germline modification.

INTRODUCTION

Spermatogonial stem cells (SSCs) divide continuously in the seminiferous tubules and provide the foundation for spermatogenesis for the lifespan of male animals (de Rooij and Russell, 2000; Meistrich and van Beek, 1993). SSCs are thought to reside in a special microenvironment called the niche and undergo self-renewal divisions in response to several cytokines, including glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2). As SSCs are the only stem cells in the germline that have self-renewal potential, they are an attractive target for germline modification. Unlike embryonic stem cells (ESCs) that are microinjected into blastocysts for germline transmission, SSCs are capable of reinitiating spermatogenesis by microinjection into the seminiferous tubules of infertile animals. Transplanted SSCs reinitiate spermatogenesis and eventually produce donor cell-derived offspring (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994).

As the proportion of SSCs in the testis is very low (estimated to be ~0.02%–0.03% of the total germ cell population) (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993), genetic manipulation of SSCs has been a difficult task. However, the development of an SSC culture system allowed *in vitro* propagation of SSCs for more than

2 years. The cultured cells, designated germline stem cells (GSCs), can be propagated in the presence of GDNF and FGF2, and appear as grape-like clusters of cells (Kanatsu-Shinohara et al., 2003). Moreover, when transplanted into the seminiferous tubules they produce offspring even after 2 years of culture (Kanatsu-Shinohara et al., 2005b). Using this system, we and others produced knockout mice and rats by genetic selection of transfected clones and subsequent transplantation (Chapman et al., 2015; Kanatsu-Shinohara et al., 2006; Sato et al., 2015; Wu et al., 2015). Thus, GSCs provide an alternative to ESCs for germline modification.

To date, genetic manipulation of SSCs has been carried out using plasmid and virus vectors. Recipient males transplanted with SSCs transduced with either type of vector sired genetically modified offspring (Kanatsu-Shinohara et al., 2005a; Nagano et al., 2001). Although these vectors allow efficient genetic manipulation, one problem associated with current genetic manipulation techniques is the limited size of the transgene. This is particularly true for virus vectors (Thomas et al., 2003). In addition, integration of the transgene may disrupt endogenous genes, which may cause insertional mutagenesis. Random integration also causes variation in transgene expression depending on the integration site. In this context, genetic manipulation with mammalian chromosome-based vectors is an

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attractive approach because mammalian artificial chromosomes do not integrate in the host genome and can express a large transgene in a physiologically regulated manner in host cells (Kazuki and Oshimura, 2011; Oshimura et al., 2015). This technique has been used not only for studies of cancer, genomic imprinting, and stem cell reprogramming but also for production of mouse models of human diseases.

Germline transmission of a mammalian-derived chromosomal vector was first reported 20 years ago by microcell-mediated chromosome transfer (MMCT) using mouse ESCs (Tomizuka et al., 1997). Surprisingly, human chromosome fragments (hCFs) could pass through meiotic division in the germline of chimeric mice and were transmitted to the next generation. Based on these observations, ESCs have been used to transfer chromosomal vectors to produce transchromosomic (Tc) mice. As it is not possible to microinject hCFs into oocytes to produce Tc mice, the ESC-based approach is currently used for introducing large DNA fragments into the germline, and hCF transfer has been used in many previous studies. For example, mouse ESCs with human chromosome 21 were used to produce a mouse model of Down's syndrome (O'Doherty et al., 2006; Shinohara et al., 2001). While this approach based on ESC manipulation has proved useful, it is widely known that ESCs are unstable in their karyotype and DNA methylation patterns (Dean et al., 1998; Liu et al., 1997; Longo et al., 1997). Therefore, chromosome-transferred ESCs often fail to undergo germline transmission after genetic selection or maintenance of ESCs, and the retention rates of mammalian-derived chromosomes in ESCs are quite variable (Harrington et al., 1997; Kazuki and Oshimura, 2011; Mandegar et al., 2011). Therefore, there is clearly a need to develop new techniques for the introduction and maintenance of large DNA fragments in the germline.

In this study, we used mouse GSCs for chromosomal transfer. Despite extensive proliferation *in vitro*, mouse GSCs were shown to maintain 40 chromosomes and stable androgenetic DNA methylation patterns (Kanatsu-Shinohara et al., 2005b), which suggested that GSCs may be a more suitable vehicle for chromosomal vectors than ESCs. Although initial attempts to establish GSC clones with hCFs by conventional MMCT failed, a mouse artificial chromosome (MAC) vector derived from mouse chromosome 11 was transferred successfully into GSCs with the retro-MMCT method, which enabled highly efficient transfer of chromosomes into many mouse and human cell lines (Suzuki et al., 2016). MAC-transferred GSCs underwent germline transmission and produced Tc mice. The use of MACs for GSC manipulation will allow new experimental strategies not only for understanding the biology of SSCs and spermatogenic cells but also for generating humanized animals and human disease models.

RESULTS

### Transfer of a MAC into Mouse GSCs by Retro-MMCT

Based on the successful transfer of hCFs into mouse ESCs in previous studies (Tomizuka et al., 1997), we attempted to transfer hCFs into mouse GSCs. In the first set of experiments, the conventional polyethylene glycol-mediated MMCT (PEG-MMCT) method was employed to transfer hCFs into mouse GSCs. After PEG-MMCT, cells were cultured with G418 on neo-resistant mouse embryonic fibroblast feeder cells (MEFs) to obtain cells containing hCFs. Despite repeated attempts, however, we obtained only a few clones that were resistant to low-dose G418 treatment, and none contained hCFs as determined by cytogenetic analysis.

