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Development of a Safeguard System Using an Episomal Mammalian Artificial Chromosome for Gene and Cell Therapy

Narumi Uno[1,2], Katsuhiro Uno[2], Shinya Komoto[2], Teruhiko Suzuki[3], Masaharu Hiratsuka[4], Mitsuhiko Osaki[5], Yasuhiro Kazuki[1,2,4] and Mitsuo Oshimura[1]

The development of a safeguard system to remove tumorigenic cells would allow safer clinical applications of stem cells for the treatment of patients with an intractable disease including genetic disorders. Such safeguard systems should not disrupt the host genome and should have long-term stability. Here, we attempted to develop a tumor-suppressing mammalian artificial chromosome containing a safeguard system that uses the immune rejection system against allogeneic tissue from the host. For proof-of-concept of the safeguard system, B16F10 mouse melanoma cells expressing the introduced H2-K(d) major histocompatibility complex (MHC class I)-allogenic haplotype were transplanted into recipient C57BL/6J mice expressing MHC H2-K(b). Subcutaneous implantation of B16F10 cells into C57BL/6J mice resulted in high tumorigenicity. The volume of tumors derived from B16F10 cells expressing allogenic MHC H2-K(d) was decreased significantly ($P < 0.01$). Suppression of MHC H2-K(d)-expressing tumors in C57BL/6J mice was enhanced by immunization with MHC H2-K(d)-expressing splenocytes ($P < 0.01$). These results suggest that the safeguard system is capable of suppressing tumor formation by the transplanted cells.
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Introduction

Cell transplantation, which involves somatic stem cells and engraftments derived from pluripotent stem cells, is expected to be an innovative technology for the progression of regenerative medicine.[1–3] Recently, numerous types of cells have been successfully generated from stem cells.[4–7] Self-renewal and multiple-lineage differentiation enable the production of functional cells to treat patients with intractable diseases including various genetic disorders. However, the risk of malignancy remains a significant concern.[8,9] Teratomas might form from immature cells, and primary tumors might be derived from injected stem cells. Tumors develop at an increased frequency in chimeric animals generated with induced pluripotent stem cells.[10–12] Moreover, neuronal tumors have formed in primates injected with pluripotent stem cell-derived neurogenic cells.[13] A striking case is a patient with ataxia telangiectasia, who developed a multifocal aggressive brain tumor following administration of neurogenic stem cells.[14] These examples strongly illustrate the need for a safeguard system, even though the cause of malignancy in such cases is unclear. The causes of malignancy include reactivation of reprogramming factors,[11] increased genomic instability,[15] frequent and nonrandom chromosomal aberrations,[16] and recurrent inactivation of tumor suppressor genes.[16–18] However, improved reprogramming procedures have greatly reduced the risk of cancer.[9] For these reprogramming procedures, nonintegrating and excisable vectors, and the exclusion of oncogenes and reprogramming agents, *i.e.*, RNA,[19] protein,[20] or small molecules,[21] are required for treatment without additional genomic alterations.[10,22–24] In addition, removal of residual pluripotent stem cells and genomic surveys for somatic mutations are crucial.[25–28] Previous safeguard systems have often involved disruption of the host genome, treatment with a prodrug,[26] or limited application of specific cell lines such as pluripotent stem cells.[29] Here, we conducted a pilot study based on the physiological barrier of immunity against an allogenic subject using a mouse artificial chromosome (MAC), a nonintegrating vector.[30] We demonstrated the feasibility of an *in vivo* safeguard system by introducing an allogenic haplotype of major histocompatibility complex (MHC) class I, which can be expressed in various tumor cells without gene disruptions and prodrugs.

Results

Construction of a safeguard system using a tumor-suppressing MAC (TS-MAC)

As an *in vivo* model of tumor rejection for the following autologous transplantations, we used the mouse melanoma cell line B16F10, For an *in vitro* model of undifferentiated cell elimination, we used mouse embryonic stem (mES) cells derived from C57BL/6J mice. MACs are maintained stably and independently from native chromosomes in mouse cell lines and individuals.[31,32] Because the introduced safeguard system required high stability, MACs were used in this study,[30,32] although human artificial chromosomes (HACs) have the potential to be

[1]Chromosome Engineering Research Center, Tottori University, Yonago, Japan; [2]Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, Yonago, Japan; [3]Stem Cell Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; [4]Department of Molecular and Cellular Biology, Faculty of Medicine, Tottori University, Yonago, Japan; [5]Division of Pathological Biochemistry, Faculty of Medicine, Tottori University, Yonago, Japan. Correspondence: Mitsuo Oshimura, Chromosome Engineering Research Center, Tottori University, 86 Nishi-cho, Yonago, Tottori 683–8503, Japan. E-mail: oshimura@med.tottori-u.ac.jp
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applied to humans. Additionally, several genes can be inserted onto a MAC and expressed under the control of the promoter. These characteristics are suitable for the construction of an *in vivo* safeguard system. MAC4 contained enhanced green fluorescent protein (*EGFP*), hygromycin resistance (*Hyg*), and 5′hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) genes, and a *loxP* site. MAC4 was combined with a phage artificial chromosome (PAC) containing *in vitro* and *in vivo* safeguard systems, and a *loxP* site following the *HPRT* gene.[33]

