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New selective amplifier genes containing c-Mpl for hematopoietic cell expansion

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Abstract

We previously developed "selective amplifier genes (SAGs)" which confer a growth advantage to transduced cells. The SAG is a chimeric gene encoding the G-CSF receptor (GCR) and the estrogen or tamoxifen (Tm) receptor and is able to expand transduced hematopoietic cells by treatment with estrogen or Tm. In the current study, we examined the in vitro efficacy of modified SAGs containing the thrombopoietin (TPO) receptor (c-Mpl) gene instead of GCR as a more potent signal generator. In addition, we constructed various mutant Mpl-type SAGs to abolish the responsiveness to endogenous TPO while retaining Tm-dependency. When Ba/F3 cells were retrovirally transduced with the Mpl-type SAGs, the cells showed Tm- and TPO-dependent growth even without IL-3. The Mpl-type SAGs induced more potent proliferation of Ba/F3 and cynomolgus $CD34^+$ cells than the GCR-type SAG. One mutant Mpl-type SAG (ΔGCRMplTmR) successfully lost the responsiveness to TPO without affecting the Tm-dependence.

Keywords: Selective amplifier gene; Hematopoietic stem cells; Mpl; Retroviral vector; Gene therapy; In vivo expansion

Hematopoietic stem cells (HSCs) are ideal targets of gene therapy for many disorders due to their self-renewal and multilineage differentiation abilities. Retroviral vectors are the most commonly used vehicles for gene transfer into HSCs. Although current retroviral vectors successfully deliver genetic materials into murine HSCs [1], the gene transfer efficiency into human HSCs is insufficient for most clinical applications [2,3]. For the achievement of clinically relevant levels of gene transfer into human HSCs with retroviral vectors, several approaches have been tried, including pseudotyping with other viral envelopes such as the gibbon ape leukemia virus envelope [4] and RD114 [5], utilizing the fibronectin fragment (CH-296) [6], and selecting and expanding successfully transduced cells in vivo [7–9]. Lentiviral vectors that can transduce nondividing cells may be more suitable gene transfer vehicles into HSCs as these cells are generally quiescent [10].

Although in vivo selection of transduced hematopoietic cells with drug-resistant genes has been studied intensively to overcome the low gene transfer efficiency into HSCs [7,8], we developed another device to directly expand transduced cells by conferring a proliferative advantage to the gene-modified cells relative to their untransduced counterparts. These genes have been designated as "selective amplifier genes (SAGs)." The prototype SAG encodes a chimeric receptor consisting of the granulocyte colony-stimulating factor (G-CSF) receptor (GCR) and the hormone-binding domain of the estrogen or tamoxifen receptor. The GCR moiety is a signal generator and the estrogen or tamoxifen receptor moiety is a molecular switch to regulate the growth signal generated from GCR. We have previously shown

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that these prototype GCR-type SAGs can selectively expand the transduced hematopoietic cells in an estrogen- or tamoxifen-dependent manner in vitro [11–13] and in vivo in mice (unpublished). We have also reported that the GCR-type SAGs expanded transduced hematopoietic cells in a nonhuman primate (cynomolgus monkey) transplantation model. However, not all treated monkeys showed an increase in transduced cells in response to estrogen or tamoxifen in vivo, suggesting that the responses of the GCR-type SAGs to exogenous drugs may be variable among monkeys [14]. More potent SAGs would be required to achieve enough in vivo expansion in nonhuman primates.

In this respect, we paid attention to the fact that the thrombopoietin (TPO) receptor, c-Mpl, is expressed in very immature hematopoietic cells and that TPO actually stimulates the growth of these cells [15–17]. The intracellular signal from c-Mpl may be thus more appropriate than that from GCR for expansion of hematopoietic stem/progenitor cells. In the present study, we constructed another class of SAGs consisting of c-Mpl and the tamoxifen receptor (TmR) (designated as Mpl-type SAG) and examined their efficacy in vitro.

Materials and methods

Cells. IL-3 dependent mouse proB cell line Ba/F3 cells [18] were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin/streptomycin (Gibco-BRL), and 10 ng/ml recombinant mouse IL-3 (rmIL-3; Gibco-BRL). The ecotropic packaging cell line BOSC23 [19] and human embryonic kidney 293T cells were maintained in DMEM containing 10% FBS.

