Biochemical and Biophysical Research Communications 442 (2013) 44–50

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Highly stable maintenance of a mouse artificial chromosome in human cells and mice

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ARTICLE INFO

*Article history:*
Received 25 October 2013
Available online 9 November 2013

*Keywords:*
Mouse artificial chromosome
Microcell-mediated chromosome transfer
Transchromosomic mouse
Gene delivery

ABSTRACT

Human artificial chromosomes (HACs) and mouse artificial chromosomes (MACs) display several advantages as gene delivery vectors, such as stable episomal maintenance that avoids insertional mutations and the ability to carry large gene inserts including the regulatory elements. Previously, we showed that a MAC vector developed from a natural mouse chromosome by chromosome engineering was more stably maintained in adult tissues and hematopoietic cells in mice than HAC vectors. In this study, to expand the utility for a gene delivery vector in human cells and mice, we investigated the long-term stability of the MACs in cultured human cells and transchromosomic mice. We also investigated the chromosomal copy number-dependent expression of genes on the MACs in mice. The MAC was stably maintained in human HT1080 cells in vitro during long-term culture. The MAC was stably maintained at least to the F8 and F4 generations in ICR and C57BL/6 backgrounds, respectively. The MAC was also stably maintained in hematopoietic cells and tissues derived from old mice. Transchromosomic mice containing two or four copies of the MAC were generated by breeding. The DNA contents were comparable to the copy number of the MACs in each tissue examined, and the expression of the *EGFP* gene on the MAC was dependent on the chromosomal copy number. Therefore, the MAC vector may be useful not only for gene delivery in mammalian cells but also for animal transgenesis.



## 1. Introduction

The introduction of large genes or gene clusters into mice allows the correct expression of transgenes by including essential remote regulatory elements [1]. Human chromosome fragments (hCFs) derived from normal fibroblasts were used as a vector for animal transgenesis, including the introduction of Mb-sized large genomic inserts into mice via microcell-mediated chromosome transfer (MMCT) technology [2,3]. Double transchromosomic (Tc) mice containing two individual hCFs carrying *IgH* and *Igk* produced antigen-specific human antibodies [4]. However, the mitotic stability of hCFs in mice varies, and large hCFs cannot be transmitted through the germline [2,5,6]. Cloning the desired genomic region into the mitotically stable hCF allowed us to generate Tc mice containing multiple large genomic inserts, which could not be cloned using conventional vectors such as plasmids and bacterial artificial chromosomes (BACs) [5,7]. However, hCFs contain several structurally undefined regions with many endogenous genes, which cause partial trisomy in cells propagating these hCFs. This may affect the physiological gene expression and the normal development. To overcome this, several groups engineered human artificial chromosomes (HACs) by random segmentation or targeted telomere-associated chromosomal fragmentation in homologous recombination-proficient chicken DT40 cells [5,8–10]. However, germline transmittable Tc mice containing multiple copies of HACs with Mb-sized large inserts have never been generated, possibly because of the instability of HACs in germ cells. Although hCFs and HACs containing large regions of genomic DNA can be autonomously maintained in Tc mice, their retention rate varies [3,6,11–14]. Thus, we constructed novel mouse artificial chromosome (MAC) vectors from a native mouse chromosome by chromosome engineering to improve the retention rate [15]. Previously, a MAC vector containing the *EGFP* gene, which can be used to monitor the cells, was used to determine its stability in vivo. The stability of this MAC in mouse tissues and hematopoietic cells was higher than that of other reported mammalian artificial

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.171

chromosomes including hCFs, HACs, and murine satellite DNA-based artificial chromosomes (mSATACs) [16]. The stability and germline transmission (GT) efficiency of hCFs, HACs, and mSATACs differed with genetic background, generation, age, and sex [4,6,12,14,17,18]. MACs will be a powerful tool to generate Tc mice carrying multiple Mb-sized genes for humanized animal models if they have high stability and GT efficiency and if multiple copies can be introduced into mice. Furthermore, if the MAC is stable in human cells, the same MAC containing a desired gene may be used for functional analysis in both mice and human cells. Therefore, we investigated: (i) MAC stability in HT1080 cells, (ii) MAC stability and GT ratio in different genetic backgrounds and sexes, (iii) MAC stability in aged mice, (iv) MAC copy number per cell in tissues, and (v) MAC copy number-dependent gene expression in tissues.