As the *in vitro* safeguard system, we constructed herpes simplex virus thymidine kinase (HSV-TK) connected to tdTomato with a P2A peptide signal sequence under the control of the nanog promoter (pNanog-HSV-TK-P2A-tdTomato). Nanog is a marker of undifferentiated ES cells.[34] A safeguard system using HSV-TK under the control of the nanog promoter via a lentiviral gene expression system has been reported previously.[26] Because the lentiviral gene expression system requires insertion of a gene expression cassette into the host cell, such gene insertion may increase the risk of tumorigenicity in cell transplantation. Therefore, we used a nonintegrative gene delivery vector, the MAC, which was maintained independently from host chromosomes. tdTomato was added to visualize the expression of HSV-TK. The P2A peptide signal in the construct can efficiently separate upstream and downstream peptides.[35]

For the *in vivo* safeguard system, we connected MHC H2-K(d) and β-2 microglobulin (*B2M*) with P2A under the control of the human telomerase reverse transcriptase (*hTERT*) promoter (phTERT-MHC H2-K(d)-P2A-B2M). hTERT is a major component of telomerase, which is a marker of unlimited proliferation in cells, and the majority of malignant tumors exhibit telomerase activity.[36–39] A haplotype of MHC class I (MHC H2-K(d)) is expressed on the surface of cells derived from Balb/c mice.[40] Thus, the transplanted cells expressing allogenic MHC H2-K(d) were rejected owing to the immune reaction of the host C57BL/6 mice.[41] In addition, MHC H2-K(d) forms a complex with B2M. Therefore, MHC H2-K(d) was linked to B2M with P2A peptide signal, so that B2M and MHC H2-K(d) would have similar expression levels. MHC H2-K(d) was expected to be present on the cell surface with B2M.

The PAC vector (pPH_pN_TK_pT_MHCK(d)) was designed for the MAC containing *in vitro* and *in vivo* safeguard systems, namely tumor suppressing-MAC (TS-MAC). Thus, the PAC and Cre-recombinase expression vector were cotransfected in Chinese hamster ovary (CHO) cells containing MAC4 to mediate site-specific recombination. CHO cells containing MAC4 that correctly recombined with the PAC vector could survive in medium containing hypoxanthine, aminopterin, and thymidine (HAT) (**Figure 1a**). Therefore, the transfected CHO cells were expanded in HAT selection medium. Fifty-eight clones were selected. To confirm that the obtained CHO clones had the expected TS-MAC, polymerase chain reaction (PCR) analysis was performed with several primer sets (**Table 1**). Among them, 21 clones showed a positive result for all primer sets (data not shown). PCR results of two representative clones, CHO/TS-MAC#1 and #2, and CHO/MAC4 as a negative control are shown in **Figure 1b**. Fluorescence *in situ* hybridization (FISH) analysis showed that TS-MAC was maintained independently from CHO chromosomes (**Figure 1c**, **Table 2**). Thus, the TS-MAC

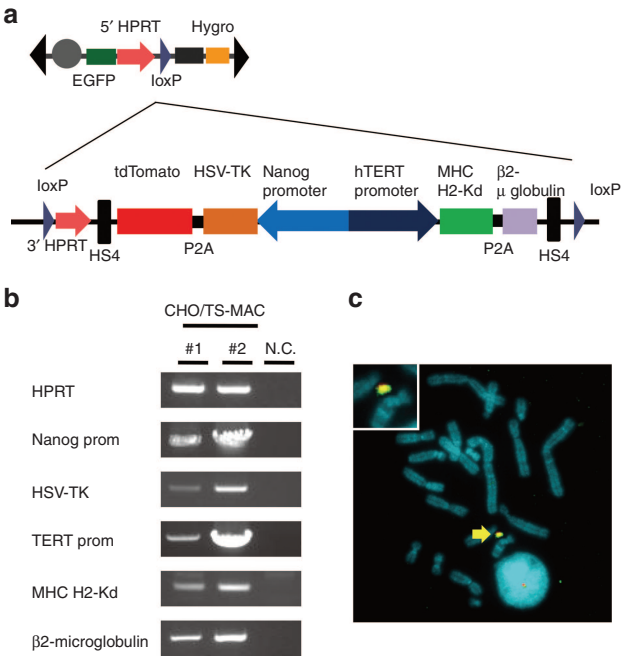

Figure 1 Construction of a tumor-suppressing mouse artificial chromosome (TS-MAC). (**a**) Diagram of the TS-MAC. The TS-MAC was constructed using MAC4, which was derived from mouse chromosome 11, and contained *EGFP* and hygromycin resistance (*Hyg*) genes, a *loxP* site, and exons 1 and 2 of the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene. pPH_pN_TK_pT_MHCK(d) was inserted into MAC4 using the Cre-*loxP* system. pPH_pN_TK_pT_MHCK(d) included HSV-TK connected to tdTomato, which was controlled by the mouse nanog promoter regulatory element, and MHC H2-K(d) connected to beta-2 microglobulin, which was controlled by the human telomerase reverse transcriptase (*hTERT*) promoter regulatory element. These two expression cassettes were aligned bi-directionally and flanked HS4 insulators. (**b**) Polymerase chain reaction (PCR) analysis of two representative Chinese hamster ovary (CHO) cell clones containing the TS-MAC and CHO cells without TS-MAC as the negative control (N.C.) with the primers listed in Table 1. (**c**) A representative image of fluorescence *in situ* hybridization (FISH) analysis of CHO/TS-MAC#1. Blue indicates 4′,6-diamidino-2-phenylindole signals. The rhodamine (red) signal indicates the MAC. The fluorescein isothiocyanate signal (green), which was observed as a yellow dot, indicates the inserted phage artificial chromosome (PAC) vector. The yellow arrow shows TS-MAC. An elongated TS-MAC is shown in the inset.

was correctly constructed in CHO cells and capable of transfer from the CHO cells to other targeted cells.