Bone marrow cells were harvested from femora of healthy adult cynomolgus monkeys reared in the Tsukuba Primate Center, National Institute of Health Japan (Tsukuba, Ibaraki, Japan). The cells were suspended in ACK lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; Wako, Osaka, Japan) to dissolve the red blood cells. Immunomagnetic selection of $CD34^+$ cells was conducted using the Dynabeads kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. The $CD34^+$ cells were frozen in a CellBanker (Nippon Zenyaku, Tokyo, Japan) and kept in liquid N_2 until use.

Plasmid construction. The plasmid pcDNA3.1-c-mpl (provided by Dr. M. Takatoku, Jichi Medical School, Tochigi, Japan) [20] containing the cDNA for human c-Mpl between the *Eco*RI and *Xba*I sites was digested with *Eco*RI and *Sac*I. The other part of c-Mpl cDNA between the *Sac*I site and C-terminal was generated by polymerase chain reaction (PCR) using the pcDNA-c-Mpl as a template with the primer pair 5′-CCC ACC TAC CAA GGT CCC TGG-3′ and 5′-CGG GAT CCA GAG GCT GCT GCC AAT AG-3′. The fragment containing the murine phosphoglycerate kinase (pgk) promoter and neomycin phosphotransferase gene (neo) (*Eco*RI–*Bam*HI) in the retroviral plasmid pMSCV2.2 (a gift from Dr. R.G. Hawley, University of Toronto, Canada) [21] was replaced with the *Eco*RI–*Sac*I fragment of the c-Mpl cDNA and the *Sac*I–*Bam*HI fragment of the c-Mpl cDNA by trimolecular ligation to construct pMSCV-Mpl. The tamoxifen receptor (TmR) cDNA was cloned from pMSCV-ΔGCRTmR [13] by PCR using the primer pair 5′-CTG GAT CCG GGC ACT TCA GGA GAC-3′ and 5′-CTG TCG ACC ACT AGT AGG AGC TCT CA-3′. The *Bam*HI–*Sal*I fragment of the TmR cDNA was ligated into the *Bam*HI–*Sal*I site of pMSCV-Mpl. The resultant plasmid expressing a chimeric MplTmR gene was designated as pMSCV-MplTmR.

The truncated Mpl mutants (Δ(99–267) = deletion of 99–267 amino acids, Δ(281–472), Δ(40–267), Δ(99–472), Δ(71–494), and Δ(40–472); Fig. 2A) were constructed by PCR using the primer pairs shown in Table 1. All PCR products were digested with *Eco*RI and *Bam*HI. The digested fragment (*Eco*RI–*Bam*HI) containing each truncated mutant and the TmR cDNA fragment (*Bam*HI–*Sal*I) were inserted into the *Eco*RI–*Sal*I site of pMSCV by trimolecular ligation to construct retroviral plasmids expressing the truncated Mpl mutants.

pMSCV-ΔGCRMplTmR was constructed as follows. The extracellular domain was from GCR cDNA lacking the ligand (G-CSF)-binding sequence (5–195 amino acids) [22], and was prepared by digesting pMSCV-ΔGCRTmR with *Hin*dIII and *Kpn*I. For the transmembrane fragment, the corresponding part of the GCR cDNA was used and prepared by PCR using pMSCV-ΔGCRTmR as a template with the primer pair 5′-GAG TGG GTA CCT GAG GCC CCT AGG-3′ and 5′-AAC TCG AGG CAG CAG AGC CAG GTC AC-3′. The cytoplasmic domain was from c-Mpl cDNA and again generated by PCR using the pcDNA-c-Mpl as a template with the primer pair 5′-AAC TCG AGA GGT GGC AGT TTC CTG CA-3′ and 5′-CGG GAT CCA GAG GCT GCT GCC AAT AG-3′. The extracellular fragment was cloned into the *Hin*dIII–*Kpn*I site of pEGFP-N1 (Clontech, Palo Alto, CA, USA) (pEGFP-ΔGCR) and then the transmembrane and cytoplasmic fragments were integrated into the *Kpn*I–*Bam*HI site of the obtained pEGFP-ΔGCR to form pEGFP-ΔGCRMpl. The fragment carrying pgk and neo (*Bgl*II–*Sal*I) in pMSCV was replaced with the ΔGCRMpl cDNA (*Bgl*II–*Bam*HI fragment from pEGFP-ΔGCRMpl) and TmR cDNA (*Bam*HI–*Sal*I fragment from pMSCV-MplTmR). The resultant construct was designated as pMSCV-ΔGCRMplTmR.