To overcome this problem, we used a MAC as a chromosome donor vector and employed the retro-MMCT method to transfer the MAC in the next set of experiments (Figure 1). The retro-MMCT method allowed transfer of a chromosomal vector into NIH3T3 cells with 26.5-fold greater efficiency than the PEG-MMCT method (Suzuki et al., 2016). In addition, because the MAC vector was constructed using normal mouse chromosome 11 (Takiguchi et al., 2014), we reasoned that MAC may be maintained more stably in GSCs because of its similarity to the endogenous genome. The MAC vector contained not only the G418-resistant gene but also the *Egfp* gene (Figure 1). In contrast to the first set of experiments, colonies of G418-resistant MAC-transferred cells were readily obtained in all four separate experiments (Figure 2A).

In total, we established four different GSC lines, all of which were analyzed for their karyotype. Cytogenetic analysis showed that all MAC-transferred GSCs contained a single MAC in addition to the endogenous 40 chromosomes (Figure 2B). Flow-cytometric analysis confirmed that all MAC-transferred GSCs expressed strong EGFP fluorescence (Figure 2C). These results showed that GSCs can be transferred with MACs.

Phenotypic Analysis of MAC-Transferred GSCs

To determine whether MAC transfer influences the phenotype of GSCs, we first examined the expression of cell-surface markers by flow cytometry (Figure 3A). We used antibodies against EPCAM, CDH1, ITGA6, ITGB1, CD9, and GFRA1, all of which are expressed on SSCs (Kanatsu-Shinohara and Shinohara, 2013). We also examined KIT, which is expressed in differentiating spermatogonia *in vivo*. GSCs transfected with an *Egfp*-expressing plasmid were used as a control. Comparison of MAC-transferred clones with *Egfp*-transfected GSCs indicated that CDH1 expression was significantly downregulated in MAC-transferred GSCs. We were unable to detect significant differences in the expression levels of the other cell-surface markers examined. We also carried out RT-PCR analysis of genes
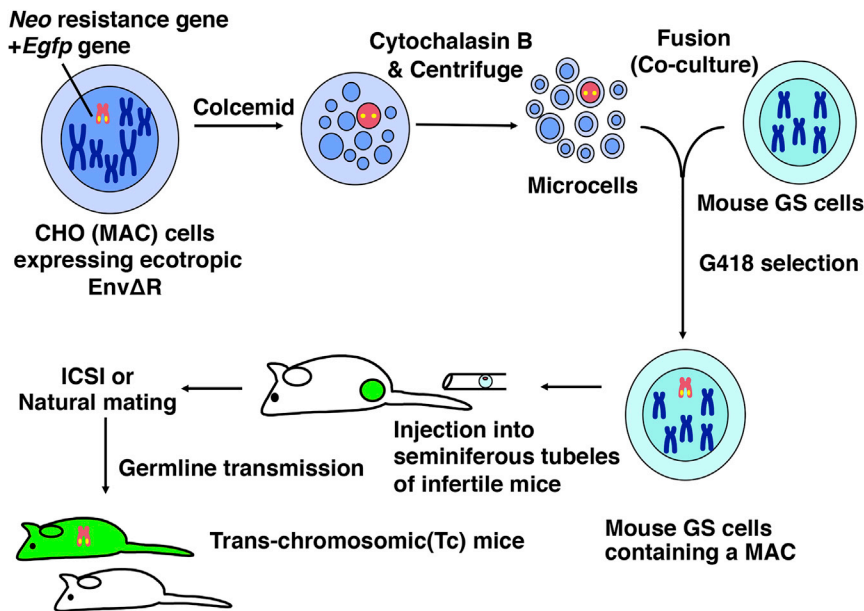
Figure 1. Experimental Procedure
GSCs were fused *in vitro* with microcells prepared from ecotropic EnvΔR-expressing CHO (MAC1) cells. The MAC-transferred GSCs were cultured on G418-resistant MEFs. G418-resistant cells were analyzed for their karyotype. Offspring were analyzed for the presence of MACs.

expressed in undifferentiated spermatogonia, including *Zbtb16* and *Neurog3* (Figure 3B). While *Bcl6b* and *Etv5* were downregulated, *Nanos2* was upregulated. Real-time PCR analysis confirmed the changes in expression levels of these genes (Figure 3C).

In addition to changes in cell phenotype, MAC-transferred GSCs also showed more active proliferation than control cells that had been transfected with a plasmid vector. While control cells expanded by 7.4-fold during 6 days, MAC-transferred GSCs expanded by 12.3-fold during the same period (Figure 3D). Consistent with this observation, we characterized two of the MAC-transferred GSC clones, and found increases in the frequency of MKI67$^+$ cells (Figure 3E). However, we did not find apparent differences in cell or colony morphology compared with control cells (Figure 2A), suggesting that the GSCs were not transformed by MAC transfer.

As MAC-transferred GSCs proliferated more actively, we also examined the expression of cell-cycle-related genes, including *Cdkn1a*, *Cdkn1b*, *Ccnd1*, *Ccnd2*, and *Ccnd3*. Although cyclin gene expression levels did not change significantly, *Cdkn1b* was upregulated (Figures 3B and 3C). MAC-transferred GSCs showed enhanced TUNEL staining, indicating that these cells undergo more extensive apoptosis (Figure 3F). These observations suggested that MAC transfer results in enhanced proliferation and apoptosis of GSCs.