Elimination of tumor cells with TS-MAC

We evaluated whether the safeguard system could be applied to suppress tumor formation *in vivo*. The TS-MAC was transferred by microcell-mediated chromosome transfer (MMCT) from CHO/TS-MAC#1 cells to the B16F10 cell line that was derived from a C57BL/6J mouse expressing MHC H2-K(b). Because C57BL6/J mice have the MHC H2-K(b) haplotype, the B16F10 cell line expressed MHC H2-K(b) protein. MHC H2-K(d) was the haplotype cloned from the Balb/c mouse. MHC H2-K(d) was completely different from MHC H2-K(b). In addition, the immune rejection force of the Balb/c mouse against transplanted tissue derived from C57BL6/J mice is higher than that of other transplantation combinations between different mouse lines.[42] Therefore, the melanoma cell line

Table 1 Primers for screening the expected fragments

Primer set No.	Gene	Primer 1 Primer 2	Sequence
No.1	HPRT junction	HPRT 400 F	TGGAGCCATAAACAAGAAGA
		HPRT 400 R	CCTTGACCCAGAAATTCCAC
No.2	pNanog	pNanog 2.5k L2	GGACCGTCTATATAAACCCGCAGTAGC
		pNanog 2.5k R2	CAAGTTTGGATCTAAGGGGCGAGAAACT
No.3	HSV-TK	TK_dw_FW3409	GCCAATACGGTGCGGTATCTGCAGG
		TK_dw_RV3810	GCCAATACGGTGCGGTATCTGCAGG
No.4	phTERT	phTERT 1.7k F	TTACCAACAGTACCGGAATGCCAAG
		phTERT 1.7k R	GGGGTGTCTTCTGGGTATCAGCG
No.5	MHC class I	MHC K(d) F	AGGAACACAGGTGGAAAAGG
		MHC P2A R	GGTCAGTGAGACAAGCACCAG
No.6	β2 micro-globulin	b2microglobuin F	ATGGGAAGCCGAACATACTG
		Primer 3 R	CTGTTATCCCTAGCGTAACTTCTAG

Table 2 Fluorescence *in situ* hybridization analysis of Chinese hamster ovary clones containing TS-MAC

Clone name	2n + 0	2n + 1	Translocated
TS-MAC #1	1	19	0
TS-MAC #2	1	18	0

B16F10 was selected as the target cell line as representative tumorigenic cells. Three clones containing the expected TS-MAC were selected by PCR analysis (**Figure 2a**). In addition, FISH analysis confirmed that the TS-MAC was maintained independently in these representative clones (**Figure 2b**, **Table 3**). Flow cytometric analysis to measure MHC H2-K(d) and H2-K(b) with specific antibodies for each MHC haplotype on the cell surface (**Figure 2c**) revealed that B16F10 cells containing TS-MAC expressed only MHC H2-K(d), which was similar to the cell line derived from the Balb/c mouse. B16F10 clones with MAC4 did not express MHC H2-K(d) or MHC H2-K(b), which is consistent with a previous report.[43]

Next, to investigate whether TS-MAC can suppress tumor formation, the B16F10 clones expressing MHC H2-K(d) (1×10^5 cells) were subcutaneously transplanted into C57BL6/J mice. The parental B16F10 cell line and B16F10 clones containing MAC4 without the MHC H2-K(d) expression cassette were prepared. First, we subcutaneously transplanted these B16F10 cells into C57BL/6J mice under the nonimmunized condition (**Figure 3**, a-1). Therefore, tumors were observed in every experiment. The volume of tumors derived from B16F10 clones containing TS-MAC was significantly decreased compared with those derived from the parental B16F10 cell line and B16F10 clones containing MAC4 expressing EGFP ($P < 0.01$; **Figure 3b**). The majority of transplanted B16F10 cells with TS-MAC formed tumors before day 12, and some suppression effects were observed. To enhance the response of immune cells against the tumorigenic cells, mice were immunized before transplantation of these B16F10 cells with cells expressing MHC H2-K(d), which were derived from the spleens of nonobese diabetic (NOD) mice, with the immunization protocol (**Figure 3**, a-2). Generally, NOD mice express MHC H2-K(d) and MHC H2-D(b) without MHC H2-L, but C57BL/6J mice express H2-K(b), H2-D(b), and H2-L(b). There is a mismatch between NOD and C57BL/6J mice for H2-K. Hence, the immunization would give C57BL/6J mice immunity against MHC H2-K(d). Tumors were not observed in 17 of 18 transplantations of B16F10 cells with TS-MAC into immunized C57BL/6J mice at 12 days. The remaining transplantation resulted in a very small tumor (0.75 mm^3) (**Figure 3b**). These results suggest that MHC H2-K(d) expressed from TS-MAC eliminated B16F10 cells by immunization against the allogenic antigen, namely MHC H2-K(d). However, among mice with transplanted B16F10 clones containing MAC4 expressing EGFP, the volume of tumors was decreased compared with that in non-immunized mice ($P < 0.01$; **Figure 3b**).

In the transplanted mice, CD3-positive T-cells were localized in the tumors (**Figure 3d**), especially at the boundary between tumor and normal tissue areas. The number of localized T-cells in an area showed an inverse correlation with the tumor volume ($R = -0.59$; **Figure 3c**). These results suggest that the tumor cells were eliminated by the immune response via T-cells.[44]

In vitro elimination of mES cells by TS-MAC

To validate the ability of TS-MAC to eliminate undifferentiated mES cells, we transferred TS-MAC from CHO/TS-MAC#2 cells to mES cells derived from a C57BL/6J mouse. PCR analysis confirmed that four clones received TS-MAC correctly (**Supplementary Figure S1a**). FISH analysis confirmed that these clones maintained TS-MAC independently from host chromosomes without disruption (**Supplementary Figure S1b**; **Table 4**). Four clones, in which the tested regions were positive, were cultured in selection medium with ganciclovir. Parental mES cells, mES cells containing MAC4, and mES cells containing TS-MAC were compared with 55 μmol/l ganciclovir treatment for 7 days. Unfortunately, the results showed that the immature cells could not be eliminated by expressing HSV-TK under the control of the nanog promoter in TS-MAC (**Supplementary Figure S1c**).