## 2. Materials and methods

### 2.1. Cell culture

HT1080 were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) plus 10% fetal bovine serum (FBS). Chinese hamster ovary (CHO) cells containing MAC1 were constructed as previously described [15]. The CHO (MAC1) cells were maintained in Ham's F-12 nutrient mixture (Invitrogen, Carlsbad, CA, USA) plus 10% FBS with 800 μg/mL G418 (Promega, Tokyo, Japan).

### 2.2. MMCT

MMCT was performed as described previously [2,19]. CHO cells containing MAC1 were used as donor microcell hybrids. 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 [15]. Briefly, HT1080 cells were fused with microcells prepared from donor hybrid CHO (MAC1) cells and selected with G418 (600 μg/mL). In each line, MAC1 was characterized by polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) analyses.

### 2.3. Quantitative genomic PCR analyses

Genomic DNA was extracted from Tc mouse tissues using a genomic DNA extraction kit (Gentra System, Minneapolis, MN, USA). Quantitative genomic PCR analyses detected the *EGFP* gene on MAC1 using the Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) and 2 × FastStart Universal SYBR Green Master (Rox) (Roche Applied Science). Brain DNA of MAC1 Tc mice was used as a positive control for normalization, while DNA from C57BL/6 mouse tissues was used as a negative control. An endogenous mouse gene, *Ravor2*, was used as an internal standard (Takara, Shiga, Japan). The following primers were used to amplify the *EGFP* gene: 5′-tctatatcatggccgacaagc-3′ and 5′-gttgtggcggatcttgaagt-3′.

### 2.4. Cytogenetic analyses

Slides of testis or cultured lymphocytes derived from MAC1 Tc mice were stained with quinacrine mustard and Hoechst 33258 or Giemsa stain to enumerate chromosomes. Images were captured using an AxioImagerZ2 fluorescence microscope (Carl Zeiss GmbH). FISH analyses were performed using fixed metaphase or interphase spreads of HT1080 cell hybrids using biotin-labeled (Roche, Basel, Switzerland) mouse COT-1 DNA (Invitrogen) essentially as described previously [2]. Chromosomal DNA was counterstained with DAPI (Sigma). Images were captured using the NIS-Elements system (Nikon, Tokyo, Japan).

### 2.5. Quantitative reverse transcription (RT)-PCR analyses

Total RNA from Tc mouse tissue specimens was prepared using ISOGEN (Nippon Gene, Tokyo, Japan), purified using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and treated with RNase-free DNase I (Wako). First-strand cDNA synthesis was performed using random hexamers and SuperScript III reverse transcriptase (Invitrogen). *β-Actin* was used as the internal control. Brain cDNA of MAC1 Tc mice was used as a positive control for normalization, while cDNA from C57BL/6 mouse tissues was used as a negative control. Quantitative RT-PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR system and 2 × EXPRESS qPCR Supermix with Premixed ROX (Life Technologies). The following primers were used: *β-actin*, 5′-ggatgcagaaggagattactgc-3′ and 5′-ccaccgatccacacagagta-3′; and *EGFP*, 5′-cctgaagttcatctgcacca-3′ and 5′-ggtcagggtggtcacgag-3′. TaqMan probe #63 and #37 were used to detect *β-actin* and *EGFP*, respectively.

### 2.6. Breeding analyses

Jcl:ICR (ICR) and C57BL/6JJcl (B6) mice were purchased from Japan Crea. Previously, we generated F1 mice by mating chimeric female mice with ICR males [15]. Briefly, F1 mice were bred with either ICR or B6 mice. In the ICR background, the mice were backcrossed with ICR until the F8 generation. In the B6 background, the mice were backcrossed with B6 until the F4 generation. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.

## 3. Results and discussion

### 3.1. Stability of MAC1 in HT1080 cells

We used the MAC1 vector with *EGFP* to monitor the gene expression and stability of the MAC. To investigate the stability of a MAC in human cells, MAC1 was transferred to the human fibrosarcoma cell line HT1080 by MMCT. Three GFP-positive clones were selected and examined. FISH analyses showed that MAC1 was present as an individual chromosome in the HT1080 cells (Fig. 1A). After the HT1080 (MAC1) cells were cultured for about 3 months with or without selection, the stability of the MAC1 and GFP expression on the MAC1 were tested. FISH analyses revealed that the MAC1 was independently and stably maintained in HT1080 cells (>90%) (Fig. 1B), and most of the HT1080 (MAC1) cells were GFP-positive (Fig. 1C). The loss of MAC1 (<10%) in FISH analyses was comparable to the ratio of the GFP-negative cells. This suggests that MAC1 is maintained stably and that the *EGFP* gene on MAC1 is expressed stably even after long-term culture in vitro without antibiotic selection. Previously, an mSATAC was created via amplicon-dependent de novo chromosome formation induced by integrating exogenous DNA sequences into centromeric/rDNA regions near the pericentric heterochromatin or acrocentric chromosomes. The mSATAC consisting of murine pericentric satellite DNA was not stable in a human cell line, but it was relatively stable in a mouse cell line [20]. Because MAC1 was stable in human cells and mouse embryonic stem (ES) cells, a mouse chromosome or MAC with a natural centromere structure may be important for stability in human and mouse cells.

