zole.[10] Rabeprazole is less affected by CYP2C19 and CYP3A4s than omeprazole or lansprazole.[11] but no study has been made on the association of *CYP2C19* and *CYP3A5* genotypes with the dose-normalized concentration of tacrolimus coadministered with rabeprazole in LDLT patients.

In liver transplantation, native intestine (recipient) and graft liver (donor) often differ in genetic background. Thus, different genotypes of intestinal and hepatic CYPs may regulate the clearance of tacrolimus.

We conducted a case-control study to compare dose-normalized concentrations of tacrolimus between LDLT patients treated with and without rabeprazole during the same post operative period, taking into consideration the genotypes of *CYP2C19* and *CYP3A5* in native intestine and graft liver, separately.

## Patients and Methods

**Patients:** The study included 55 *de novo* adult LDLT patients and their 55 corresponding donors enrolled in this study between February 2004 and December 2008, with prior written informed consent. The patients (all Japanese) receiving tacrolimus (Prograf®, Astellas Pharma Inc., Tokyo, Japan) with (n = 38) and without (n = 17) rabeprazole (Pariet®, Eisai Co. Ltd, Tokyo, Japan) at 10 mg/day, were studied on days 22–28 post transplantation.

The study was in accordance with the Declaration of Helsinki and its amendments and approved by *the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee*.

**Dosage regimen of tacrolimus and measurement of tacrolimus concentrations:** The basic immunosuppression regimen consisted of tacrolimus with low-dose steroids.[12] Tacrolimus was administered orally at 0.075 mg/kg every 12 hours from the evening of postoperative day 1.[12,13] The target of whole-blood trough concentration of tacrolimus was set between 10 and 15 ng/mL during the first 2 weeks. Steroid treatment was started at graft reperfusion at 10 mg/kg, with gradual reduction from 2 $mg \cdot kg^{-1} \cdot day^{-1}$ to 0.3 $mg \cdot kg^{-1} \cdot day^{-1}$ during the first 2 weeks after surgery. The dosage of tacrolimus was adjusted on the basis of whole-blood trough concentrations measured 12 hours after the evening dosage every day, using a semiautomated microparticle enzyme immunoassay (IMx®; Abbott, Tokyo, Japan).[13]

**Evaluation of drug interactions between tacrolimus and rabeprazole:** Because the oral administration of rabeprazole started approximately two weeks after surgery, we evaluated data on postoperative days 22–28. The clinical course of all patients was stable. The average dose-normalized blood concentration of tacrolimus during this observation period was assessed as the concentration/dose (C/D) ratio [(ng/mL)/(mg/day)] of tacrolimus for each patient and used for analysis.

We excluded data obtained during treatment with a temporal high-dose steroid injection against acute cellular rejection due to inducement of the intestinal expression of CYP3A4.[14] We also excluded data during concomitant use of azoles, potent inhibitors of CYP3A and P-glycoprotein,[15] for prophylaxis of fungal infection.

**Genotyping:** Genomic DNA was extracted from the peripheral blood of transplant patients or donors with a Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI). Because two *CYP2C19* variant alleles, *CYP2C19*2* and *CYP2C19*3*, account for the poor metabolizer phenotype in the Japanese,[16] detection of the wild-type allele (**1*) and these two variant alleles was performed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.[16,17] The *CYP3A4*1B* allele is very rare in Japanese,[18] but the *CYP3A5*3* allele resulting in functional CYP3A5 deficiency is reported as the major variant allele in the Japanese population, with a 0.759 frequency.[19] Therefore, in this study we genotyped *CYP3A5*3* polymorphism as described previously.[18,20,21]

**Classification of patients:** The patients and their corresponding donors were separately classified into 3 groups on the basis of the *CYP2C19* genotype as follows: *CYP2C19*1/*1* (extensive metabolizers, EMs), *CYP2C19*1/*2* or *CYP2C19*1/*3* (intermediate metabolizers, IMs), and *CYP2C19*2/*2*, *CYP2C19*3/*3*, or *CYP2C19*2/*3* (poor metabolizers, PMs).[22] As for the *CYP3A5* genotype, patients were allocated into 2 groups as follows: *CYP3A5*1/*1* or *CYP3A5*1/*3* (CYP3A5 expressors) and *CYP3A5*3/*3* (non-expressors).

**Statistical analysis:** The C/D ratio of tacrolimus was compared for control and rabeprazole groups using the Mann-Whitney *U* test. Data are expressed as the median and range or means ± standard deviation (SD), depending on data type. For all analyses, a two-tailed $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad PRISM, version 4 (GraphPad Software, San Diego, CA).

## Results

**Table 1** shows patient characteristics in each group. The control (n = 17) and rabeprazole (n = 38) groups were similar in age, graft-to-recipient weight ratio, ABO incompatibility and proportion with hepatocellular carcinoma. Although the rabeprazole group had a higher gender ratio, younger donors and a lower proportion of females, no significant differences were observed between the two groups.

For the *CYP2C19* genotype, **1*, **2*, and **3* alleles were found at 55.5%, 28.2%, and 16.3% in the graft liver, and 55.5%, 29.1%, and 15.4% in the native intestine, respectively. Then, the EMs (**1/*1*), IMs (**1/*2* or **1/*3*) and PMs (**2/*2*, **2/*3*, or **3/*3*) of CYP2C19 accounted for

38.2% (n = 21), 34.5% (n = 19), and 27.3% (n = 15) in the graft liver, and 32.7% (n = 18), 45.5% (n = 25), and 21.8% (n = 12) in the native intestine, respectively. For the *CYP3A5* genotype, **1* and **3* alleles were found at 22.7% and 77.3% in graft liver and 20.0% and 80.0% in native intestine, respectively. The frequencies of *CYP3A5* expressors (**1/*1* and **1/*3*) and *CYP3A5* non-expressors (**3/*3*) were 40.0% (n = 22) and 60.0% (n = 33) in graft liver, and 36.4% (n = 20) and 63.6% (n = 35) in native intestine, respectively.