Discussion

Gene introduction with a nonintegrating vector is generally thought to avoid the risk of tumor development through insertion of transgenes in a host genome.[45] Nonintegrating viral vectors such as Sendai virus and adeno-associated viruses often have low long-term stability. In addition, these

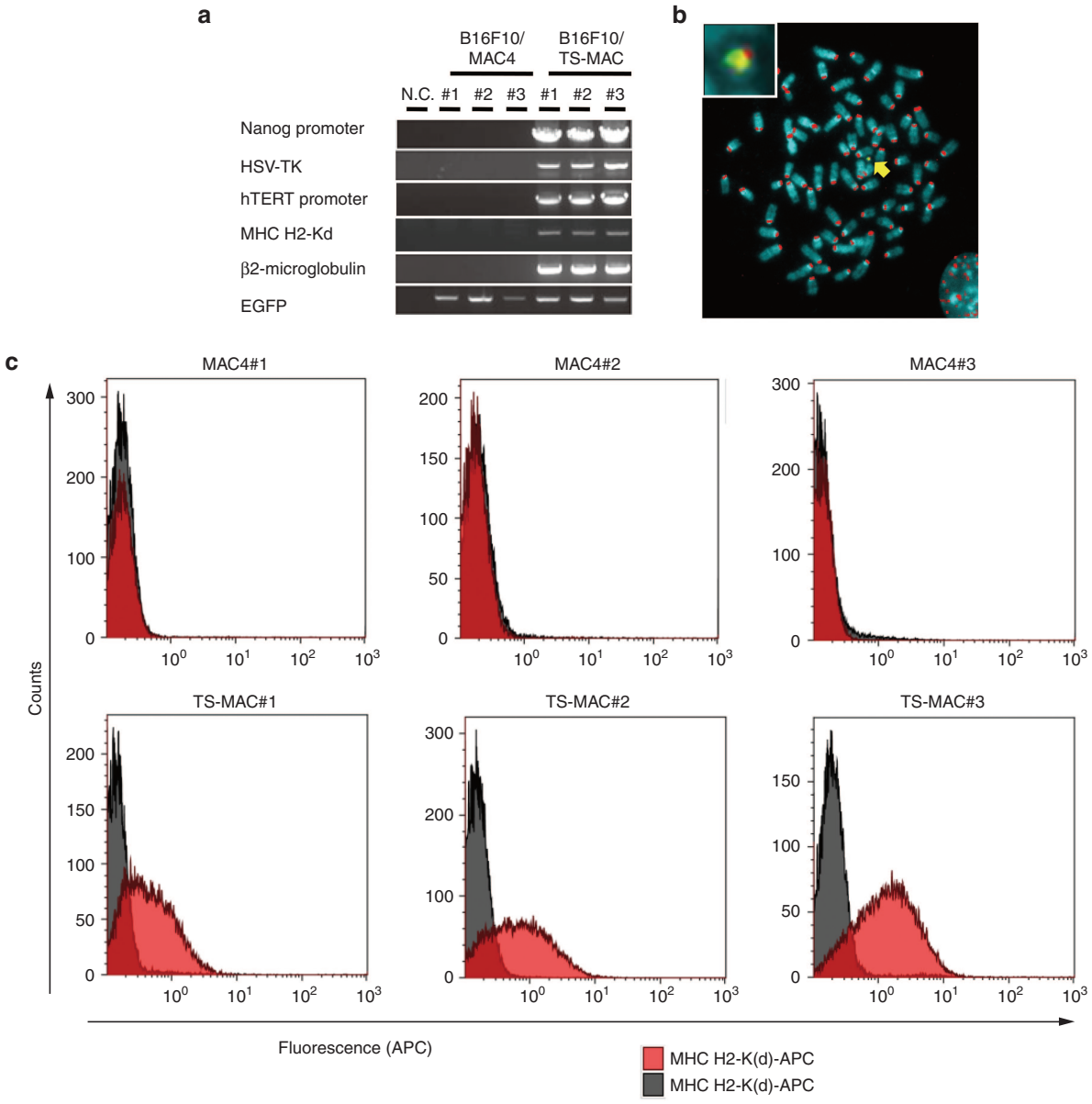

Figure 2 Generation of B16F10 clones containing TS-MAC or MAC4. (**a**) PCR analysis with the primers for B16F10 clones containing TS-MAC or MAC4 from microcell-mediated chromosome transfer (MMCT). (**b**) A representative image of FISH analysis of B16F10/TS-MAC#1. Blue indicates 4′,6-diamidino-2-phenylindole signals. The rhodamine (red) signal indicates the centromere sequence of mouse chromosomes. The fluorescein isothiocyanate signal (green), which was observed as a yellow dot, indicates the inserted PAC vector. The yellow arrow shows TS-MAC. An elongated TS-MAC is shown in the inset. (**c**) Flow cytometric analysis of B16F10 clones containing TS-MAC or MAC4 using antibodies against MHC H2-K(d) or -K(b). The gray histogram shows the counts of MHC H2-K(b) polyclonal mouse anti-goat IgG conjugated with allophycocyanin (APC) as a negative control. Red histograms show the counts of MHC H2-K(d) detected by a polyclonal mouse anti-goat IgG conjugated with APC.