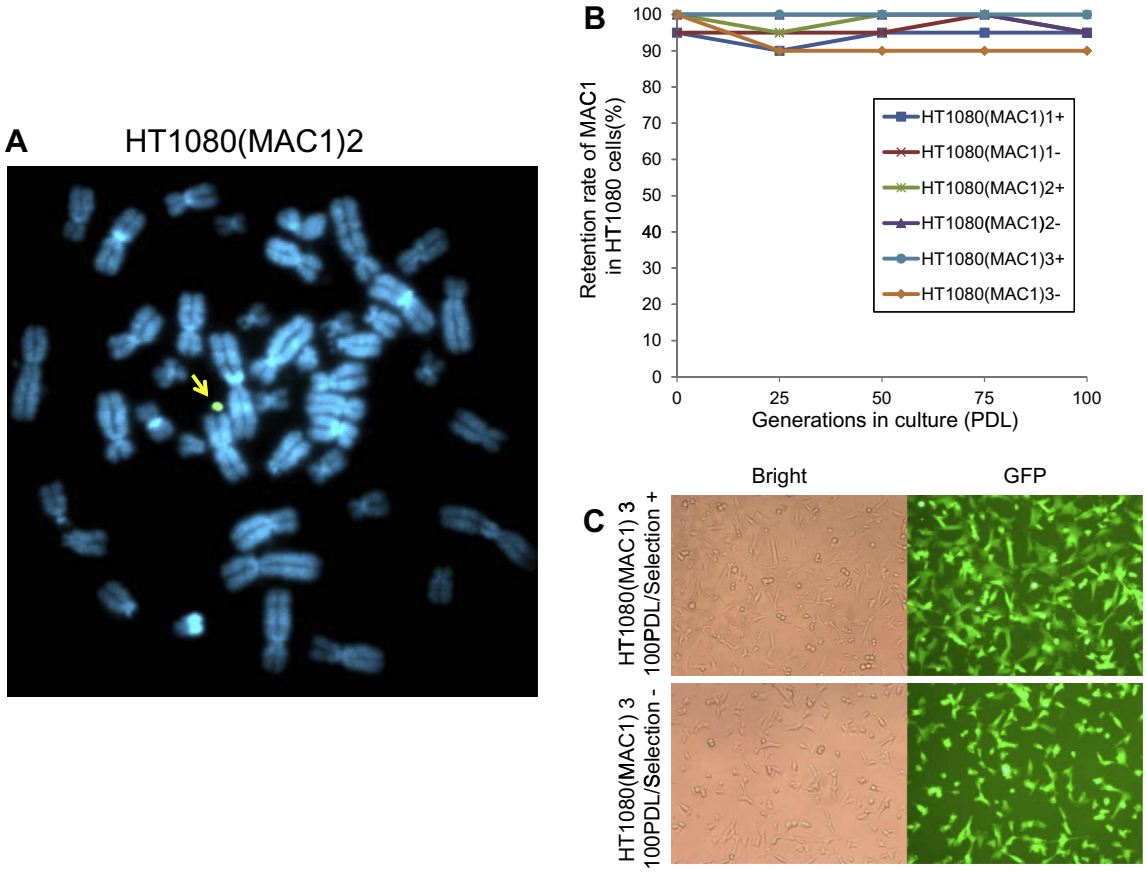


**Fig. 1.** Stability of MAC1 in HT1080 cells. (A) FISH analyses of HT1080 (MAC1) cells. An arrow indicates MAC1. (B) Mitotic stability of MAC1 in HT1080 (MAC1) cells. (C) GFP expression after long-term culture. Phase-contrast (left panel) and GFP-fluorescence (right panel) micrographs are shown.