**Influence of concomitant administration of rabeprazole on the pharmacokinetics of tacrolimus, according to *CYP2C19* genotype:** To investigate whether intestinal *CYP2C19* polymorphisms affect the pharmacokinetics of tacrolimus coadminisitered with rabeprazole, patients were divided on the basis of the *CYP2C19* genotype (**Fig. 1A**). For EMs/IMs, no significant difference was found in the median (range) C/D ratio of tacrolimus between the control and rabeprazole groups [2.71 (1.00–6.15) versus 2.55 (0.96–9.25); $P = 0.85$]. For PMs, there was no significant difference in the median C/D ratio of tacrolimus between the control and rabeprazole groups [4.92 (2.44–7.00) *versus* 3.82 (2.00–7.31); $P = 0.68$].

Classification based on the graft liver *CYP2C19* genotype (**Fig. 2A**), indicated no significant differences in the C/D ratio of tacrolimus between these groups (EMs/IMs, $P = 0.52$; PMs, $P = 0.51$).

**Influence of concomitant administration of rabeprazole on the pharmacokinetics of tacrolimus, according to the *CYP3A5* genotype:** Because the *CYP3A5* genotypes of both transplant patients and donors are important for the oral clearance of tacrolimus in liver transplantation,[18,20,21,23,24] we examined the effects of rabeprazole on the C/D ratio of tacrolimus, stratified according to intestinal and graft liver *CYP3A5* genotype (**Figs. 1B, 2B**).

In CYP3A5 expressors, there were no significant differences in the median C/D ratio of tacrolimus for the control and rabeprazole groups for intestinal [2.35 (1.00–7.00) *versus* 2.31 (0.99–7.31); $P = 0.97$] and graft liver genotype classification (2.44 (1.00–6.15) *versus* 2.81 (0.99–7.31); $P = 0.87$).

**Table 1.** Patient characteristics (n = 55)

| Variables | Control (n = 17) | Rabeprazole (n = 38) | *P* value |
|---|---|---|---|
| Age, y | 55.1 ± 12.7 | 54.2 ± 7.1 | 0.74 |
| Female, % | 35.3 | 47.4 | 0.40 |
| Body weight, kg | 65.4 ± 12.9 | 63.9 ± 12.7 | 0.71 |
| Graft-to-recipient weight ratio, % | 1.10 ± 0.35 | 0.98 ± 0.24 | 0.14 |
| ABO incompatibility, % | 23.5 | 26.3 | 0.80 |
| Hepatocellular carcinoma, % | 64.6 | 71.0 | 0.64 |
| Donor age, y | 45.0 ± 13.8 | 39.3 ± 12.8 | 0.14 |
| Donor female, % | 52.9 | 47.4 | 0.70 |

Data are expressed as numbers or means ± SD.

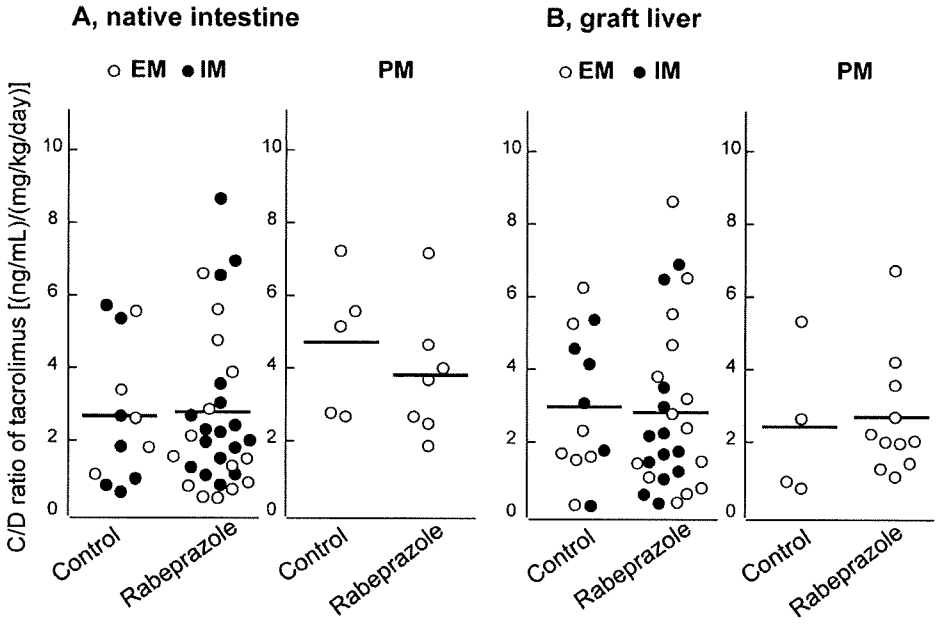


**Fig. 1.** Effects of rabeprazole on the C/D ratio of tacrolimus in LDLT patients during postoperative days 22–28
Patients were categorized based on intestinal *CYP2C19* (A) and *CYP3A5* (B) genotypes. Each bar indicates a median value. *P* values were determined by the Mann-Whitney *U* test.