Karyotype Stability of MAC-Transferred GSCs

One of the problems with ESCs is the instability of the karyotype (Liu et al., 1997; Longo et al., 1997). In contrast, the karyotype of GSCs is very stable even after long-term

culture or genetic selection (Kanatsu-Shinohara et al., 2005b, 2006). However, it was considered possible that GSCs may not accept exogenous chromosomes and only maintain the endogenous 40 chromosomes. To examine this issue, we compared the number of endogenous chromosomes and MACs after culture of MAC-transferred GSCs (Table 1). We used ESCs that had been transferred with the same MAC for comparison of chromosomal stability (Takiguchi et al., 2014). The ESC line, TT2F with the 39, XO karyotype is a derivative of the male-derived ESC line, TT2 with the 40, XY karyotype (Uchida et al., 1995). It was previously reported that hCFs transferred into TT2F can undergo more efficient germline transmission than those transferred into TT2 (Tomizuka et al., 1997; Uchida et al., 1995). At culture initiation, all GSCs contained a single MAC, and 75%–90% of the cells had the 40, XY karyotype in the host chromosome. Thus, the modal karyotype of the three GSC lines was 41, XY, +MAC. On the other hand, all three ESC lines contained one to two copies of the MAC in more than 90% of the cells at initiation of culture, and >70% of the cells had the 39, XO karyotype in the host chromosome, which was of original ESCs (Uchida et al., 1995). Thus, the modal karyotype of the ESCs was 40, XO, +MAC or 41, XO, +MAC, +MAC. We cultured three different lines of each cell type without G418 for 50 population doublings, which took ~84 and ~30 days for GSCs and ESCs, respectively.

Even after consecutive culture, EGFP fluorescence levels in the GSCs did not change significantly after 50 doublings, suggesting stable maintenance of MAC in GSCs. Consistent with this observation, karyotype analysis of the cells showed that >95% of the GSCs stably maintained the
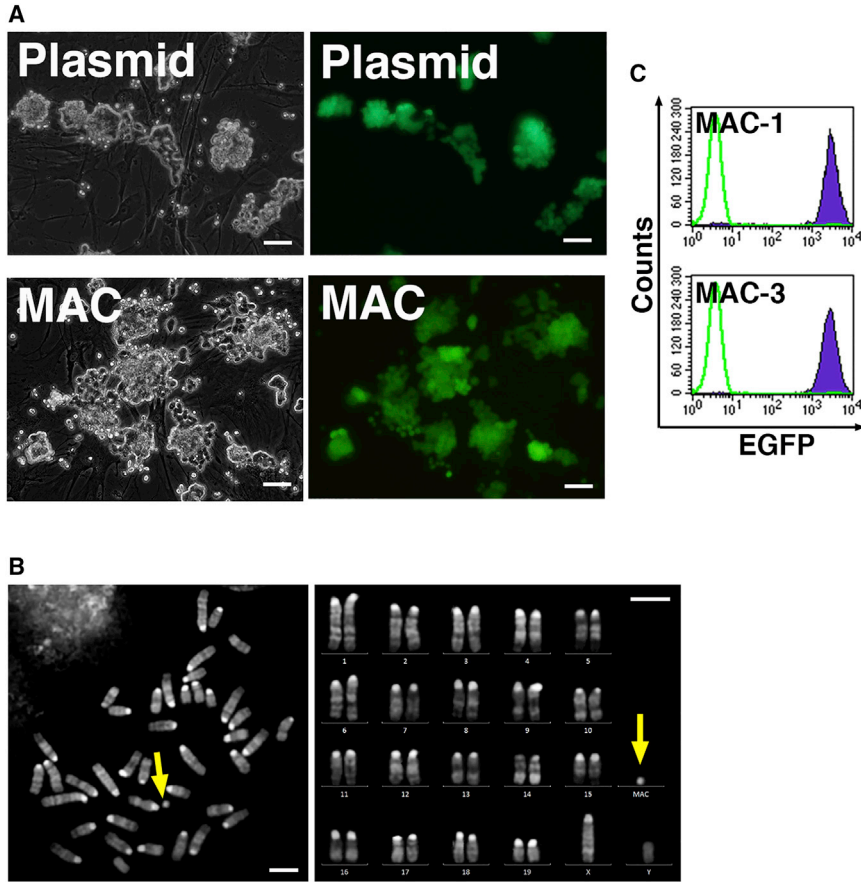
Figure 2. Analysis of GS Microcell Hybrids Containing MACs

(A) Appearance of MAC-transferred GSCs. Scale bars, 50 μm.

(B) Metaphase spread of GSCs with one copy of the MAC. Arrows indicate the MAC. Scale bars, 5 μm.

(C) Flow-cytometric analysis of EGFP fluorescence.

MAC and >80% of the cells were 41, XY, +MAC. In contrast, ESCs showed a significant variation in the karyotype. All three lines became aneuploid after culture, and only 10%–20% of the cells had the 39, XO karyotype in the host chromosome, which is the karyotype of the parental ESCs. Although one of the lines contained the MAC in all cells, two other lines showed decreased retention rates with only 70%–75% of the cells containing the MAC. These results confirmed the unstable karyotype of ESCs and suggested that GSCs are superior as a vehicle for MAC propagation.