### *3.2. Stability and germline transmission ratio of MAC1 in different genetic backgrounds and sexes*

Previously, the stability and GT efficiency were shown to differ with genetic background and sex [4,6]. Thus, breeding analyses were performed to determine the stability and GT efficiencies of MAC1 through the male and female germline in ICR and B6 genetic backgrounds. The F8 generation in the ICR line and the F4 generation in the B6 line were generated by sequentially breeding with either the ICR or B6 strain, respectively. To investigate the retention rate of MAC1 in female or male founder-derived Tc mouse lymphocytes in each genetic background and generation, flow cytometry (FCM) analysis was used to determine the GFP-expressing cell ratios. The ratios of GFP-expressing cells in lymphocytes with MAC1 were very high in F3 (male and female) and F8 (female) Tc mice from male and female ICR founder derivatives, and in F2 (male and female) and F4 (female) Tc mice from male and female B6 founder derivatives (Fig. 2A). These data suggest that MAC1 is extremely stable in multiple genetic backgrounds, founder derivatives, and generations. To date, the Tc progeny have not exhibited any overt phenotypic abnormalities.

Next, we investigated the GT efficiency in female and male Tc mice in each genetic background and generation. The GT efficiency was 54.4% and 23.4% in female and male ICR derivatives, respectively, and 45.8% and 28.8% in female and male B6 derivatives, respectively (Fig. 2B and Supplementary Table S1). These data suggest that the GT ratio in female, but not male, derivatives is mendelian (50%). Cytogenetic analyses of the testis of Tc mice with MAC1 showed perfect retention of MAC1 in meiosis I cells and an equal number of meiosis II cells with and without MAC1 (Supplementary Fig. S1 and Supplementary Table 2). These results suggest that MAC1 is mitotically and meiotically stable in mice.

The expected GT efficiency in Tc mice that are hemizygous for MAC1 is 50% when the mitotic stability of MAC1 is perfect and it is properly segregated in meiosis. Consistent with the observation that MAC1 is more stable in mouse ES cells or mice than human-derived chromosomal vectors such as CV (a ring chromosome from human chromosome 1), W23 (hCF2), SC20 (hCF14), and 21HAC, the transmission efficiency of MAC1 in females was perfect (45.8–54.4%), while that in males was 23.4–28.8%. Previously a mini-chromosome, ST1, containing mouse major and minor satellite sequences and human alphoid DNA sequences was transmitted to offspring from females at a frequency approaching 50%; however, the GT efficiency from male mice was about 20% [11,21]. ST1 was very stable in mouse tissues, and the stable retention of ST1 in meiosis I cells was observed. This observation was consistent with the GT efficiency of MAC1 and the stability in testis in this study. Nevertheless, the lower GT efficiency in males than in females may be caused by the delayed spermatocyte maturation or the sperm competition effect on the fertilization capacity (normal vs. MAC/ST1-containing sperms). However, the factors determining the GT efficiency of MAC1 from male mice remain unknown. Detailed analyses, such as FISH of sperm to confirm the presence of MAC1, are required to understand the difference in the transmission rate of MAC1 in males and females.

Furthermore, the GT efficiencies in Tc mice containing hCF2 (W23) were 8–12% in males and 22–33% in females, and the efficiencies in Tc mice containing SC20 (hCF14) were 32–34% in males and 33–38% in females [4]. In contrast to the gradual loss of W23 (hCF2), SC20 (hCF14), which was used to construct λ-HAC, ΔHAC,