Table 1
Primers for construction of Mpl deletion mutants

Mutants	Primers	Sequences
Δ(99–267)	A	5′-CCGTTAACTCCTGGGGATCCTGGTCC-3′
	B	5′-CCGTTAACCTGGTCTGGAAACTGGCA-3′
Δ(40–267)	A	Described above
	C	5′-CCGTTAACCTTCAGGGGCTCTGAGTC-3′
Δ(281–472)	D	5′-CCGTTAACCCCTGGAGCTCGTGGTCG-3′
	E	5′-CCGTTAACCAGGTCCACAGTCACAGG-3′
Δ(99–472)	D	Described above
	B	Described above
Δ(40–472)	D	Described above
	C	Described above
Δ(71–494)	E	5′-CCGAATTCATGTGGAGCTCGTGGTCG-3′
	F	5′-CCGAGCTCCAGCAAGTGAGGTCCT-3′

pMSCV-ΔGCRTmR-ires-EGFP, pMSCV-MplTmR-ires-EGFP, and pMSCV-ΔGCRMplTmR-ires-EGFP were derived from pMSCV-ΔGCRTmR, pMSCV-MplTmR, and pMSCV-ΔGCRMplTmR, respectively, by inserting the *Xho*I–*Cla*I fragment containing ires-EGFP derived from the *Xho*I–*Cla*I digenst of pMSCV-ires EGFP to the *Sal*I–*Cla*I sites [13].

Retroviral vectors. To obtain ecotropic retroviral vectors, we transfected BOSC23 cells with mouse stem cell virus (MSCV)-based retroviral plasmids (derivatives from pMSCV) using the Transfection MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The supernatants harvested at 48–72 h after transfection exhibited titers of 1×10^6/ml as assessed by RNA dot-blot. To obtain amphotropic retroviral vectors, we transfected 293T cells with MSCV-based retroviral plasmids along with pCL-Ampho (Imgenex, San Diego, CA) using the Transfection MBS Mammalian Transfection Kit (Stratagene). The supernatants harvested at 48–72 h after transfection showed titers of 1×10^6/ml as assessed by RNA dot-blot.

Transduction and culture. Ba/F3 cells were suspended at a density of 1×10^5 cells/ml in 1 ml viral supernatant containing 10 ng/ml rmIL-3 and transferred to six-well plates coated with 20 μg/cm^2 of RetroNectin (Takara Bio, Shiga, Japan) [8]. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 h. During this period, the culture medium was replaced with fresh viral supernatant every 12 h. As an untransduced control, Ba/F3 cells were similarly cultured in medium not containing virus. Transduced or untransduced Ba/F3 cells were then cultured in DMEM containing 10% FBS in the presence or absence of 10 ng/ml rmIL-3, 10 ng/ml recombinant human (rh) TPO (provided by Kirin Brewery, Tokyo, Japan) or 10^{-7} M 4-hydroxytamoxifen (OH-Tm, an active metabolite of Tm; Sigma, St. Louis, MO).

Cynomolgus CD34$^+$ cells were placed in six-well plates coated with 20 μg/cm^2 of RetroNectin and cultured for 24 h at 37 °C with 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 50 ng/ml rhIL6 (provided by Ajinomoto, Osaka, Japan), 100 ng/ml rhSCF (provided by Amgen, Thousand Oaks, CA), 100 ng/ml rhFlt-3 ligand (Research Diagnostic, Flanders, NJ), and 100 ng/ml rhTPO. The cells were resuspended in 1 ml viral supernatant containing cytokines as described above at a density of 1×10^5 cells/ml. During the transduction, the culture medium was replaced with fresh viral supernatant every 12 h. Mock transduction was performed using medium not containing retroviral vectors.

Western blot analysis. Approximately 5×10^4 untransduced or transduced cells were suspended in 2× Tris–SDS SepraSol (Owl, Woburn, MA) and then boiled at 100 °C for 5 min. The protein samples were resolved on a 7.5% SDS–polyacrylamide gel (Multigel 7.5; Daiichi, Tokyo, Japan) followed by electroblotting to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was incubated with blocking buffer (Block Ace; Dainippon, Osaka, Japan) and then with anti-ER antibody MC-20 (Santa Cruz, Santa Cruz, CA) for 1 h at room temperature. The membrane was washed with the blocking buffer five times and incubated with goat phycoerythrin (PE)-conjugated anti-rabbit IgG antibody (Cedarlane, Ont., Canada) for 1 h at room temperature. After washing the membrane five times with the blocking buffer, the fusion proteins with TmR were visualized by an ECL system (Amersham, Little Chalfont, UK).