Table 3 Fluorescence *in situ* hybridization analysis of B16F10 clones containing TS-MAC

Clone name	2n + 0	2n + 1	Translocated
TS-MAC #1	5	15	0
TS-MAC #2	0	20	4
TS-MAC #3	1	19	0

viral vectors sometimes activate immune reaction via their viral antigens.[46,47] In contrast, HACs and TS-MACs are non-integrating vectors that show long-term stability with independent maintenance from host chromosomes.[31,48,49] Thus, MACs may be a new tool for gene and cell therapies.[30,50–52] Here, we showed that a TS-MAC suppressed the growth of a strongly malignant tumor cell line, namely mouse melanoma cells, using the *hTERT* promoter that functions in tumors.[53] Although the expression level of MHC H2-K(d) among the clones was similar to that in CD19-positive B-cells derived from a C57BL/6J mouse (data not shown), the MHC H2-K(d) expression level from the TS-MAC was sufficient to suppress tumor growth under certain conditions.

Only one of the 18 transplantations into immunized C57BL/6J mice resulted in a very small tumor at day 12

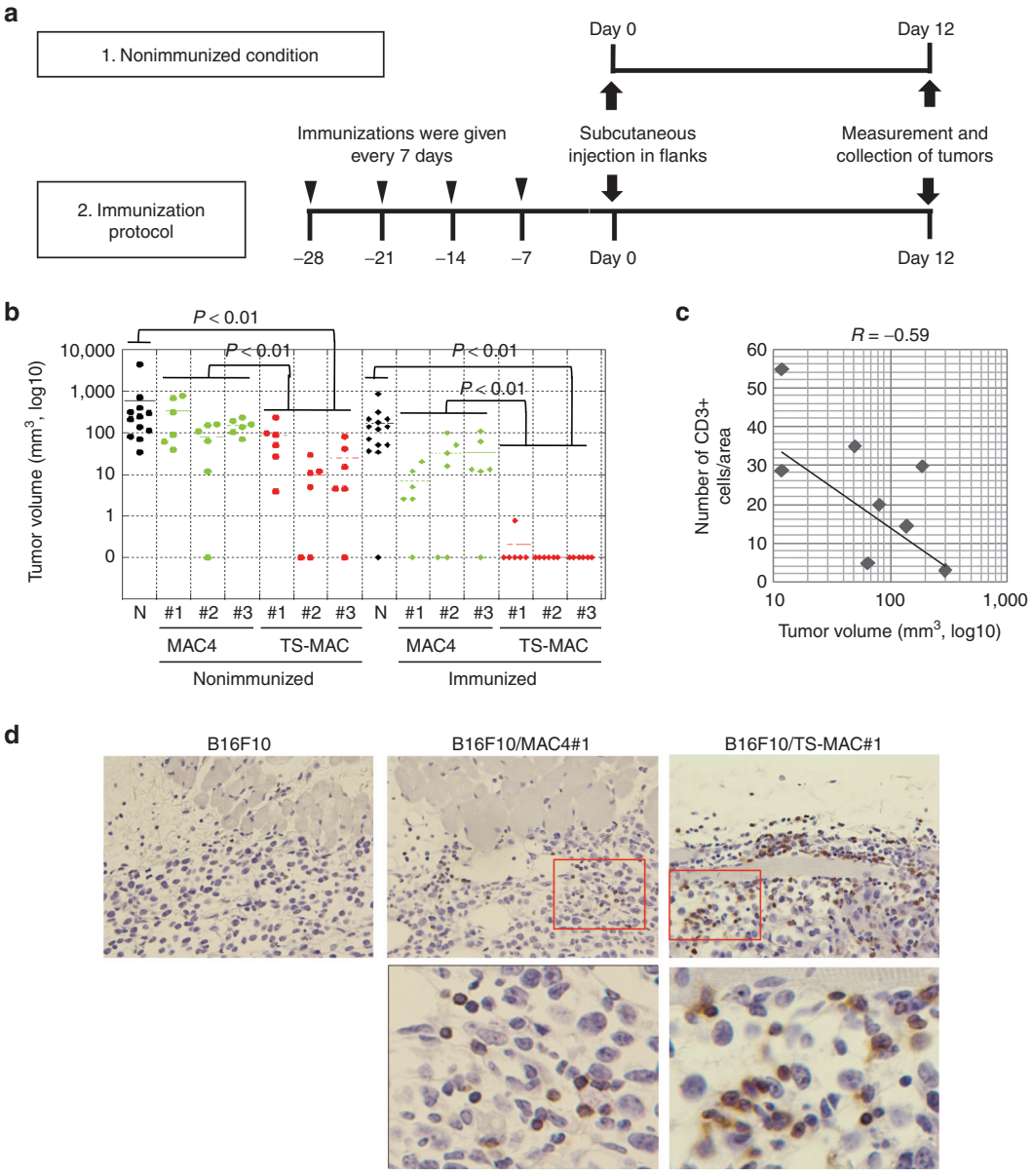

Figure 3 Tumor formation assay with B16F10 clones containing TS-MAC or MAC4. (**a**) Schema of the tumor formation assay with or without immunization. A-1 shows the nonimmunized condition without immunization. A-2 shows the immunization protocol. In the immunized protocol, splenocytes from NOD mice were injected on days –28, –21, –14, and –7. Then, each B16F10 clone was injected on day 0. On day 12, the volume of each tumor was measured. (**b**) Comparison of tumor volumes among B16F10 cells. Black dots in lane N, parental B16F10 cell line; green dots, B16F10/MAC4#1–3; red dots, B16F10/TS-MAC#1–3. (**c**) Comparison of the number of CD3-positive cells in an area of each tumor. (**d**) Immunohistochemical examination of CD3+ cells in each clone. The number of CD3+ cells was distinctly higher in tumors derived from B16F10/TS-MAC than those derived from B16F10/MAC4 or B16F10 cells. Enlarged images of the red boxed areas are shown below. Photographs were taken using a 40× objective lens.