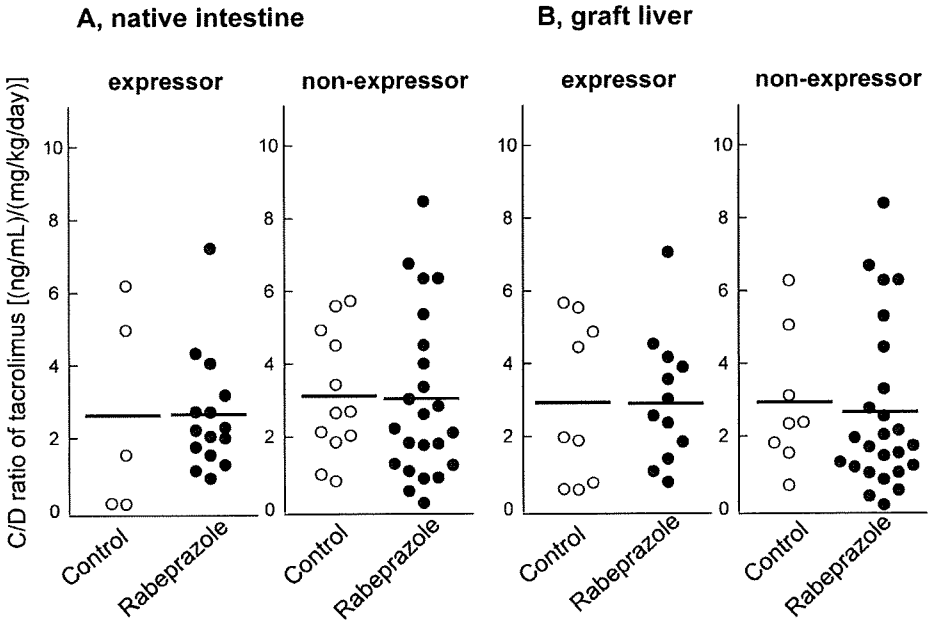


**Fig. 2.** Effects of rabeprazole on the C/D ratio of tacrolimus in LDLT patients during the postoperative days 22–28
Patients were categorized on the basis of the graft liver *CYP2C19* (A) and *CYP3A5* (B) genotypes. Each bar indicates median values. *P* values were determined by the Mann-Whitney *U* test.

Similarly, in CYP3A5 non-expressors, no significant differences were observed in the median C/D ratio of tacrolimus for the control and the rabeprazole groups for intestinal and graft liver genotype classification ($P = 0.89$ for native intestine; $P = 0.56$ for graft liver).

## Discussion

This study investigates the effects of rabeprazole on the pharmacokinetics of tacrolimus, taking into consideration the *CYP2C19* and *CYP3A5* genotypes in LDLT patients (native intestine) and their corresponding donors (graft liver), separately. In liver transplantation, the genotypes of drug-metabolizing enzymes generally differ between native intestine and graft liver. Thus, the different genotypes of intestinal and hepatic CYPs may regulate the clearance of tacrolimus. Rabeprazole was found to little affect the dose-normalized concentration of tacrolimus, regardless of the *CYP2C19* and *CYP3A5* genotypes in native intestine or graft liver. To the best of our knowledge, this study indicates for the first time the little effect of rabeprazole on the pharmacokinetics of tacrolimus in LDLT patients, irrespective of the *CYP2C19* and *CYP3A5* genotypes in native intestine or graft liver.

The C/D ratio of tacrolimus coadministered with omeprazole was previously found significantly higher in adult LDLT patients with two variants (**2* or **3*) for intestinal CYP2C19 than intestinal wild-type homozygotes and heterozygotes, but the extent of increase was attenuated by carrying the wild-type allele in graft liver even when patients were *CYP3A5*1* non-carriers[10] Also the *CYP2C19* polymorphisms in native intestine and graft liver little influenced interactions between tacrolimus and lansoprazole, but *CYP3A5*1* non-carriers showed higher tacrolimus concentration/dose ratios than *CYP3A5*1* carriers.[10] Previous results indicated that CYP2C19 PMs in native intestine and graft liver are more susceptible to the inhibitory effects of omeprazole on tacrolimus metabolism, whereas CYP3A5 non-expressors are more susceptible to the inhibitory effects of lansoprazole on tacrolimus metabolism.[10] Our results reveal little impact of CYP2C19 and CYP3A5 in native intestine and graft liver on the dose-adjusted concentration of tacrolimus coadministered with rabeprazole (**Figs. 1 and 2**). Thus the contribution of CYP2C19 as well as CYP3A4/5 to the metabolism of rabeprazole may be minor. They are consistent with the finding that rabeprazole is predominantly transformed to the thioether metabolite in a nonenzymatical manner.[11] The difference in magnitude of CYP2C19- and CYP3A4/5- mediated metabolism among PPIs may reflect the degree to which PPIs affect the dose-adjusted concentration of tacrolimus.

There are marked interethnic differences in the incidence of the PM phenotype. The frequency of occurrence is much greater in Japanese (17% to 23%) than Caucasians (3% to 5%).[16] Therefore, the possibility of increased concentration of tacrolimus coadministered with omeprazole may be higher in Japanese PM subjects than in white PM subjects. In contrast, the *CYP3A5*3* allele was frequently detected in Caucasians, 60–90% of whom were CYP3A5 non-expressors, whereas we found that

among Japanese, 63.6% of transplant patients and 60.0% of donors were non-expressors, suggesting high possibility of increased concentrations of tacrolimus in CYP3A5 non-expressors among Caucasian as well as Japanese subjects. Importantly, using rabeprazole, patients homozygous for the *CYP3A5*3* allele or *CYP2C19* variant alleles may be at less risk for exceeding the therapeutic upper limit of tacrolimus concentrations during treatment for these types of peptic ulcers.

Because anti-tacrolimus antibody reacts with minor metabolites including 31-O-demethyl tacrolimus (M-II) and 15-O-demethyl tacrolimus (M-III) but not 13-O-demethyl tacrolimus (M-I) as well as the unchanged form the present data on blood concentrations of tacrolimus may include concentrations of M-II and M-III. However, we could not specifically measure unchanged tacrolimus in routine therapeutic monitoring. Therefore, we could not exclude the possibility of overestimation of blood concentration of tacrolimus due to the cross-reactivity of M-II and M-III owing to technical limitations.