Flow cytometry. To assess the EGFP expression, transduced cells were washed and subjected to FACSCalibur flow cytometry (Becton–Dickinson, Palo Alto, CA) using excitation at 488 nm and fluorescence detection at 530±30 nm. Untransduced cells served as negative controls.

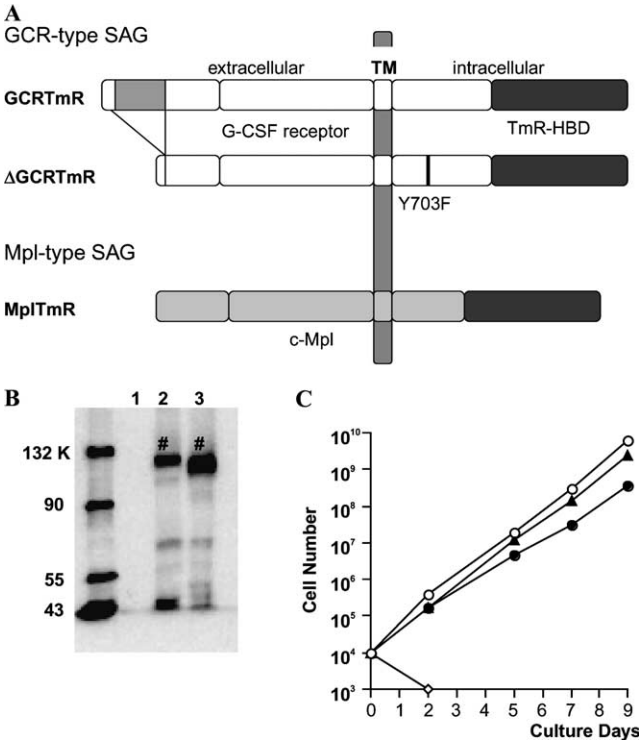

Fig. 1. Construction of modified SAGs. (A) GCRTmR is a GCR-type SAG consisting of the murine G-CSF receptor (GCR) and the tamoxifen receptor (TmR). In ΔGCRTmR, the ligand (G-CSF)-binding domain of GCR was deleted to abolish the responsiveness to G-CSF. In addition, the tyrosine residue at the 703rd amino acid was replaced with phenylalanine to hamper the differentiation signal. Mpl-TmR is an Mpl-type SAG consisting of the full-length human c-Mpl and the TmR, which responds to both TPO and Tm. TM, transmembrane. (B) Western blot analysis of transduced Ba/F3 cells. Ba/F3 cells were transduced with the retroviral vector expressing ΔGCRTmR or MplTmR. Lysates of untransduced Ba/F3 cells (lane 1), Ba/F3 cells transduced with the ΔGCRTmR vector (lane 2) or with MplTmR vector (lane 3) were immunoblotted with anti-ER antibody. (C) Growth of Ba/F3 cells expressing MplTmR in suspension culture in the presence (open circle) or absence (open diamond) of IL-3 (10 ng/ml), TPO (closed triangle) (10 ng/ml) or Tm (closed circle) (10^{-7} M). Cell counts are shown in a log scale.

Results and discussion

The structures of SAGs in this study are shown in Fig. 1A. The prototype SAG is a chimeric gene encoding GCR and the estrogen receptor hormone-binding domain [11]. In GCR, the tyrosine residue at the 703rd amino acid was replaced with phenylalanine to prevent the generation of a differentiation signal [12]. In the hormone-binding domain of the estrogen receptor, a mutation (G525R) was introduced to hamper the binding of endogenous estrogen to the SAGs retaining the responsiveness to synthetic hormones such as tamoxifen (Tm) [13,23]. The GCR-type SAG containing this mutant estrogen receptor (Tm receptor, TmR) is designated as GCRTmR. To remove the responsiveness to endogenous G-CSF from GCRTmR, the ligand (G-CSF)-binding domain was deleted (ΔGCRTmR). The new SAG in this study of Mpl-type, the Mpl-type SAG, MplTmR, is a chimeric gene in which the GCR moiety of GCRTmR is replaced by c-Mpl. Thus, MplTmR was

designed to generate growth signals in response to both TPO and Tm.