Functional Analysis and Germline Transmission of MAC-Transferred GSCs

As SSCs account for only 1%–2% of GSCs and the slightly modified cell phenotype suggested changes in SSC concentration by MAC transfer (Kanatsu-Shinohara and Shinohara, 2013), we carried out spermatogonial transplantation (Brinster and Zimmermann, 1994). We chose one of the MAC-transferred GSC lines, which was cultured *in vitro* for 44 days. After dissociation into single cells by digestion with trypsin, cells were microinjected into the seminiferous tubules of infertile mice. GSCs that had been trans-

fected with the *Egfp*-expressing plasmid were used as controls. Analysis of transplants showed colonization of MAC-transfected GSCs and 83.3 ± 28.9 colonies per 10^5 transplanted cells (n = 9) (Figures 4A and 4B). This was comparable with those produced by control GSCs, which produced 105.0 ± 26.9 colonies per 10^5 cells (n = 9).

Because these results confirmed SSC activity of GSCs, we set out to produce Tc mice. All three lines of MAC-transferred GSCs were transplanted into infertile mice. Two months after transplantation, one of the recipient testes was collected to assess the degree of spermatogenesis. Immunohistochemical analysis of recipient testes showed normal differentiation of transplanted MAC-transferred GSCs, and peanut agglutinin (PNA)-expressing haploid cells were found (Figure 4C). No tumors were found in the recipient testes despite extensive proliferation *in vitro*. These results suggested that MAC transfer does not influence spermatogonial differentiation.

Three months after transplantation, three recipient mice were euthanized and the testes collected for microinsemination to produce offspring from MAC-transferred GSCs. The testes were refrigerated overnight before microinsemination on the next day. To recover spermatogenic cells, we
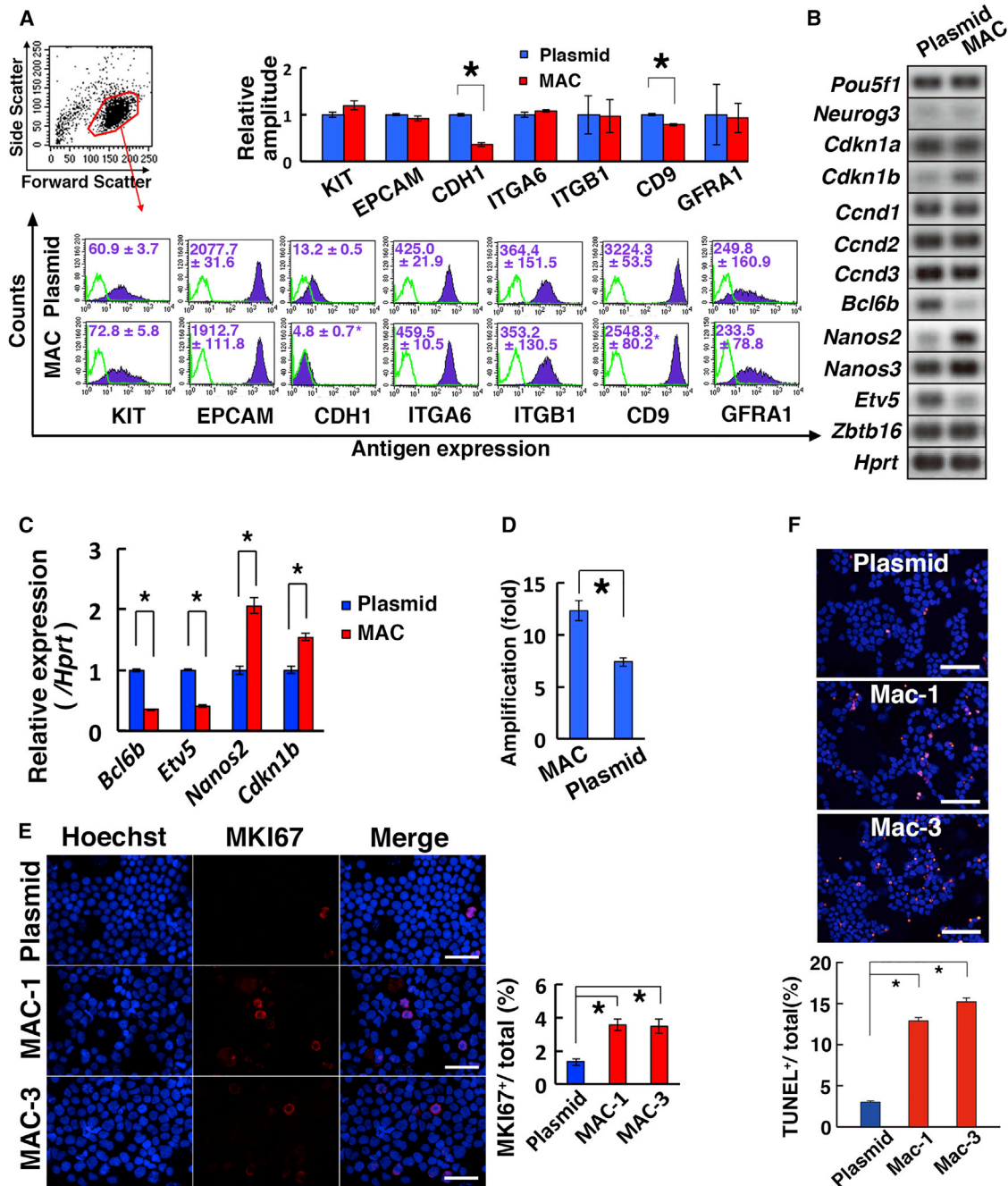
Figure 3. Phenotypic Analysis of MAC-Transferred GSCs

(A) Flow cytometric analysis of cell-surface markers (n = 3). Results of three independent experiments.

(B) RT-PCR analysis of spermatogonia markers (n = 4).

(C) Real-time PCR analysis. Results of four independent experiments.

(D) Enhanced proliferation of MAC-transferred GSCs (n = 6). Cells were cultured for 6 days. Results of six independent experiments.