In conclusion, this study demonstrates that the *CYP2C19* genotype, in native intestine or graft liver, little affects the dose requirement of tacrolimus coadministered with rabeplazole in LDLT patients. CYP3A5 in native intestine little affects the pharmacokinetics of tacrolimus coadministered with rabeprazole in LDLT patients. The present findings suggest a safer dosing and monitoring of tacrolimus coadministered with rabeprazole early on after liver transplantation regardless of the genotyping of *CYP2C19* and *CYP3A5* in transplant patients and their donors.

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# Direct Hematological Toxicity and Illegitimate Chromosomal Recombination Caused by the Systemic Activation of CreER$^{T2}$ [1]

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**The CreER$^{T2}$ for conditional gene inactivation has become increasingly used in reverse mouse genetics, which enables temporal regulation of Cre activity using a mutant estrogen binding domain (ER$^{T2}$) to keep Cre inactive until the administration of tamoxifen. In this study, we present the severe toxicity of ubiquitously expressed CreER$^{T2}$ in adult mice and embryos. The toxicity of Cre recombinase or CreER$^{T2}$ in vitro or in vivo organisms are still less sufficiently recognized considering the common use of Cre/*loxP* system, though the toxicity might compromise the phenotypic analysis of the gene of interest. We analyzed two independent lines in which CreER$^{T2}$ is knocked-in into the Rosa26 locus (R26CreER$^{T2}$ mice), and both lines showed thymus atrophy, severe anemia, and illegitimate chromosomal rearrangement in hematopoietic cells after the administration of tamoxifen, and demonstrated complete recovery of hematological toxicity in adult mice. In the hematopoietic tissues in R26CreER$^{T2}$ mice, reduced proliferation and increased apoptosis was observed after the administration of tamoxifen. Flow cytometric analysis revealed that CreER$^{T2}$ toxicity affected several hematopoietic lineages, and that immature cells in these lineages tend to be more sensitive to the toxicity. In vitro culturing of hematopoietic cells from these mice further demonstrated the direct toxicity of CreER$^{T2}$ on growth and differentiation of hematopoietic cells. We further demonstrated the cleavage of the putative cryptic/pseudo *loxP* site in the genome after the activation of CreER$^{T2}$ in vivo. We discussed how to avoid the misinterpretation of the experimental results from potential toxic effects due to the activated CreER$^{T2}$. *The Journal of Immunology,* 2009, 182: 5633–5640.**

Conditional gene inactivation using the Cre/*loxP* system has become increasingly used in reverse mouse genetics (1–6). This system takes advantage of the bacteriophage P1 Cre-recombinase ability to catalyze the excision of a DNA sequence flanked by *loxP* sequences. Inactivation of the target gene in conditional knockout mice is regulated depending on the expression pattern of Cre recombinase under the control of tissue-specific promoters (7, 8). However, to analyze gene functions in adult mice, additional temporal control of gene inactivation is indispensable to circumvent problems such as embryonic lethality or developmental abnormalities arising from the early onset of Cre recombinase activity.

Recently, temporal regulation of Cre recombinase activity has been accomplished using tetracycline-controlled gene expression and IFN-inducible expression (9, 10). Another approach uses engineered recombinase fused to the mutated ligand-binding domain of the estrogen receptor (ER$^{T2}$),[3] which does not bind endogenous estradiol but is highly sensitive to the synthetic ligand tamoxifen (TM) or its metabolite 4-hydroxytamoxifen (11). The fusion protein is inactivated by binding to heat shock proteins, until the administration of TM, when it is released from the complex, becomes active and excises *loxP*-flanked DNA regions. Several transgenic mouse lines have been generated that express CreER$^{T2}$ fusion genes under the control of tissue-specific promoters, which show ligand-dependent recombination in certain cell types (12–17).

It has long been assumed that the expression of Cre recombinase does not adversely affect the physiology of the host cell, despite several reports alarming the toxicity of Cre recombinase. High levels of Cre expression have been reported to be toxic in some mammalian cells. Mouse embryonic fibroblasts, NIH3T3 cells, and some human cell lines can be sensitive to the continuous presence of Cre (18–20). Regarding the adverse effect of Cre in vivo, aberrant chromosomal rearrangement in spermatids and male

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A.Y.H., T.I., A.N., T.O., and H.K. performed experiments; A.N.E., A.J.M., and J.R. generated R26CreER$^{T2}$ mice; M.M., T.H., T.N., T.K., and M.Y. analyzed results and made the figures; and M.Y., M.M., and H.K. designed the research and wrote the paper.

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[3] Abbreviations used in this paper: ER$^{T2}$, mutated ligand-binding domain of the estrogen receptor; TM, tamoxifen; WT, wild type; 4-OHT, 4-hydroxytamoxifen.



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infertility has been reported in transgenic mice expressing Cre in postmeiotic spermatids (21). Another group reported dilated cardiomyopathy in their transgenic mice expressing Cre under the control of $\alpha$-myosin H chain (22). Glucose impairment was also reported in commonly used transgenic mice expressing Cre under the control of the insulin 2 promoter (23).

CreER$^{T2}$ was first expected to avoid adverse effects of Cre recombinase by keeping Cre recombinase inactive until the administration of TM. However, very recently, Naiche et al. (24) reported that systemic activation of CreER$^{T2}$ results in lethal anemia and widespread apoptosis in embryos. In their report however, it is unclear whether the toxicity is the direct effect of CreER$^{T2}$ or not.