To evaluate the functions of these SAGs in vitro, we used Ba/F3 cells as targets. Murine pro-B cell growth is dependent on IL-3 and the cells enter the apoptotic cycle in the absence of the cytokine [24]. Neither TPO nor Tm supported their growth (data not shown). To generate ecotropic retroviral vectors expressing the ΔGCRTmR or MplTmR gene, we transfected BOSC23 cells with pMSCV-ΔGCRTmR or pMSCV-MplTmR. The culture supernatants were used for infection of Ba/F3 cells. The transgene expression was confirmed by Western blotting with anti-ER (lanes 2 and 3 in Fig. 1B), while endogenous ER was not detected in the parental Ba/F3 cells (lane 1). The blots indicated that the molecular weights of ΔGCRTmR and MplTmR were approximately 120 kDa (lane 2) and 115 kDa (lane 3), respectively, as predicted in their molecular design. The Ba/F3 cells expressing ΔGCRTmR (BaF3/ΔGCRTmR) showed hydroxytamoxifen (an active metabolite of Tm)-dependent growth as it has been reported [13]. The Ba/F3 cells expressing MplTmR (BaF3/MplTmR) proliferated in the presence of either TPO or Tm as well as IL-3 (Fig. 1C), suggesting that the Mpl-type SAG (MplTmR) is able to induce the proliferation of Ba/F3 cells in a TPO- or Tm-dependent manner.

To avoid responses to endogenous TPO, we deleted the extracellular domain of c-Mpl. The response of transduced cells to endogenous TPO should be avoided to strictly control the growth signal generated from the Mpl-type SAG. Since putative TPO-binding sites of Mpl have not been described yet, we generated retroviral vectors which express various mutant SAGs lacking the extracellular domain of c-Mpl; Δ(99–267), Δ(281–472), Δ(40–267), Δ(99–472), Δ(71–494), and Δ(40–472) as shown in Fig. 2A and examined which mutant most efficiently abolished the responsiveness to TPO without losing the reactivity to Tm. Ba/F3 cells were transduced with each of such truncated MplTmR-expressing vectors. The expression of each SAG was confirmed by Western blotting with anti-ER (Fig. 2B). However, the expression of these mutants in Ba/F3 cells resulted in a total loss of responsiveness not only to TPO but also to Tm (data not shown). The deletion in the extracellular domain of c-Mpl seemed to hamper the dimerization of the SAG protein.

Regarding the GCR-type SAGs, the deletion in the extracellular domain of the GCR moiety successfully lost responsiveness to G-CSF while remaining responsive to steroids [11,13]. Based on these observations we replaced the extracellular portion of MplTmR with that of the GCRTmR, which lacks the natural ligand (G-CSF)-binding ability to solely knock out the TPO-binding ability without spoiling the growth dependency on Tm, and thus obtained another mutant Mpl-type SAG designated as ΔGCRMplTmR (Fig. 3A). A retroviral vector expressing ΔGCRMplTmR was generated and Ba/F3 cells were stably transduced with the vector. Western blotting confirmed a transgene expression at the molecular weight of approximately 110 kDa (Fig. 3B). The Ba/F3 cells expressing ΔGCRMplTmR now successfully showed Tm-dependent growth, while they did not show any response to G-CSF or TPO (Fig. 3C). Thus, the new SAG can now be strictly controlled only by treatment with a synthetic hormone such as Tm.