(E) Immunostaining of GSC culture using anti-MKI67 antibody. Results of five independent experiments (n = 5).

(F) TUNEL staining. Results of five independent experiments (n = 5).

Counterstain: Hoechst 33342 (E and F). Scale bars, 20 μm (E and F). *p < 0.05. See also Tables S1 and S2.

Table 1. Stability of Host Chromosomes and MAC Retention Rate after Culture

Population Doubling	Cell Line	Copy Number of MAC												MAC Retention Rate (%)	Stability of Host Chromosomes[a] (%)	N	Modal Karyotype
		0			1					2							
		≤38	39	40	≤38	39	40	41	≥42	≤38	39	40	41				
0	ES MAC-4	0	1	0	1	16	1	0	0	1	0	0	0	95	85	20	40, XO, +MAC
0	ES MAC-23	0	0	0	2	7	2	0	0	0	9	0	0	100	80	20	41, XO, +MAC, +MAC
0	ES MAC-26	1	1	0	4	13	1	0	0	0	0	0	0	90	70	20	40, XO, +MAC
25	ES MAC-4	0	5	0	1	11	3	0	0	0	0	0	0	75	80	20	40, XO, +MAC
25	ES MAC-23	0	0	0	0	4	12	2	0	0	1	1	0	100	25	20	41, XO, +MAC
25	ES MAC-26	0	2	1	0	10	3	3	0	0	1	0	0	85	65	20	40, XO, +MAC
50	ES MAC-4	0	1	2	0	1	5	0	1	0	0	0	0	70	20	10	41, XO, +MAC
50	ES MAC-23	0	0	0	0	2	15	3	0	0	0	0	0	100	10	20	41, XO, +MAC
50	ES MAC-26	0	0	5	0	2	10	2	0	0	0	1	0	75	10	20	41, XO, +MAC
0	GS MAC-1	0	0	0	0	2	18	0	0	0	0	0	0	100	90	20	41, XY, +MAC
0	GS MAC-2	0	0	0	0	4	15	1	0	0	0	0	0	100	75	20	41, XY, +MAC
0	GS MAC-3	0	0	0	0	2	17	1	0	0	0	0	0	100	85	20	41, XY, +MAC
25	GS MAC-1	0	0	1	0	0	19	0	0	0	0	0	0	95	100	20	41, XY, +MAC
25	GS MAC-2	0	0	1	0	0	18	1	0	0	0	0	0	95	95	20	41, XY, +MAC
25	GS MAC-3	0	0	0	0	1	19	0	0	0	0	0	0	100	95	20	41, XY, +MAC
50	GS MAC-1	0	0	1	0	0	15	4	0	0	0	0	0	95	80	20	41, XY, +MAC
50	GS MAC-2	0	0	0	0	0	20	0	0	0	0	0	0	100	95	20	41, XY, +MAC
50	GS MAC-3	0	0	0	0	0	20	0	0	0	0	0	0	100	95	20	41, XY, +MAC

[a]The karyotype of host ES and GSCs was 39, XO and 40, XY, respectively. The ratio shows alteration of host karyotype excluding MAC.

dissected and dissociated tubules with EGFP fluorescence by repeated pipetting. We collected elongated spermatids and spermatozoa, which were microinjected into oocytes. A total of 135 embryos were constructed and 88 two-cell embryos were transferred into the oviducts of pseudopregnant females (Table 2). All females produced offspring (total of 27 offspring; 9 males and 18 females) (Figure 4D). We euthanized one of the female offspring and analyzed EGFP expression from the MAC. Analysis of the offspring showed variable levels of fluorescence in different parts of the body, including the brain, heart, intestine, kidney, liver, lung, skeletal muscle, spleen, ovary, and thymus (Figure 4E). Multicolor fluorescent *in situ* hybridization (mFISH) analysis of bone marrow cells confirmed the independent presence of MACs without integration into the host chromosome (Figure 4F). In addition to microinsemination experiments, we were also able to obtain offspring from recipient mice by natural mating. As we used busulfantreated recipients, it is possible that they may have been born from endogenous spermatozoa of busulfan-treated mice. However, 7 of 16 offspring showed EGFP fluorescence under UV light, which confirmed the donor cell origin (Figure 4G).

To confirm germline transmission of Tc mice, we used F1 offspring born after microinsemination and tested their fertility. Both male and female F1 offspring were able to produce Tc offspring. Taken together, these results showed that Tc mice produced from MAC-transferred GSCs were fertile.