Table 4 Fluorescence *in situ* hybridization analysis of B6ES cell clones containing TS-MAC

	Number of host chromosome + MAC			
Clone name	**39+1**	**40+0**	**40+1**	**40+2**
TS-MAC #01	1	0	19	0
TS-MAC #02	1	0	19	0
TS-MAC #03	1	4	15	0
TS-MAC #04	0	3	16	1

(**Figure 3b**). It is possibly due to the loss of the transferred TS-MAC in one-quarter of in the TS-MAC #1 clone (**Table 3**).

The reason we did not use mES cells for the *in vivo* study is that mES cells were not suitable for the proof of this concept because mES cells do not express MHC class I on their cell surface because they lack chaperones for MHC class I, such as Transporter associated with antigen processing.[54,55] In this study, B16F10 cells, a malignant melanoma cell line, were used for the proof-of-concept as a model of malignant tumors formed by transplanted cells for cell therapy.

Various promoters are available to control the expression of MHC H2-K(d) to eliminate tumor cells. Many cell types,

including stem cells, immortalized cells, and somatic cells, are being applied to cellular therapies. To adapt this concept of a safeguard system using an allogenic antigen expressed by a tumor-specific promoter in transplanted tissue, a safeguard system requires a suitable promoter to control the antigen expression in the expected tumor cells. Some promoters can achieve a high level of gene expression, whereas others may result in a low expression level. Our data showed that a promoter resulting in low expression could eliminate tumor cells, thereby strongly supporting the idea that an extensive array of promoters might be suitable for this safeguard system.

To induce stronger effects, immunization was performed with splenocytes from NOD mice. Splenocytes present not only MHC H2-K(d) but also other minor antigens. Hence, it is possible that the immune system responded to other antigens, and that the response was not specific to MHC H2-K(d). Because the immunization was crude, an experiment using purified MHC H2-K(d) protein as the immunogen should be performed in the future.

The growth suppression of B16F10 cells expressing EGFP and parental B16F10 cells in immunized mice might have been the result of an upregulated immune reaction against antigens including EGFP, the drug-resistance protein encoded on MAC4, or cancer antigens.

Requiring immunization to eliminate tumor cells completely may be impractical. Therefore, natural immunization, in which the patients have already been inoculated with viral and bacterial antigens, may be ideal. These higher immunogenic alternative antigens are expected to be effective to adapt this type of safeguard system to practical use. MHC H2-K(d) was derived from mice. To adapt the safeguard system using MHC class I, a human leukocyte antigen could be used in a safeguard system for humans.

Unfortunately, the results of our *in vitro* elimination system using HSV-TK under the control of the nanog promoter showed that the system did not act as expected. The *in vitro* elimination system did not express enough HSV-TK to kill B6ES cells, whereas overexpressed HSV-TK on a HAC eliminates tumorigenic cells[48] and undifferentiated cells.[56] A safeguard system using HSV-TK under the control of the nanog promoter has been reported previously.[28] This discrepancy might be caused by the method of gene transfer using MACs or viral vectors. The system in this study included only one copy of the gene expression cassette. However, gene introduction using a viral vector would result in higher gene expression because several copies of the transgene are introduced into a cell. Therefore, to generate an efficient cell elimination system, loading of multiple copies on the TS-MAC or enhanced expression may resolve the issue of low expression. However, it is necessary to mention the safety of gene introduction mechanisms. MACs can be maintained independently in host cells, but viral vectors are integrated into the genome. Because insertion of a viral vector has potential oncogenicity, there is a paradoxical risk of tumorigenicity in the elimination of a tumor derived from contaminating ES cells in cellular therapy. On the other hand, MACs have been shown to be safe through the generation of transchromosomic mice.[32]

Finally, an advantage of our safeguard system is that it does not require a pro-drug to eliminate tumorigenic cells via suicide genes using a tumor-specific promoter and allogenic MHC class I with the MAC. Regenerative medicine with gene and cell therapies has the potential to benefit our health in the near future. Therefore, the concept of our safeguard system will be helpful to solve the safety issues related to gene and cell therapies, as well as induction of pluripotent stem cells using episomal MACs.[56,57]

Materials and Methods

Plasmid construction. A PAC, pPH_pN_TK_pT_MHCK(d), was constructed, which contained *HSV-TK* under the control of the mouse nanog promoter, and MHC H2-K(d) and *B2M* under the control of the *hTERT* promoter. The PAC vector also contained a *loxP* site and exons 3–9 of the *HPRT* gene to mediate site-specific recombination by Cre recombinase.

A MAC vector named MAC4 (refs. [31,32]) was constructed previously and contained the *EGFP* gene under the control of the CAG promoter, *Hyg*^{r}, and a *loxP* site adjacent to exons 1–2 of the *HPRT* gene.