In this study, we demonstrated widespread hematological toxicity of ubiquitously expressed CreER$^{T2}$ in adult mice as well as in embryos. We analyzed two independent mouse lines in which CreER$^{T2}$ is inserted into the R26 locus (R26CreER$^{T2}$ mice), and both lines showed reduced proliferation, increased apoptosis, and illegitimate chromosomal rearrangement in hematopoietic cells after the administration of TM possibly due to the toxicity of CreER$^{T2}$. Flow cytometric analysis revealed that CreER$^{T2}$ toxicity affected several hematopoietic lineages, and that immature cells in these lineages tend to be more sensitive to the toxicity. In vitro culturing of hematopoietic cells from these mice further demonstrated the direct toxicity of CreER$^{T2}$ on growth and differentiation of hematopoietic cells. We further demonstrated the in vivo cleavage of the putative cryptic/pseudo *loxP* site in the genome after the activation of CreER$^{T2}$, which results in the illegitimate chromosomal rearrangement. These results emphasize the critical importance of including control mice carrying the Cre gene to avoid the misinterpretation of the results.

We further demonstrated that these hematological abnormalities in adult R26CreER$^{T2}$ mice recover spontaneously after 1 mo, which ensures the availability of this mice in reverse mouse genetics provided appropriate control mice are included.

## Materials and Methods

### *Animal use*

One line of R26CreER$^{T2}$ mice was purchased from ARTEMIS Pharmaceuticals (17). Another line of R26CreER$^{T2}$ mice was generated by Regeneron Pharmaceuticals using Velocigene technology (25), essentially as described. The R26 locus was heterozygous for the CreER$^{T2}$ knock-in in all experiments. Both lines were derived on a mixed 129/Svj and C57BL/6J background, and the contribution of 129/Svj was the same in every experiment. R26R Cre reporter mice (R26R mice) (26) were purchased from The Jackson Laboratory. All animal experiments were performed in accordance with the Institutional Guidelines, and were in accordance with National Institutes of Health guidelines.

### *Administration of TM*

TM (Sigma-Aldrich) was dissolved in a sunflower oil/ethanol (9/1) mixture at 3.0 mg/ml. In adult R26CreER$^{T2}$ mice at 8 to 12 wk, 35 mg/kg or 175 mg/kg TM was administered orally for indicated days depending on the experiments. One to four days later depending on experiments, mice were sacrificed and subjected to experiments. Wild-type (WT) littermates administered with the same amount of TM were used as controls. For R26CreER$^{T2}$ embryos, 200 mg/kg TM was administered i.p. into pregnant mothers at E14.5, or 150 mg/kg TM was administered at both E13.5 and E14.5 depending on the experiments.

### *Antibodies*

The following Abs were used: anti-Ter119, anti-Mac1 (M1/70), anti-Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), anti-CD19 (1D3), anti-IgM (R6-60.2), anti-CD3 (145-2C11), anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD25 (PC61), and anti-c *kit* (2B8) were obtained from BD Pharmingen. Anti-Ter119, anti-Mac1, anti-Gr-1, anti-B220, anti-NK1.1, anti-CD3, anti-CD4, and anti-CD8, were used as Lin markers. Anti-Ter119 (BD Pharmingen) and anti-Ki67 (Novocastra) Abs were used for immunostaining.

### *Histological studies*

The organs were fixed in Tris-buffered 10% formalin solution and embedded in paraffin. Sections (2 $\mu$m) were stained with H&E. $\beta$-gal staining was performed as previously described (27). For immunostaining, the specimens were fixed in 4% paraformaldehyde at 4°C overnight, serially soaked in 10, 20, and 30% sucrose/PBS and embedded in OCT compound (Tissue Tek, Sakura Finetech) and 6-$\mu$m sections were prepared. The sections were immunostained as previously described (28–30).

### *TUNEL staining*

Sections were subjected to the TUNEL staining using the in situ apoptosis detection TUNEL kit (MK500, Takara) and visualized by reaction with 3,3′-diaminobenzidine (SK-4100; Vector) for 1 min.

### *Coculture of hematolopoietic cells with stromal cells*

To assess the effect of CreER$^{T2}$ activation on the differentiation of hematopoetic stem cells, Lin$^-$c-*kit*$^+$ cells were collected from the bone marrow of R26CreER$^{T2}$ mice and WT littermates, cultured on a monolayer of TSt-4 cells (31), and administered with 1 $\mu$M of 4-OHT (Sigma-Aldrich) at various time points. We analyzed the differentiation of the Lin$^-$c-*kit*$^+$ cells by examining the expression of Ter119 for erythroid potential, CD19 for B cell potential, and Mac-1 for myeloid potential. To assess the effect of CreER$^{T2}$ activation on the proliferation of differentiated hematopoetic cells, Mac-1$^+$ cells, and CD19$^+$ cells were collected, cocultured with TSt-4 cells, and administered with 4-OHT.

### *Chromosomal number and karyotype analysis*

R26CreER$^{T2}$ mice and WT littermates were treated with vehicle or TM for 5 consecutive days, and were sacrificed 3 days after the last administration. Bone marrow cells of these mice were cultured and chosen randomly for chromosomal number analysis (47 cells for R26CreER$^{T2}$ mice treated with TM, 20 cells for WT mice treated with TM, and 50 cells for R26CreER$^{T2}$ mice treated with vehicle) and for karyotype analysis (14 cells for R26CreER$^{T2}$ mice treated with TM, 4 cells for WT mice treated with TM, and 7 cells for R26CreER$^{T2}$ mice treated with vehicle) as previously described (32).

### *Quantification of rearranged cryptic loxP site in the genome by real-time PCR*

Primers were designed around the candidate locus for cryptic/pseudo *loxP* site to detect the amount of intact genome. Sequences for primers were described in corresponding figure legend. Real-time PCR was performed with a 7700 Sequence Detection System (Applied Biosystems) using SYBR Green PCR amplification reagent (Applied Biosystems), and the results were normalized with the amount of GAPDH genome.