DISCUSSION

Manipulation of the germline has been mostly limited to cells found in females: oocytes, fertilized eggs, or blastocysts. Although oocyte/egg manipulation can be applied to a wide range of animals, their genetic manipulation has been limited due to their small numbers and significant
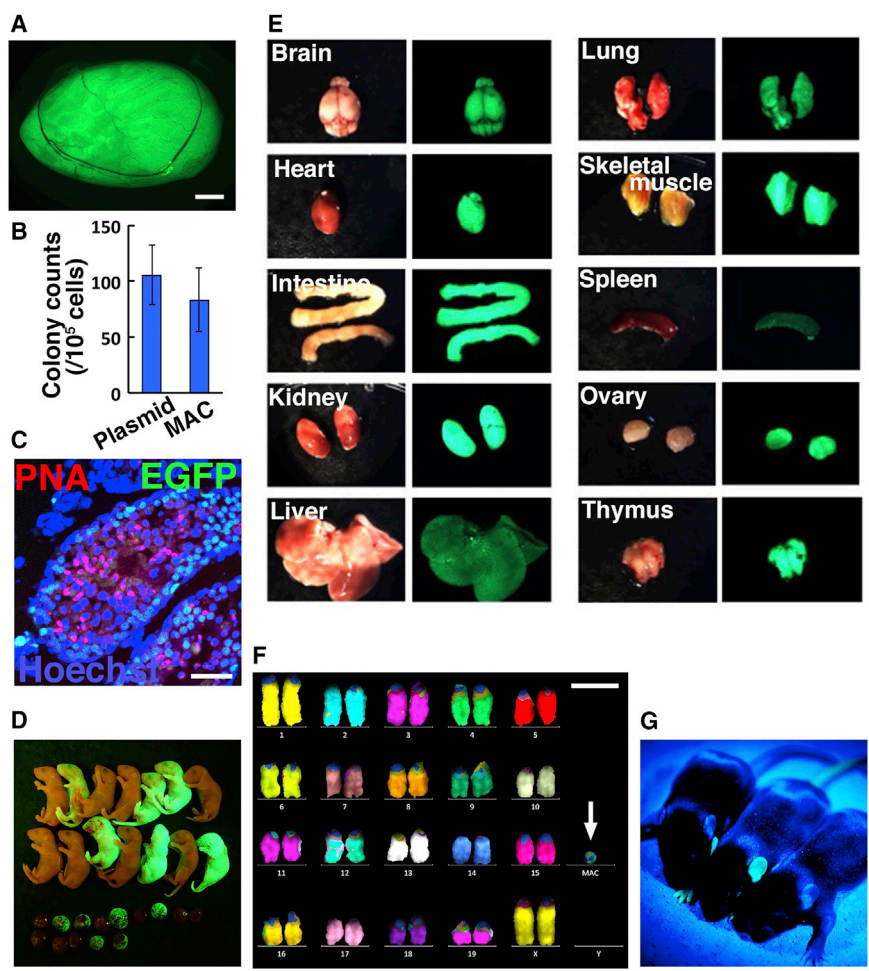
Figure 4. Functional Analysis of MAC-Transferred GSCs

(A) Macroscopic appearance of a recipient testis. Green fluorescence indicates colonies originating from transplanted GSCs.

(B) Colony counts (n = 9). Results of four transplantation experiments.

(C) Lectin staining of a recipient testis showing normal-appearing spermatogenesis.

(D) Offspring born from the transplanted GSCs, showing fluorescence under UV light.

(E) EGFP expression in various organs in the offspring.

(F) mFISH analysis of bone marrow cells from offspring. Arrow indicates MAC.

(G) Offspring born by natural mating.

Counterstain: Hoechst 33342 (C). Scale bars, 1 mm (A), 50 μm (C), and 5 μm (F). See also Table S1.

variation in microinjection efficiency. In mice, ESCs, which are derived from the inner cell mass of the blastocyst, are widely used because they proliferate actively for precise genetic manipulation. However, ESCs often show genetic and epigenetic abnormalities. In contrast to traditional approaches based on oocytes/eggs or ESCs, SSCs derived from the male testes may facilitate the development of new means of manipulation. Genetic modification of SSCs was first reported by retroviral infection, with approximately 5%–10% of offspring carrying the transgene by natural mating of recipient mice (Nagano et al., 2001). Although transgenes were randomly integrated into the host genome in these experiments, gene targeting and genetic selection techniques improved the transgenic production efficiency to 50% and allowed the introduction of site-specific mutations in SSCs (Kanatsu-Shinohara et al., 2006). More recently the CRISPR/Cas9 system has been applied to SSCs, and knockout mice and rats have been generated (Chapman et al., 2015; Sato et al., 2015; Wu et al., 2015). While these technical developments allowed sophisticated genetic manipulation of SSCs,

mammalian chromosomal vectors have several unique advantages over previous approaches because they can stably express large megabase-sized transgenes at a stable expression level without integrating into the host genome. As such large DNA fragments cannot be transferred into eggs via MMCT, ESCs have been used in the transfer of chromosome vectors, but the feasibility of mammalian chromosomal vectors in SSCs has not been explored.

Our initial attempts to introduce mammalian artificial chromosomes failed when we used hCFs. Due to previous success with mouse ESCs in the production of Tc mice, we initially thought it reasonable to use the same chromosomal vector for MMCT into GSCs. However, despite repeated attempts, we were unable to obtain any clones after conventional PEG-MMCT using hCFs. This problem was resolved by addressing the transfer method and the type of chromosome vector used. The retro-MMCT method uses the envelope protein of murine leukemia virus as a fusogen, which enables highly efficient chromosome transfer from donor Chinese hamster ovary (CHO) cells to murine leukemia virus-permissive recipient cells (Suzuki et al.,

Table 2. *In Vitro* Microinsemination Using Spermatogenic Cells Regenerated in Recipient Mouse Testes

Recipient Mice	Type of Microinsemination	No. of Embryos Cultured	No. of Embryos Transferred (%)	No. of Embryos Implanted (%)	No. of Pups (%)	EGFP Fluorescence (%)
20009	ICSI/ELSI	32	24 (75.0)	16 (50.0)	11 (34.4)	3 (9.4)
24623	ICSI	35	13 (37.1)	9 (25.7)	2 (5.7)	1 (2.9)
25005	ICSI	68	29 (42.6)	21 (30.9)	14 (20.6)	6 (8.8)[a]

Elongated spermatids or sperm were collected from three recipient mice. Embryos were cultured for 24 hr and transferred at the 2-cell stage. ICSI, intracytoplasmic sperm injection; ELSI, elongated spermatid injection.
[a]One of the offspring was dead at the time of birth.