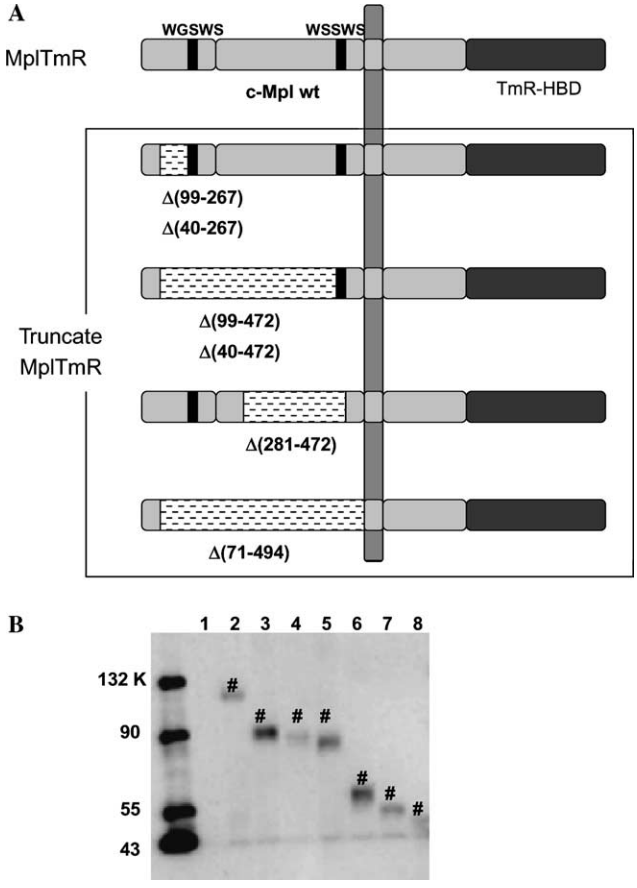

Fig. 2. Deletion of the TPO-binding sites from MplTmR. (A) A series of truncated MplTmR mutants. (B) Western blot analysis of transduced Ba/F3 cells. Ba/F3 cells were transduced with the retroviral vectors expressing truncated Mpl-TmR mutants. The cell lysates were immunoblotted with anti-ER antibody. Lane 1, untransduced Ba/F3 cells; lane 2, Ba/F3 cells transduced with the intact MplTmR gene; and lanes 3–8, Ba/F3 cells transduced with the deletion mutant genes (Δ(99–267), Δ(281–472), Δ(40–267), Δ(99–472), Δ(71–494), and Δ(40–472), respectively).

To compare the Mpl- and GCR-type SAG for their abilities to expand hematopoietic cells, we transduced Ba/F3 cells with retroviral vectors expressing these SAGs and examined the Tm-dependency of cell growth (Fig. 3D). The Mpl-type SAG (MplTmR) induced more potent proliferation of Ba/F3 cells in response to Tm than the GCR-type SAG (ΔGCRTmR). Although the proliferation rates induced by the mutant Mpl-type SAG lacking the TPO-binding ability (ΔGCRMplTmR) were lower than those induced by the parental Mpl-type