### *Statistical analysis*

Data are presented as means ± SD. Statistical significance was assessed by nonpaired, nonparametric Student's *t* test.

## Results

### *Systemic recombination in adult and embryo R26CreER$^{T2}$ mice*

First, we tested the recombination efficiency of R26CreER$^{T2}$ mice using R26R Cre reporter mice (26) (R26R mice), which express *lacZ* after Cre-mediated excision of a *neo* cassette. Adult R26R/R26CreER$^{T2}$ mice were treated with 175 mg/kg body weight TM for 5 consecutive days, and the recombination of the *lacZ* reporter was analyzed 4 days after the last administration (Fig. 1*A*). Southern analysis of genomic DNA from different organs showed up to 50% recombination (50% in the liver, 30% in the kidney), without detectable background activity in untreated animals, as reported previously (17) (data not shown). Whole-mount tissues from R26R/R26CreER$^{T2}$ mice demonstrated strong $\beta$-gal expression in almost all tissues (Fig. 1*A*) except for brain (data not shown). No background recombination was observed in R26R/R26CreER$^{T2}$ mice treated with vehicle (data not shown). The recombination efficiency was also tested during embryogenesis. Female R26R mice mated with male R26CreER$^{T2}$ mice bearing E14.5 embryos were injected i.p. with 200 mg/kg TM. Two days later, tissues from E16.5 embryos were stained with X-gal, demonstrating that

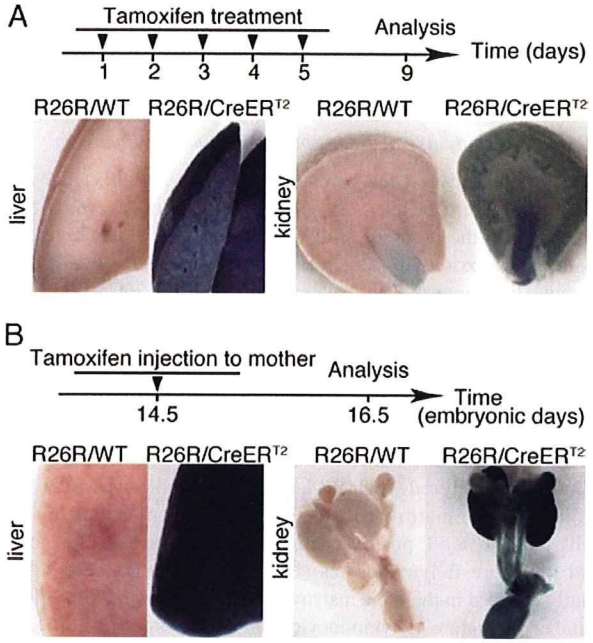


**FIGURE 1.** TM-inducible LacZ expression in R26R/R26CreER$^{T2}$ mice. *A*, Whole-mount *LacZ* staining of liver and kidney of R26R/R26CreER$^{T2}$ adult mice. R26R/R26CreER$^{T2}$ mice and R26R/WT littermates (R26R/WT) were orally administered 175 mg/kg of TM for 5 consecutive days. Four days later, mice were sacrificed and subjected to X-Gal staining. Only the double transgenic mice exposed to TM showed X-Gal positive staining. *B*, Whole mount *LacZ* staining of tissues in R26R/R26CreER$^{T2}$ embryos. Male R26CreER$^{T2}$ mice were mated with female R26R mice, and pregnant females with E14.5 embryos were injected i.p. with 200 mg/kg TM. Two days later, tissues from E16.5 embryos were stained with X-gal. Only the tissues from double transgenic mice exposed to TM showed X-Gal positive staining.

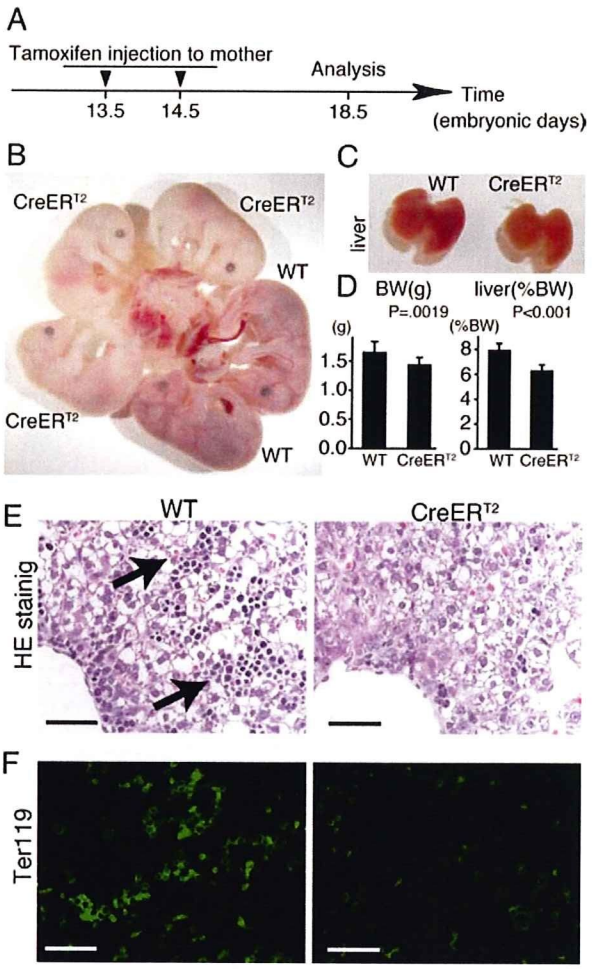


**FIGURE 2.** Severe anemia in R26CreER$^{T2}$ embryos after the administration of TM. *A*, Pregnant female mice were administered 150 mg/kg TM i.p. at E13.5 and E14.5 and sacrificed at E18.5. *B*, R26CreER$^{T2}$ embryos without floxed alleles were anemic compared with WT embryos. These embryos were treated with TM simultaneously. *C*, The liver of R26CreER$^{T2}$ embryos was anemic and smaller than those of WT littermates. *D*, Body weight and liver weight normalized to body weight were lower in R26CreER$^{T2}$ embryos ($n = 5$). *E*, Erythroblasts in the embryonic liver (arrow) decreased significantly in R26CreER$^{T2}$ embryos. *F*, Ter119$^{+}$ cells in the embryonic liver decreased in R26Cre-ER$^{T2}$ mice. Bar = 100 $\mu$M.