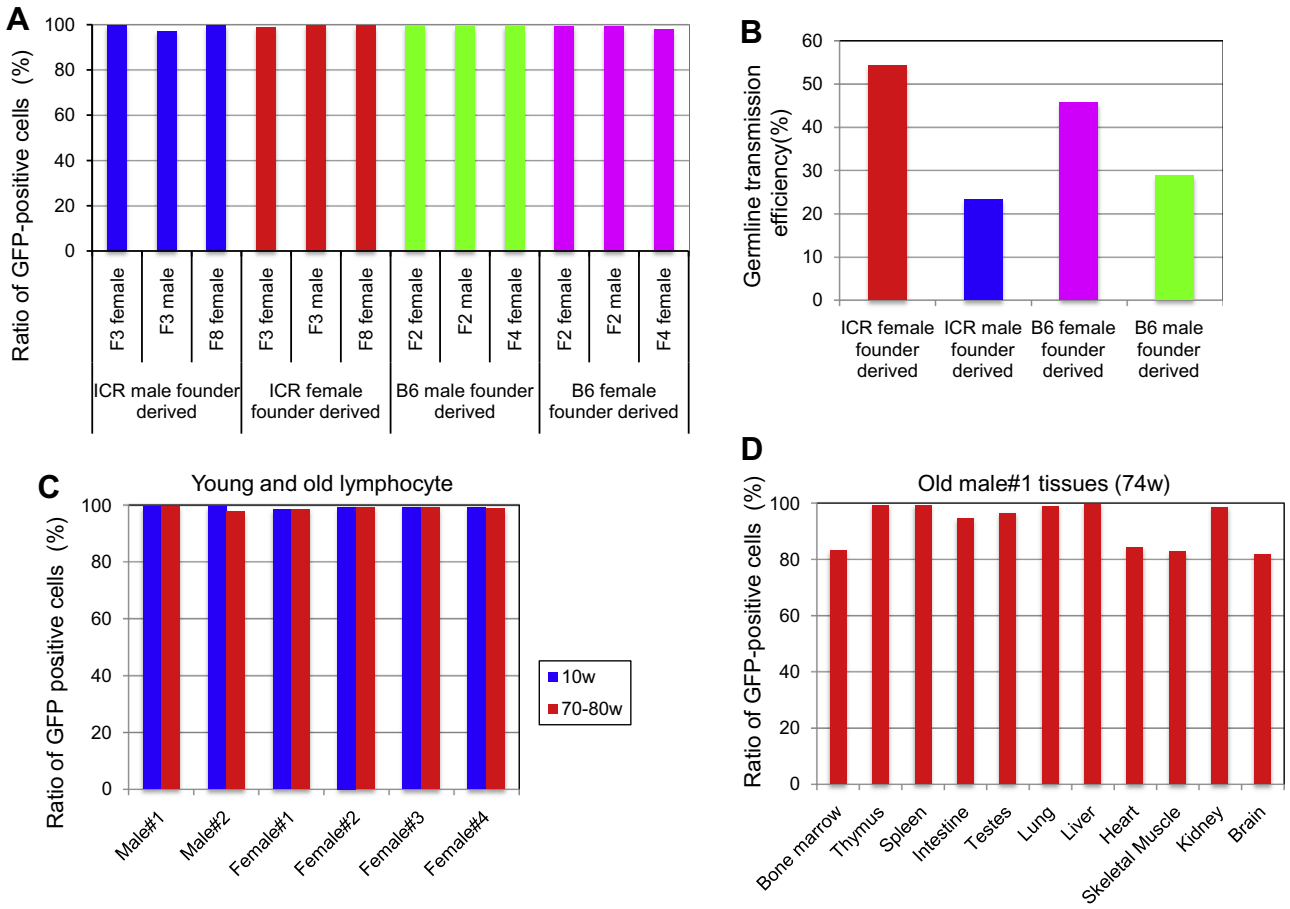


**Fig. 2.** Stability of MAC1 in vivo. (A) Stability of MAC1 in lymphocytes from different sexes, founder derivatives, and generations. (B) Germline transmission efficiency of MAC1 in different genetic backgrounds and sexes. (C) Stability of MAC1 in lymphocytes from young and aged Tc mice. (D) Stability of MAC1 in several tissues from the old Tc mouse. Representative data using old male #1 are shown. The results were confirmed in old male #2 (data not shown).

and CYP3A-HAC, was very stable [5,7,22]. Additionally, the GT efficiencies in Tc mice containing GCH1-HAC were 14–16% in males and 37–40% in females [14], whereas the GT efficiencies in Tc mice containing a CV from human chromosome 1 were 31% in males and 35% in females [23]. Previously, Drost et al. reported that 62 and 25 cell divisions in mice were required to generate mature sperm and oocytes, respectively [24], indicating that the GT efficiency may be higher in females than in males. Thus, the GT ratio may be unique for each hCF, HAC, and MAC, although the GT efficiency of hCFs, HACs, and MACs may be dependent on the sex of the founder and the stability in vitro.

### 3.3. Stability of MAC1 in aged mice

To investigate the long-term stability of MAC1 in vivo, young and old hematopoietic cells were analyzed using FCM. The ratios of GFP-expressing cells with MAC1 in lymphocytes were very high in 10-week-old and 70–80 week-old mice (Fig. 2C). Furthermore, the ratios of GFP-expressing cells with MAC1 in several tissues from the old Tc mouse were clearly high, which was consistent with reports from young Tc mouse tissues (Fig. 2D) [15]. Although a previous report showed that an mSATAC was gradually lost during aging, possibly because of its stability in mouse cells [17], the stability of MAC1 in lymphocytes and tissues was not affected by aging, suggesting that genes inserted into MACs are stably expressed and that the MAC vector is consistently maintained in mice.