A nanog promoter regulatory element, which was the sequence 2.6 kbp upstream of the first ATG in the mouse *nanog* gene, was inserted into pGEM-Stm L as an *Asc*I-*Sal*I fragment.[34] The *hTERT* promoter, which was 1.7 kbp upstream of the first ATG in the *hTERT* gene, was inserted into hTERT prom4-Lu.[58] The complete DNA sequence of MHC H2-K(d)[40] and *B2M*, which were linked by a self-cleaving peptide derived from porcine teschovirus-1 (P2A) was synthesized by Genescript (Piscataway, NJ). A plasmid vector with an acceptor site for the *nanog* promoter was synthesized and named the pNanog acceptor vector. The *HSV-TK* gene was amplified from ×6.1[18] by PCR with primers TKwt_Fw (ccatggcttcgtaccccggccatc) and TKwt_Rv (aacctagggttagcctccccatctcccgt). Then, *HSV-TK* cDNA was inserted into the *Nco*I and *Nhe*I sites of the pNanog acceptor vector after digestion with *Nco*I and *Avr*II. The cDNA of tdTomato was amplified by PCR with primers tdTomato (ATGGATCCACCGG TccCGCCACCATGGT) and pB4_Asc-Cla_Fw (GCCGTA CGCTAGCATCGATCCTTAT) from pCMV-tdTomato (Clontech Laboratories, Mountain View, CA) and inserted into the *Age*I and *Pvu*I sites of the pNanog acceptor vector. Then, 2.6 kbp of the *nanog* promoter sequence was inserted into the pNanog acceptor vector at *Asc*I and *Sal*I sites. A synthesized DNA fragment from the synthe_homing endonuclease vector (Integrated Device Technology: IDT, San Jose, CA) was inserted into the pinsB4 vector at the *Asc*I and *Avr*II sites. Then, the DNA fragment containing pNanog-HSV-TK-P2A-tdTomato was inserted into the pinsB4 vector at the *Asc*I and I-*Sce*I sites. This plasmid vector was named pinsB4_pNanog promoter HSV-TK-P2A-tdTomato.

To construct pinsB4_phTERT_MHC K(d), the ICeuI-hTERT-ISceI vector was synthesized by IDT. Then, a DNA fragment of the *hTERT* promoter sequence was inserted into the ICeuI-hTERT-ISceI vector at the *Kpn*I and *Nco*I sites, and the MHC K(d)-2A-β-2 microglobulin DNA fragment was inserted into the *Pvu*I site of the ICeuI-hTERT-ISceI vector. A DNA fragment of the synthesized HNMCS vector was

inserted into pinsB4. A DNA fragment containing the *hTERT* promoter sequence was inserted into the pinsB4 vector from the ICeuI-hTERT-ISceI vector using the I-*Sce*I and I-*Ceu*I sites. This vector was named pinsB4_phTERT promoter MHC H2-K(d).

Next, a DNA fragment from pinsB4_phTERT promoter MHC H2-K(d) was inserted into the pinsB4_pNanog promoter HSV-TK-P2A-tdTomato at the *Fse*I and I-*Ceu*I sites, resulting in pinsB4_pN_TK_pT_MHCK(d).

Finally, a fragment of pPH3-9, which contained exons 3–9 of the *HPRT* gene and a *loxP* site, was inserted into pinsB4_pN_TK_pT_MHCK(d) using the *Asc*I and *Avr*II sites, constructing pPH_pN_TK_pT_MHCK(d) (**Figure 1a**).

Cell culture. CHO cells containing MAC4 or TS-MAC were maintained in Ham's F-12 nutrient mixture (Wako Pure Chemical Industries, Osaka, Japan) with 10% fetal calf serum (FCS; Biowest SAS, Nuaillé, France) and 800 µg/ml hygromycin B (Hyg) (Wako Pure Chemical Industries). mES cells, which were newly established from a C57BL/6J/Slc mouse, were cultured in mES medium consisting of Knockout Dulbecco's modified Eagle's medium (DMEM) containing 17.5% FCS, 1% sodium pyruvate, 1% minimum essential medium non-essential amino acids, 1% L-glutamine, 0.1 mmol/l 2-mercaptoethanol, 0.1% penicillin streptomycin (all purchased from Thermo Fisher Scientific, Waltham, MA), and 2,000 U/ml leukemia inhibitory factor (ES Gro; Millipore, Billerica, MA). Following the transfer of each MAC vector, B6ES cells were cultured in mES cell medium with 300 µg/ml Hyg. B16F10 cells were cultured in DMEM containing 10% FCS. B16F10 cells containing MAC4 or TS-MAC were cultured in DMEM with 300 µg/ml Hyg.

Microcell-mediated chromosome transfer. To transfer MAC vectors, we performed microcell-mediated chromosome transfer.[59] Donor CHO cells (4×10^6 cells) expanded in T-25 flasks (Thermo Fisher Scientific) were treated with Ham's-F12 medium containing 20% FCS and 0.1 µg/ml colcemid (Thermo Fisher Scientific) at 37 °C for 48 hours to induce micronucleation. Then, the culture medium was refreshed, and the cells were incubated for another 24 hours. The T-25 flask containing the CHO cells with micronuclei was filled with DMEM containing 10 µg/ml cytochalasin B (Sigma-Aldrich, St. Louis, MO) and centrifuged for 1 hour using an Avanti HP-26XP, JLA-10,500 rotor (Beckman Coulter Life Sciences, Indianapolis, IN) at 11,900 × *g* to form microcells. The pellet including microcells was collected and sequentially filtered through 8-, 5-, and 3-µm pore-size filters to purify the microcells. Microcell pellets were collected by centrifugation at 760 × *g* with a tabletop centrifuge (Kubota, Tokyo, Japan). To introduce the MAC vector into mES cells, 5×10^6 mES cells were collected and layered on the microcell pellets. To fuse the microcells and mES cells, the pellets were mixed and treated with polyethylene glycol 1500 (PEG1500) (Roche, Basel, Switzerland) containing 10% dimethyl sulfoxide Hybrimax (Sigma-Aldrich). Finally, the fused cells were expanded on a feeder layer and cultured in mES medium containing 300 µg/ml Hyg. To introduce the MAC vector into B16F10 cells, 2×10^6 B16F10 cells were prepared in a 6-cm dish (Corning Incorporated, Corning, NY). The microcell pellets were suspended in 2 ml DMEM containing 0.05 mg/ml phytohemagglutinin P (Sigma-Aldrich). This suspension was seeded on the dish containing B16F10 cells. Then, the cells were treated with PEG1500 containing 10% dimethyl sulfoxide for 20 minutes to fuse the microcells and B16F10 cells. These fused cells were incubated for 24 hours at 37 °C in DMEM containing 10% FCS and 300 µg/ml Hyg.