only the tissues from double transgenic mice exposed to TM showed $\beta$-gal expression (Fig. 1*B*).

### *Severe anemia observed in R26CreER$^{T2}$ embryos after the administration of TM*

We first noticed the toxicity of R26CreER$^{T2}$ mice when we tried to knockdown the expression of BMP-4 in embryogenesis using R26CreER$^{T2}$ mice, and administered TM to pregnant BMP-4$^{flox/flox}$ mice (33) bearing BMP-4$^{flox/flox}$; R26CreER$^{T2}$ embryos and BMP-4$^{flox/flox}$; WT embryos. In this experiment, 150 mg/kg TM was administered for 2 consecutive days (Fig. 2*A*) to achieve complete recombination in both alleles in BMP-4$^{flox/flox}$ mice. Four days after the last injection, we analyzed the embryos, and observed severe anemia in BMP-4$^{flox/flox}$; R26CreER$^{T2}$ embryos, but not in BMP-4$^{flox/flox}$; Cre $^{-}$ embryos (data not shown). To test whether the phenotype in BMP-4$^{flox/flox}$; R26CreER$^{T2}$ embryos was due to the deletion of BMP-4 gene or due to the systemic activation of CreER$^{T2}$, we administered the same amount of TM to pregnant WT mice bearing R26CreER$^{T2}$ embryos and WT embryos without a floxed allele. Four days later, R26CreER$^{T2}$ embryos without a floxed allele showed severe anemia as well (Fig. 2*B*), indicating that the anemia was not due to the deletion of floxed alleles, but is due to the toxicity of CreER$^{T2}$. The livers of R26CreER$^{T2}$ embryos looked pale (Fig. 2*C*), and body weight as well as liver weight of R26CreER$^{T2}$ embryos was lower compared with those of WT embryos (Fig. 2*D*). R26CreER$^{T2}$ embryos treated with vehicle did not show anemia, or the reduction in body weight or liver weight. Histological analysis demonstrated the colonization of erythroblasts in the liver in WT embryos in late embryogenesis, while the number of erythroblast was significantly reduced in R26CreER$^{T2}$ embryos (Fig. 2, *E* and *F*). These hematological changes in R26CreER$^{T2}$ embryos were already evident at E16.5 (supplementary Fig. 1),[4] while the body weight reduction was not observed yet.

### *Thymus atrophy and hematological abnormality observed in R26CreERT2 adults after the administration of TM*

Next, we administered TM to adult R26CreER$^{T2}$ mice and WT littermates according to the protocol shown in Fig. 3*A*. R26CreER$^{T2}$ mice administered TM developed severe thymus atrophy, but not R26CreER$^{T2}$ mice treated with vehicle, nor WT mice treated with TM (Fig. 3*A*). Thymus weight normalized to body weight was significantly reduced in R26CreER$^{T2}$ mice treated with TM (Fig. 3*A*),

[4] The online version of this article contains supplemental material.