2016). Several MAC vectors with major and minor satellites of mouse origin have been developed by several groups (Shen et al., 2000; Takiguchi et al., 2014; Telenius et al., 1999). For example, the MAC vector, ST1, was not maintained stably in the tissue of the offspring; while a high retention rate was found in the liver and prostate, and testis and kidney showed retention below 60% (Shen et al., 2000). The MAC vector used in this study, MAC1, was originally derived from the normal mouse chromosome 11 and possesses a native mouse centrosome (Takiguchi et al., 2014). When MAC1 was introduced into mice via ESCs, it was more stably maintained in mouse tissues compared with previously described mammalian artificial chromosomal vectors, and MAC1 was stably maintained at least in F8 offspring (Kazuki et al., 2013), showing remarkable stability during germline transmission. Thus, we postulated that the appropriate combination of transfer method and type of chromosomal vector would improve the transfer of exogenous chromosomes into GSCs.

The success of MAC transfer into GSCs was clearly demonstrated by *in vitro* drug selection and stable EGFP fluorescence without apparent abnormalities in colony morphology. However, the MAC-transferred GSCs not only showed decreased CDH1 expression but also proliferated more actively with an increased proportion of apoptotic cells. To the best of our knowledge, there have been no previous reports of similar growth promotion and apoptosis following transfer of mammalian artificial chromosome vectors. This occurred although the MAC contained virtually no endogenous genes. This effect of the MAC appears somewhat similar to *Myc* transfection, which also promoted proliferation and apoptosis (Kanatsu-Shinohara et al., 2014a, 2016; Morimoto et al., 2012). Although it is not yet clear why MAC transfer promoted proliferation and apoptosis, we speculated that cells that received the MAC may have expressed higher levels of cationic amino acid transporter 1 (SLC7A1), which is required for successful fusion. It is possible that GSCs expressing higher levels of SLC7A1 proliferate more actively than the other cells because this molecule transports amino acids, such as arginine and lysine, which may stimulate GSC proliferation and has been suggested to play a role in regeneration of the liver (Aulak et al., 1996). Further studies will be required to determine why MAC transfer resulted in enhanced cell proliferation, which may be useful to improve relatively slow GSC proliferation.

The most important finding of this study was the marked contrast between GSCs and ESCs in the maintenance of MACs and endogenous chromosome number. Instability of ESC karyotype has often been noted in both mouse and human ESCs (Draper et al., 2004; Liu et al., 1997; Longo et al., 1997), and this is a critical problem of these cells for germline engineering and regenerative medicine. Consistent with previous observations, the results of the present study demonstrated the karyotype instability of MAC-transferred ESCs. In this sense, stable maintenance of both MACs and endogenous chromosomes in GSCs is very attractive for germline transgene expression. In particular, because Tc mice can be generated directly in the F1 generation by fertilization between MAC-bearing sperm and wild-type oocytes, it is possible to obtain Tc mice in the F1 generation. In contrast, the ESC-based approach requires mating of chimeric animals, which may not always contain the chromosomal vector in the germline. By genetically modifying the MAC components and comparing the efficiency of MAC maintenance, our approach based on GSCs will also be useful for understanding the molecular machinery of the chromosome maintenance system in GSCs, which appears to be different from that of ESCs.

The next important goal is to apply MACs to GSCs from other species. Rat SSCs are the next obvious target because they can undergo germline transmission in both mouse and rat testes (Hamra et al., 2005; Shinohara et al., 2006). While rat ESCs are also derived from the inner cell mass in a manner similar to mouse ESCs, the technical difficulties associated with culture and drug selection have limited their usage (Tong et al., 2010). As rats are widely used in physiology and brain science, the application of MACs to rat GSCs would provide new experimental possibilities. However, rat GSCs are also more difficult to handle

because they proliferate more slowly and are sensitive to drug selection, and we were unable to obtain offspring from GSCs after homologous recombination (Kanatsu-Shinohara et al., 2011). Nevertheless, mutant rats were generated with the CRISPR/Cas9 technique using SSCs (Chapman et al., 2015). The development of a chromosome transfer technique in rats will facilitate the development of improved models of human diseases and would complement other methods of site-specific gene modification.

While previous studies were based on chromosomal integration, this study established a method for the stable reliable maintenance of MACs in SSCs, which provides an alternative approach for the introduction of large DNA fragments into the germline. Our observations suggested that the GSC-based MAC transfer method has several advantages over traditional methods using ESCs because of their greater karyotype stability and direct Tc offspring production in the F1 generation. In addition to extending this system to other animal species to allow the development of new techniques for germline manipulation, this technique may also be useful for understanding the genetic factors associated with human male infertility that often occur as a result of chromosomal abnormalities. Such studies will contribute not only to our understanding of the biology of stem cells and spermatogenesis, but also to the generation of humanized animals and human disease models.

EXPERIMENTAL PROCEDURES

Cell Culture

GSCs were established from 7- to 10-day-old DBA/2 pup testes, as described previously (Kanatsu-Shinohara et al., 2003). The cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA), which was supplemented with 10 ng/mL human FGF2, 15 ng/mL recombinant rat GDNF (both from Peprotech, London, UK), and 1% fetal bovine serum (FBS) (Kanatsu-Shinohara et al., 2014b). We also used a GSC line transfected with pCAG-*Egfp2* as a control (Kanatsu-Shinohara et al., 2005a). Cultures were maintained on mitomycin C-treated MEFs. G418 treatment was carried out as described previously (Kanatsu-Shinohara et al., 2005a).