FISH. Metaphase chromosomes were prepared from colcemid-treated cell cultures by hypotonic treatment with 0.075 mol/l KCl and methanol/acetate (3:1) fixation. FISH was carried out using mouse cot-1 DNA labeled with digoxigenin (Roche) and the PAC vector, which was inserted into the MAC vector labeled with biotin (Roche).[31] The DNA was labeled using a nick translation kit (Roche). Hybridization of the probe and immunochemical staining were performed using the Ventana XT-Discovery System (Roche). The digoxigenin-labeled DNA was detected with an anti-digoxigenin-rhodamine complex (Roche), and the biotin-labeled DNA was detected using avidin conjugated with fluorescein isothiocyanate (Roche). The chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). Metaphase images were captured digitally with a CoolCubeI CCD camera mounted on a fluorescence microscope (Axio Imager, Z2; Carl Zeiss, Jena, Germany). Images were processed using ISIS software provided with the microscope.[31]

Plasmid transfection and gene loading to the MAC vector. Construction of the MAC4 vector has been reported previously.[31] To insert a safeguard system into MAC4, 8 µg pPH_pN_TK_pT_MHCK(d) was cotransfected with 0.1 µg pBS-185 expressing Cre recombinase into 2×10^6 CHO cells containing MAC4 using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The transfected CHO cells were incubated for 2 h, transferred to Ham's F-12 culture medium with 10% FCS, and incubated for 24 hours. The transfected cells were expanded in Ham's F12 medium with 10% FCS and HAT supplements (Sigma-Aldrich) that were added to the medium according to the manufacturer's instructions.

Genomic PCR analysis. Genomic DNA was extracted using a Gentra Puregene Cell kit (Qiagen, Valencia, CA). PCR analyses were carried out with Ex taq or LA taq (Takara Bio, Shiga, Japan). The primers (Sigma-Aldrich) are described in **Table 1**. The primer sets for detection of EGFP have been described elsewhere.[48]

Flow cytometry. Cells (1×10^6) were resuspended in 100 µl phosphate-buffered saline (PBS; Nissui, Tokyo, Japan) containing 1% FCS and incubated with 1 µl anti-MHC H2-K(d) (116603; BioLegend, San Diego, CA) or H2-K(b) (116503; BioLegend) for 1 hour at 4 °C. The cells were washed with PBS, resuspended in 100 µl PBS, and incubated with allophycocyanin-conjugated goat anti-mouse IgG (Poly4053; Biolegend) for 1 hour at 4 °C. After washing with PBS, flow cytometric analysis was performed using a Gallios Flow Cytometer (Beckman Coulter Life Sciences) and Kaluza software.

Immunization with splenocytes. The spleen of a NOD/ShiJcl mouse (CLEA Japan, Tokyo, Japan) was removed and homogenized in a 1.5-ml tube. After washing with PBS, the sample was filtered through a 40-μm pore-size filter (BD Biosciences, San Jose, CA). Then, 1×10^6 cells were suspended in 100 μl PBS and injected subcutaneously into both hind limbs of a mouse once per week for a total of four times in a month.

Tumor formation assay. Transplanted cells were collected by trypsinization, counted, and diluted to 1×10^6 cells/ml. A cell suspension (100 μl) was injected into both flanks of C57BL6/J mice (CLEA Japan) with or without immunization. After 12 days, the tumor volume was measured. The observed tumors were surgically excised and fixed in 10% formalin neutral buffer solution (Wako Pure Chemical Industries). All animal experiments were approved by the Animal Care and Use Committee of Tottori University. The tumor volume (mm^3) was defined as (height × length × width). Statistical analysis was performed using the Mann–Whitney U-test.

Immunohistochemistry. Specimens were fixed with 10% formalin and embedded in paraffin. As the primary antibody, we used a rat polyclonal antibody raised against CD3 (1:100; AbD serotec, Oxford, UK). Briefly, paraffin-embedded sections were dewaxed with xylene and hydrated gradually. Antigen retrieval was performed by incubation in 10 μg/ml Proteinase K (Sigma-Aldrich)/PBS(-) for 30 minutes. Endogenous peroxidase activity was blocked by immersing the sections in 0.3% hydrogen peroxide/methanol for 30 minutes. The sections were reacted with the primary antibody overnight at 4 °C and then treated with a Histofine Simple Stain Rat MAX-PO kit (Nichirei Biosciences, Tokyo, Japan) for 30 minutes at room temperature. Immunoreactions were visualized with diaminobenzidine (Wako Pure Chemical Industries). The sections were counterstained with hematoxylin. Photographs were taken using a CCD camera mounted on a microscope (Nikon, Tokyo, Japan). Images were processed using the software provided with the microscope.

Supplementary Material

Figure S1. *In vitro* elimination of ES cells containing the TS-MAC by ganciclovir treatment.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)