Trisomy of human chromosome 21 results in Down syndrome, a disorder that affects many aspects of physiology including hematopoiesis [25,26]. Previously, the most significant problem of Down syndrome mouse models was that the introduced human chromosome 21 was not mitotically stable in proliferative mouse tissues such as spleen and bone marrow, making further analysis of its effects on hematopoietic phenotypes difficult [3,13]. Because the MAC vector was stably maintained in proliferative hematopoietic cells, Down syndrome model mice using the MAC vector may represent the hematopoietic phenotypes.

### 3.4. Cytogenetic analysis in Tc mice with two copies of MAC1

To generate Tc mice homozygous for MAC1 (Tc (MAC1)2), male and female Tc mice with a single MAC1 per cell (Tc (MAC1)1) were crossed. Cytogenetic analyses of the bone marrow of Tc (MAC1)2 mice showed that the karyotype was 42, XY, +MAC1, +MAC1, and that two copies of MAC1 were retained independently without integration into the host genome (Fig. 3A, B). Next, meiosis I chromosomes in the testis were investigated by Hoechst 33258 staining. Metaphase spreads with or without pairing of two MAC1s in the testis were observed (Fig. 3C, D). MAC1s were paired or closely associated in 30% of the examined metaphase spreads, and they were unpaired or dissociated in 70% of the examined metaphase spreads. These data suggest that MAC1s can be unpaired or that they separate earlier than other chromosomes, because of their small size, resulting in sperm with two copies of MAC1 following

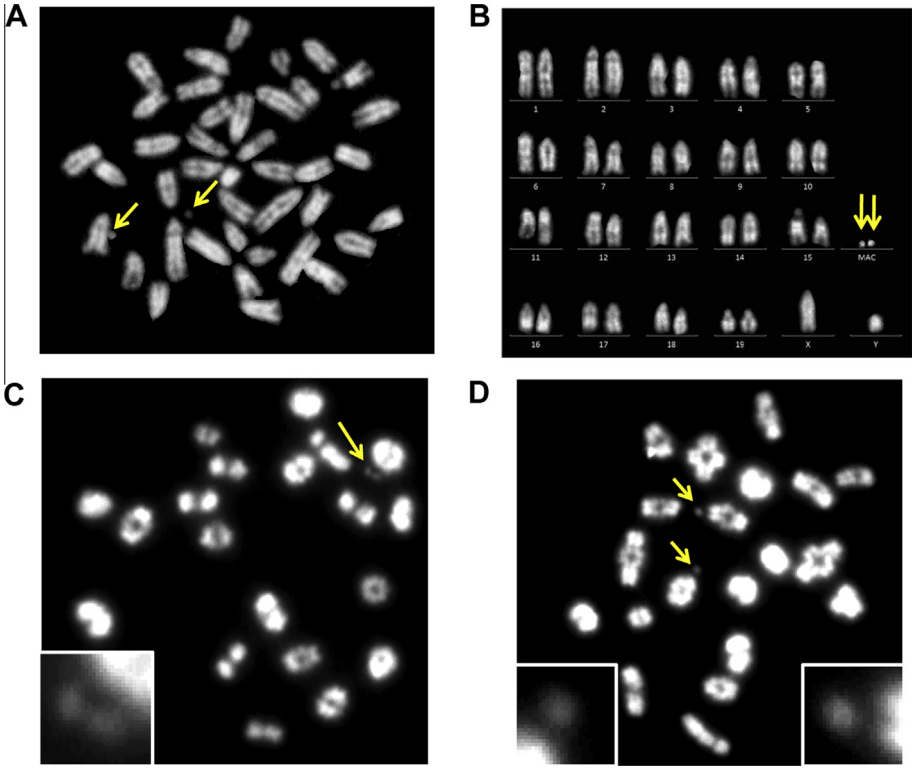


**Fig. 3.** Cytogenetic analysis of Tc mice with two copies of MAC1. (A) Metaphase spread of Tc (MAC1) 2 bone marrow. (B) Karyotype of Tc (MAC1) 2 bone marrow-derived chromosomes. (C, D) Meiosis I metaphase spreads from Tc (MAC1) testis with two copies of MAC1, and the inset shows an enlarged image of MAC1. Arrows indicate MAC1.

non-disjunction. Therefore, we performed breeding analyses to generate mice with more than two copies of MAC1.