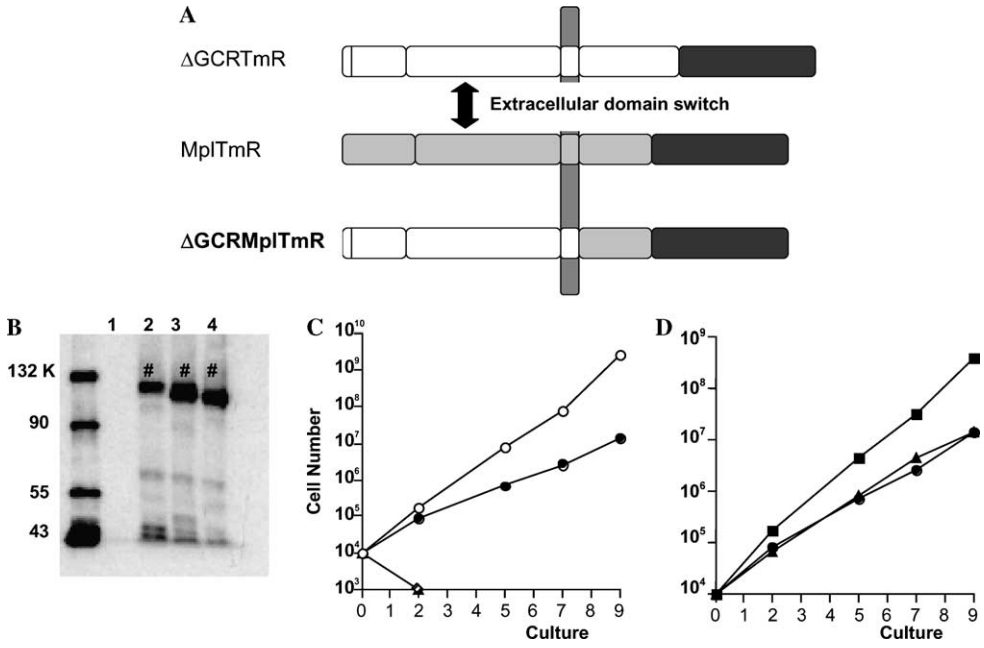

Fig. 3. Construction of a mutant MplTmR (ΔGCRMplTmR) lacking the TPO-responsiveness without affecting the Tm-responsiveness. (A) Replacing the extracellular domain of MplTmR with that of ΔGCRTmR. (B) Western blot analysis of transduced Ba/F3 cells. The cell lysates were immunoblotted with anti-ER antibody. Lane 1, untransduced Ba/F3 cells; lanes 2–4, Ba/F3 cells transduced with the ΔGCRTmR, MplTmR, and ΔGCRMplTmR genes, respectively. (C) Growth of Ba/F3 cells expressing ΔGCRMplTmR in suspension culture in the presence (open circle) or absence (open diamond) of IL-3 (10 ng/ml), in the presence of TPO (closed triangle)(10 ng/ml), or in the presence of Tm (closed circle) (10^{-7} M). (D) Growth of Ba/F3 cells transduced with the ΔGCRTmR (closed triangle), MplTmR (closed square), or ΔGCRMplTmR gene (closed circle) in the presence of Tm (10^{-7} M).

SAG (MplTmR), they were still comparable to those induced by the GCR-type SAG (ΔGCRTmR). The Ba/F3 cells expressing the Mpl-type SAGs (either MplTmR or ΔGCRMplTmR) showed long-term proliferation in the presence of Tm even without IL-3, and the cells died upon removal of Tm from the culture media (data not shown). Thus, our SAG can now be strictly controlled only by treatment with a synthetic hormone such as Tm. This finding suggests that Tm can strictly control the growth signal in the transduced Ba/F3 cells.

We constructed bicistronic retroviral plasmids which express the Mpl-type SAG (MplTmR or ΔGCRMplTmR) gene as the first cistron and the enhanced green fluorescent protein (EGFP) gene as the second cistron (designated as pMSCV-MplTmR-ires-EGFP or pMSCV-ΔGCRMplTmR-ires-EGFP). To generate amphotropic retroviral vectors, 293T cells were transfected with the retroviral plasmids along with a helper plasmid expressing the amphotropic envelope. Cynomolgus $CD34^+$ bone marrow cells were transduced with these amphotropic retroviral vectors by the supernatant transduction method. Following transduction, the cells were cultured in medium containing Flt-3, TPO or Tm. As shown in Fig. 4A, while the fractions of GFP^+ cells did not increase in the $CD34^+$ cells transduced with the GCR-type SAG retroviral vector (MSCV-ΔGCRTmR-ires-EGFP) in the presence of Flt-3 or TPO, the fraction expanded by about 6-fold (8–47%) during the first week's culture in the presence of Tm, but subsequently decreased to 5% during the second week's culture. On the other hand, as shown in Fig. 4B, the fraction of GFP^+ cells transduced with the Mpl-type SAG retroviral vector (MSCV-MplTmR-ires-EGFP) increased by 2-fold (23–50%) during the first week's culture in the presence of Tm, and the fraction did not decrease during the second week's culture. Stimulation of the transduced cells with the natural ligand TPO induced a remarkable increase in GFP^+ cells, and over 90% of cells became GFP^+ cells after a two-week culture with TPO. The fraction of GFP^+ cells transduced with the mutant Mpl-type SAG lacking the TPO-binding ability (MSCV-ΔGCRMplTmR-ires-EGFP) also increased by about 2-fold (12–26%) during the second week's culture (Fig. 4C). We repeated this experiment four times and all the results suggest that the Mpl-type SAG can expand transduced $CD34^+$ cells more efficiently than the GCR-type SAG.

In this study, we utilized the c-Mpl signal transduction domain to develop another class of SAGs (Mpl-type SAGs) and examined their ability in vitro using murine Ba/F3 cells and cynomolgus bone marrow $CD34^+$ cells. We have shown that the intracellular signal from the Mpl-type SAGs is more potent than that from the GCR-type SAGs for the expansion of these cells. Blau et al. [25] also showed that the signal generated from c-Mpl, GCR, and Flt-3