MMCT

PEG-MMCT and retro-MMCT were performed as described previously (Suzuki et al., 2016; Tomizuka et al., 1997). A9 cells containing human chromosome 21 and CHO cells containing MAC1 and expressing ecotropic EnvΔR were used as donor microcell hybrids in the PEG-MMCT and retro-MMCT method, respectively. The structure of MAC1, which consists of a centromere from mouse chromosome 11, *Egfp* flanked by HS4 insulators, PGKneo, 3′HPRT-loxP site, PGKpuro, and telomeres, was described in detail previously (Takiguchi et al., 2014). In brief, GSCs were fused with microcells prepared from A9 (hChr.21) and Eco-CHO (MAC1) cells

and selected with G418 (40 μg/mL). In each line, MAC-transferred GSCs were characterized by cytogenetic analyses.

Cytogenetic Analyses

Slides of GSCs with MACs and bone marrow derived from Tc mice with MACs were stained with quinacrine mustard and Hoechst 33258 to enumerate chromosomes. Images were captured using an AxioImagerZ2 fluorescence microscope (Carl Zeiss, Jena, Germany). mFISH analyses were performed using fixed metaphase spreads of bone marrow derived from Tc mice with MACs. Procedures for the denaturation of metaphase chromosomes and mFISH probes (MetaSystems, Altlussheim, Germany), hybridization, post-hybridization washes, and fluorescence staining were performed in accordance with the manufacturer's instructions. Metaphase images were captured digitally with a cooled CCD camera using the ISIS mFISH software program (MetaSystems), processed, and stored for subsequent analysis.

Animals and Spermatogonial Transplantation

For busulfan treatment, 4- to 5-week-old C57BL/6 (B6) × DBA/2 F1 (BDF1) mice underwent intraperitoneal injection with busulfan (44 mg/kg; Japan SLC, Shizuoka, Japan). For quantification of germ cell colonies after spermatogonial transplantation, 4- to 5-week-old W mice were used (Japan SLC). Spermatogonial transplantation was carried out by microinjection into the seminiferous tubules of infertile mice via the efferent duct (Ogawa et al., 1997). Approximately 10 μL or 4 μL was administered into the testes of BDF1 or W mice, respectively, because the latter were smaller. Each injection filled 75%–85% of the seminiferous tubules. All busulfan-treated recipient mice were used 4–8 weeks after busulfan treatment. Approximately 10^6 cells were microinjected into the seminiferous tubules of each testis for offspring production, whereas 2×10^3 were transplanted for colony counting. The Institutional Animal Care and Use Committee of Kyoto University and Tottori University approved all of the animal experimentation protocols.

Analysis of Recipient Testes

For assessment of colony counts, recipients were euthanized 2 months after transplantation, and donor cell colonization was examined under UV light. Germ cell clusters were defined as colonies when the entire basal surface of the tubule was occupied and the cell clusters were at least 0.1 mm in length.

Immunohistochemistry and Lectin Immunostaining

Testis samples were fixed in 2% paraformaldehyde for 3 hr and embedded in Tissue-Tek OCT compound for cryosectioning. Staining of cryosections was carried out by treating the samples with 0.1% Triton X-100 in PBS. After immersion in blocking buffer (0.1% Tween 20, 1% BSA, and 1% goat serum in PBS) for >1 hr, samples were incubated with PNA at 4°C overnight.

TUNEL Staining

GSCs were incubated in PBS/0.1% Triton X-100/0.1% sodium citrate for 2 min, and labeled using an *in situ* cell death detection kit, TMR red (Roche Applied Science, Mannheim, Germany),

according to the manufacturer's protocol. Cells were counter-stained with Hoechst 33342 (Sigma, St. Louis, MO) and analyzed under a fluorescence microscope.

Flow Cytometry

GSCs were dissociated using Cell Dissociation Buffer (Invitrogen). Cells were analyzed with a FACS-Calibur system (BD Bioscience, Franklin Lakes, NJ). After three washes with PBS supplemented with 1% FBS, samples were incubated with the indicated primary antibody. The samples were washed twice and secondary antibodies were added for detection. Samples in which the primary antibodies were omitted served as a control. The antibodies used in the study are listed in Table S1.

Real-Time PCR Analysis

Total RNA was isolated using TRIzol (Invitrogen), and first-strand cDNA was synthesized using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) and used for RT-PCR. For real-time PCR, the StepOnePlus Real-Time PCR system and FastStart Universal SYBR Green PCR Master Mix (Roche, Basel, Switzerland) were used according to the manufacturer's protocol (Applied Biosystems, Warrington, UK). Transcript levels were normalized relative to those of *Hprt*. PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each reaction was performed in duplicate. PCR primer sequences are listed in Table S2.

Microinsemination

Testes that had been injected with EGFP-expressing donor cells were refrigerated overnight and used for microinsemination on the day after collection, as described previously (Ogonuki et al., 2006). The seminiferous tubules containing EGFP fluorescence were dissected and dissociated by repeated pipetting using a glass needle under UV light. Microinsemination was performed using spermatozoa or elongated spermatids into BDF1 oocytes. After *in vitro* culture, two-cell-stage embryos were transferred into the oviducts of day-1 ICR pseudopregnant mice (CLEA Japan, Tokyo, Japan). Offspring were born by cesarean section on day 19.5.

Statistical Analyses

Significant differences were determined by Student's t tests. Multiple comparison analyses were carried out using ANOVA followed by Tukey's honestly significant Difference test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.08.012.

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