### 3.5. Cytogenetic, genomic and expression analyses in Tc mice with multiple copies of MAC1

Copy number control of MAC1 by breeding would be useful if the expression of genes on MAC1 shows a gene-dosage effect. Tc (MAC1)2 mice were fertile, and crossing Tc (MAC1)2 mice resulted in offspring with up to four copies of MAC1 per cell. Hoechst 33258 staining analyses of cultured lymphocytes showed that Tc (MAC1)1, Tc (MAC1)2, and Tc (MAC1)4 contained one, two, and four copies of MAC1, respectively (Fig. 4A–C). The quantitative genomic PCR analyses showed that relative DNA contents of the *EGFP* gene on MAC1 were copy number dependent in the nine tissues examined (Fig. 4D). The quantitative RT-PCR analyses showed that the relative expression of the *EGFP* gene on MAC1 was also copy number dependent (Fig. 4E). However, the *EGFP* expression in brain, heart, lung, and skeletal muscle was lower than that in thymus, liver, spleen, kidney, and small intestine, which might be consistent with the CAG promoter activity in each tissue for *EGFP* expression [27]. Although Tc mice containing up to four copies of the CV were generated previously, the stability of CV in several mouse tissues was lower than that of MAC1 [15,28]. Because two and four copies of MAC1 were stably maintained, the *EGFP* expression in the tissues was copy number dependent. Thus, the MAC will be useful for generating Tc mice with multiple copies of a desired genomic region or combination of genes.

### 3.6. FCM analysis in hematopoietic cells in Tc mice with multiple copies of MAC1

To determine copy number-dependent gene expression in hematopoietic cells, FCM analyses were performed using Tc (MAC1)1, Tc (MAC1)2, and Tc (MAC1)4 mice. The *EGFP* gene driven by the CAG promoter on MAC1 was expressed in CD4+ T lymphocytes, CD8+ T lymphocytes, and CD19+ B lymphocytes in a copy number-dependent manner (Fig. 4F), suggesting that multiple copies of MAC1 were stably maintained in vivo and that the copy number of MAC1 could be controlled to attain the desired gene expression level.

We showed that MAC1 was stably maintained in both human cells and mice. Previously, the stability of 21HAC was shown to be lower than that of MAC1 in mouse cells, and the 21HAC vector was maintained stably in human cells [15,29]. Thus, the MAC vector may be a universal vector for gene expression in mammalian cells. If the same copy number control of a desired gene without integration is needed in both mouse and human cells, MACs with the gene may be a useful tool when the conventional gene delivery system is not appropriate [16]. Previously, Kuroiwa et al. transferred HACs to cow fibroblasts and generated Tc cows by the nuclear transfer technique [22,30]. Using the same cloning techniques, we can test the MAC stability in several species. Because multiple copies of MAC1 were transmitted through the germline and stably maintained in vivo, it will be useful to generate mouse models containing a combination of large genes, such as the drug-metabolizing enzymes CYP3A cluster (~300 kb), CYP2C (~400 kb), and UGT2 cluster (~1.5 Mb). Taken together, the MAC vector, which was developed from a natural mouse chromosome using a top-down approach, will be useful to deliver large and multiple genes into human cells and to generate humanized mouse models.

## Acknowledgements

We wish to thank Kaori Adachi, Yumiko Kumura, Kayo Fujimoto, Kazuki Tanaka, Naoyo Kajitani, Toko Yoshino, Hiromichi Kono, Yukako Sumida, Manami Iitsuka, Madoka Fukuura, Noriyasu Oko,