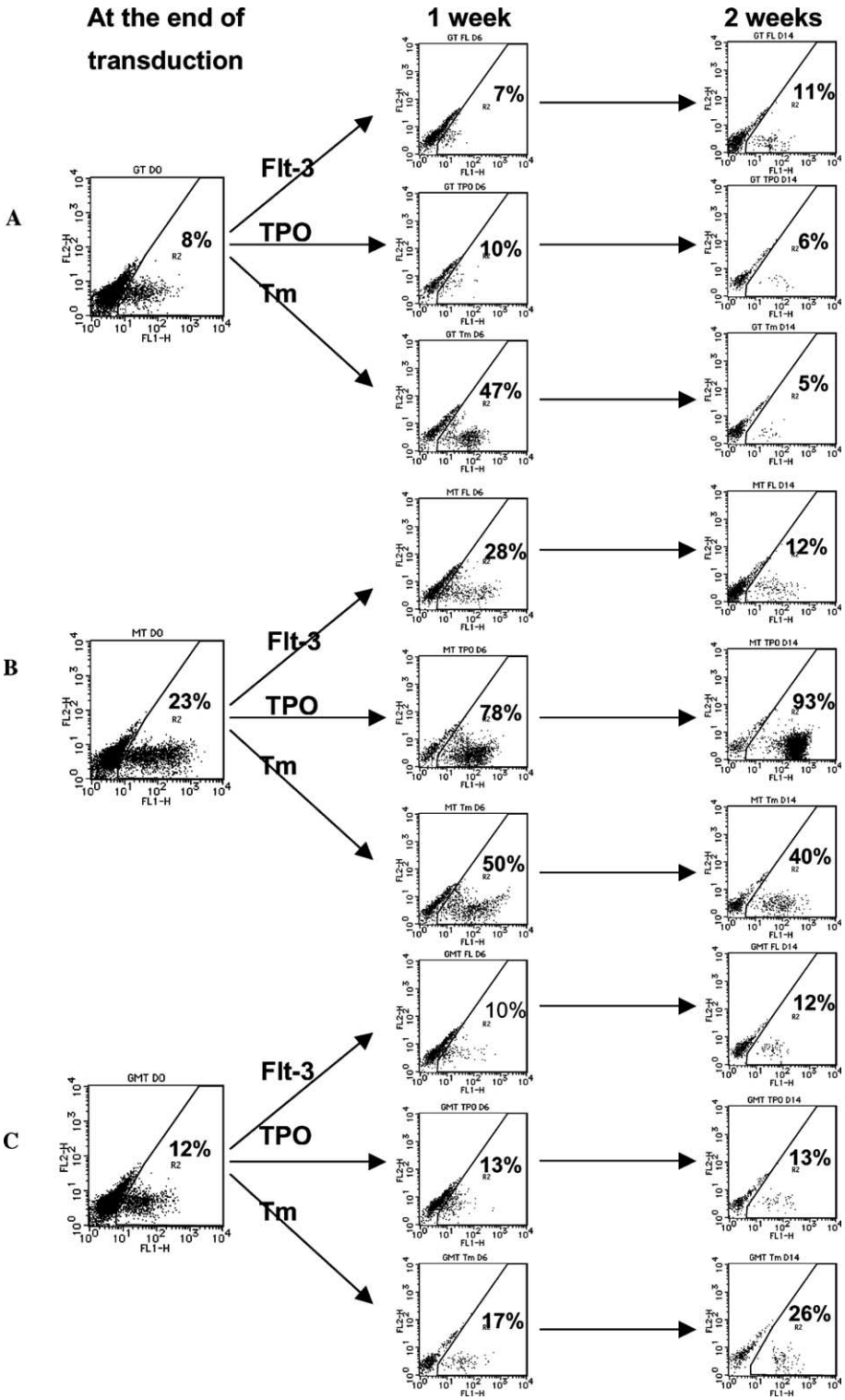

Fig. 4. Tm-induced expansion of cynomolgus bone marrow CD34$^+$ cells transduced with (A) MSCV-ΔGCRTmR-ires-EGFP, (B) MSCV-MplTmR-ires-EGFP, or (C) MSCV-ΔGCRMplTmR-ires-EGFP. The transduced CD34$^+$ cells were cultured with Flt-3 ligand (100 ng/ml), TPO (100 ng/ml), or Tm (10^{-7} M). The cells were analyzed for EGFP expression by flow cytometry on the indicated weeks after transduction. The values represent percentage of EGFP$^+$ cells.

supported the growth of Ba/F3 cells, but that only Mpl among them supported the sustained growth of transduced murine bone marrow cells. Taken together, the intracellular signal by c-Mpl was confirmed to be suitable for the expansion of immature hematopoietic cells.

However, the growth signal of the Mpl-type SAGs generated by Tm was considerably attenuated compared to that generated by the natural ligand TPO (Fig. 4B). The Tm-mediated dimerization of MplTmR may be less efficient than the TPO (natural ligand)-mediated dimerization. Since c-Mpl is a membrane receptor while the estrogen or tamoxifen receptor is a nuclear one, they may not be so compatible for the dimerization. Now, we are constructing further modified SAGs, in which a signal generator and a molecular switch are both membrane proteins.

Acknowledgments

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