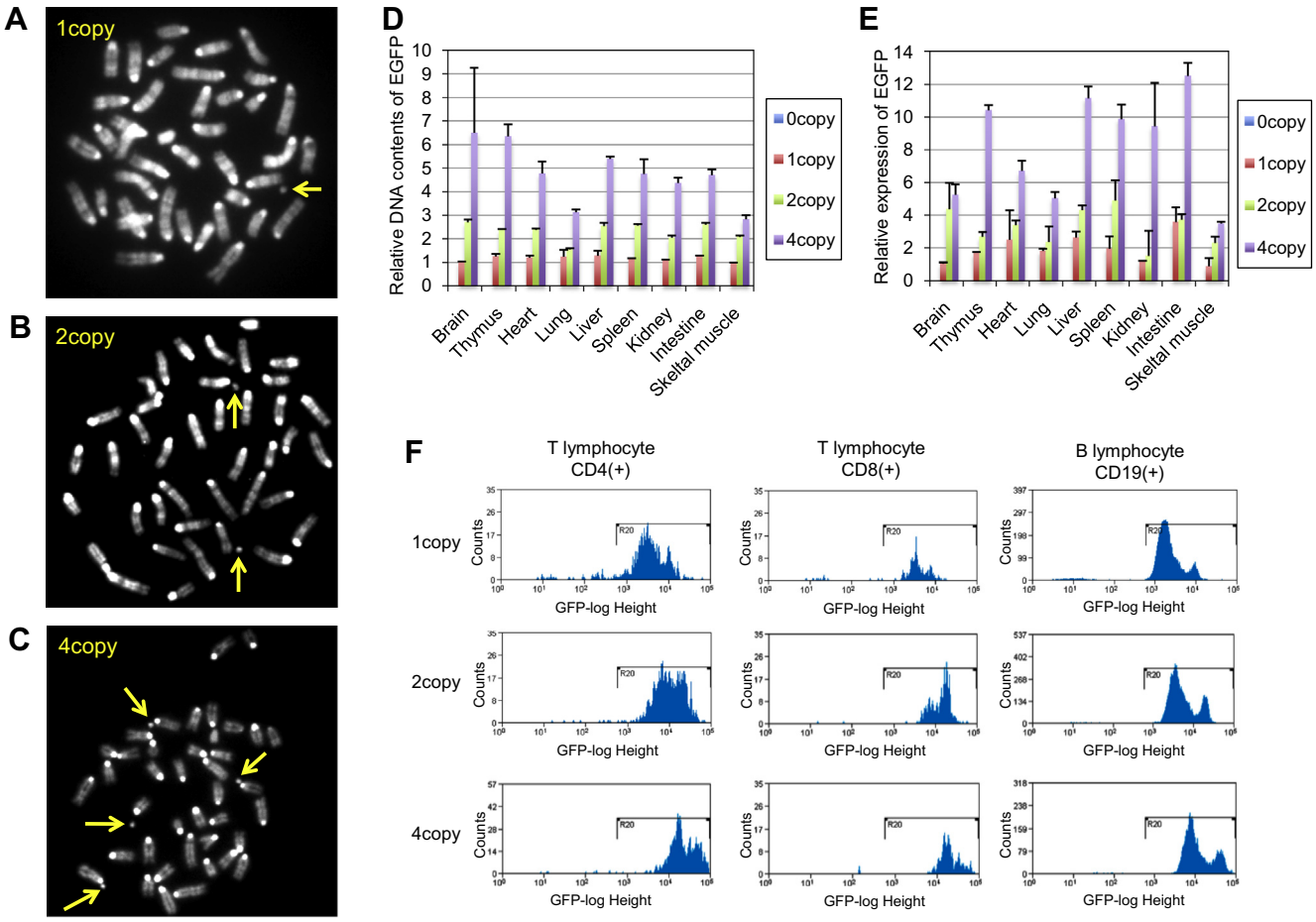


**Fig. 4.** Copy number-dependent *EGFP* expression in Tc mice. (A–C) Cytogenetic analysis of cultured lymphocytes from the Tc mouse with one, two, and four copies of MAC1. Arrows indicate MAC1. (D) Quantitative genomic PCR analyses detected *EGFP* DNA from MAC1 in the Tc mouse tissues. The relative DNA contents were normalized to the endogenous mouse *Ravor2* gene. Data are the means of three independent experiments (±S.D.). (E) Quantitative RT-PCR analyses detected *EGFP* expression from MAC1 in the Tc mouse tissues. The relative expression was normalized to the endogenous mouse *β-actin* gene expression. Data are the means of three independent experiments (±S.D.). (F) FCM analysis in CD4+ T cells, CD8+ T cells, and CD19+ B cells from Tc mice with one, two, and four copies of MAC1.

and Ayako Takami for technical assistance, and Yuji Nakayama and Eiji Nanba for critical discussions. This study was supported in part by the Funding Program for Next Generation World-Leading Researchers (NEXT Program) from the Japan Society for the Promotion of Science (JSPS) (Y.K.), JST, CREST (M.O.), and Regional Innovation Strategy Support Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) (M.O.).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.171.

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