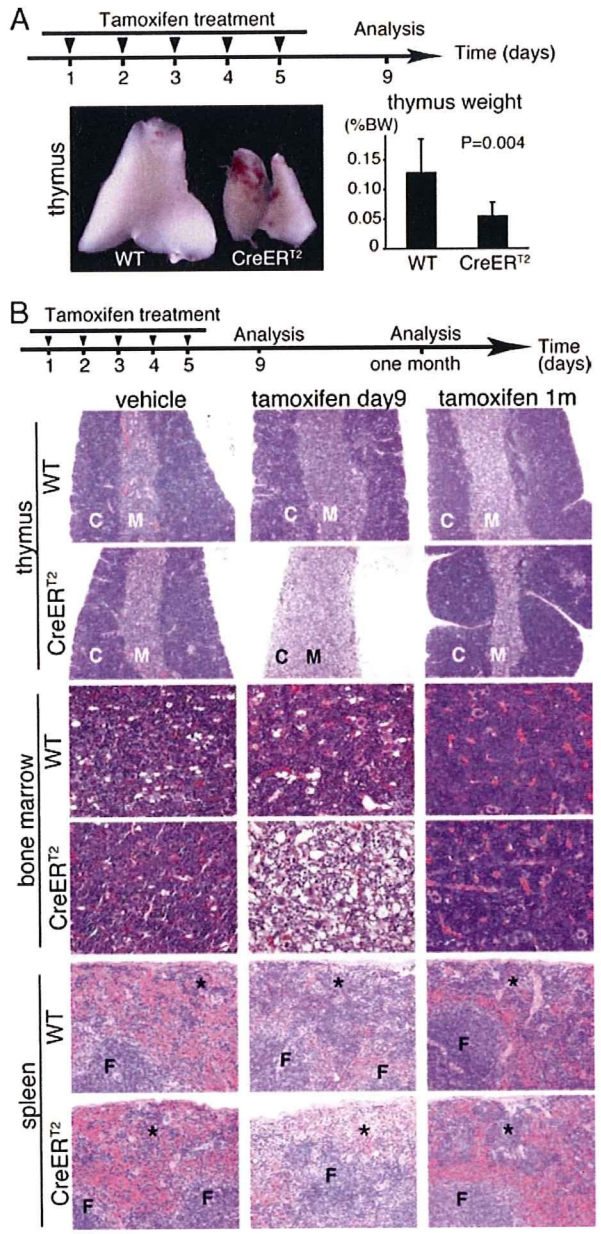


**FIGURE 3.** Thymus atrophy and hematological abnormality in R26CreER$^{T2}$ adults after the administration of TM. *A*, Eight-week-old R26CreER$^{T2}$ mice and WT littermates were treated with 175 mg/kg of TM orally for 5 consecutive days. Animals were analyzed 4 days after the administration. Representative thymus atrophy and the reduction of thymus weight normalized to body weight in R26CreER$^{T2}$ embryos were shown ($n$ = 5). *B*, Representative histological findings in the thymus, bone marrow, and spleen after 9 days, and 1 mo. R26CreER$^{T2}$ mice exhibited thymus cortical atrophy, hypocellular bone marrow, and decrease of erythroblasts in the red pulp of the spleen at day 9, while these changes were significantly diminished after 1 mo. Cell density in the follicle of the spleen was not changed. C, cortex; M, medulla; F, follicle; *, erythroblasts in red pulp of spleen.

which was consistent with the reduced cell density in the cortical region of the thymus (Fig. 3*B*). R26CreER$^{T2}$ mice treated with TM also exhibited hypocellular bone marrow, and a decrease of erythroblasts in the red pulp of the spleen (Fig. 3*B*, *), but the cell density in the white pulp of the spleen was not changed. We also analyzed whether the strains recover from the hematological abnormality, and demonstrated that the extent of recovery from the hematological toxicity greatly differed among individual mice 2 wk after the administration of TM (supplementary Fig. 2), while all R26CreER$^{T2}$ mice recovered completely after 1 mo (Fig. 3*B*).

### *CreER$^{T2}$ toxicity affected multiple hematopoietic lineages*

We further analyzed the hematopoietic lineages affected by the toxicity. Adult R26CreER$^{T2}$ mice and WT littermates were treated according to the protocol used in Fig. 3, which exerts severe hematological toxicity in R26CreER$^{T2}$ mice. Numbers of cells in the thymus, bone marrow, and spleen decreased in R26CreER$^{T2}$ mice after TM treatment (Fig. 4*A*).

FACS analysis in the thymus demonstrated that CD4$^+$CD8$^+$ double positive cells were significantly reduced in R26CreER$^{T2}$ mice (Fig. 4*B*, DP). In addition, the numbers of the cells in double negative subsets in c-*Kit*/CD25 profiles of Lin$^-$ fraction were reduced in R26CreER$^{T2}$ mice.

We also analyzed Ter119/Mac-1, Gr-1 profile and B220/IgM profile of bone marrow cells (Fig. 4*B*). The numbers of myeloid cells (Mac-1, Gr-1 positive cells), erythroblasts (Ter119$^+$ cells) and immature B lymphocytes (B220$^+$/IgM$^-$ cells) were significantly reduced in the bone marrow of R26CreER$^{T2}$ mice, while the number of mature B lymphocytes did not change (Fig. 4*B*). Together with that the number of CD4$^+$CD8$^+$ double positive cells was significantly reduced in the thymus of R26CreER$^{T2}$ mice, immature cells might be more sensitive to the toxicity of CreER$^{T2}$.

To analyze the toxicity in the peripheral tissues, we further examined Ter119/Mac-1, Gr-1 profile and B220/IgM profile in the spleen (Fig. 4*B*). Similar to the results in the bone marrow cells, the numbers of myeloid cells and erythroblasts decreased in the spleens of R26CreER$^{T2}$ mice, but not the number of mature B lymphocytes.

### *Increased apoptosis and attenuated proliferation in the hematopoietic tissues of R26CreER$^{T2}$ mice after the administration of TM*

To define the nature of the toxicity of CreER$^{T2}$, we analyzed apoptosis and cell proliferation in the hematopoietic tissues in adult R26CreER$^{T2}$ mice treated with TM according to the protocol in Fig. 3. These mice were sacrificed at the last day of administration, when viable cells still remain in the hematopoietic tissues (Fig. 5). The numbers of Ki67-positive cells were reduced both in thymus and spleen of R26CreER$^{T2}$ mice, while the numbers of TUNEL-positive cells were increased in spleen, but not in thymus of R26CreER$^{T2}$ mice.

Considering high rate of apoptosis during thymocyte maturation, we postulate that the loss of immature thymocytes in R26CreER$^{T2}$ mice (Fig. 4) might reduce the number of "native" apoptosis, and mask the increased apoptosis due to the toxicity.

Therefore, we conclude that the toxicity of CreER$^{T2}$ is due to attenuated proliferation and increased apoptosis.

### *Direct toxicity of CreER$^{T2}$ in hematopoietic cells*

To exclude the possibility that the hematological abnormality observed in R26CreER$^{T2}$ mice is caused secondarily to unknown systemic disorders, we analyzed the direct effect of TM on hematopoietic cells obtained from R26CreER$^{T2}$ mice. First, we isolated lineage marker negative (Lin$^-$) c-*kit*$^+$ cells from bone marrow and cultured these cells with erythropoietin to induce differentiation into erythroid cells in the presence or absence of 4-hydroxytamoxifen (4-OHT). Ter119$^+$ cells were not generated when 4-OHT was administered to the cells from R26CreER$^{T2}$ mice (Fig. 6*A*). Next, we cultured Lin$^-$c-*kit*$^+$ cells on a monolayer of stromal cell line TSt-4, which efficiently supports the generation of B and myeloid

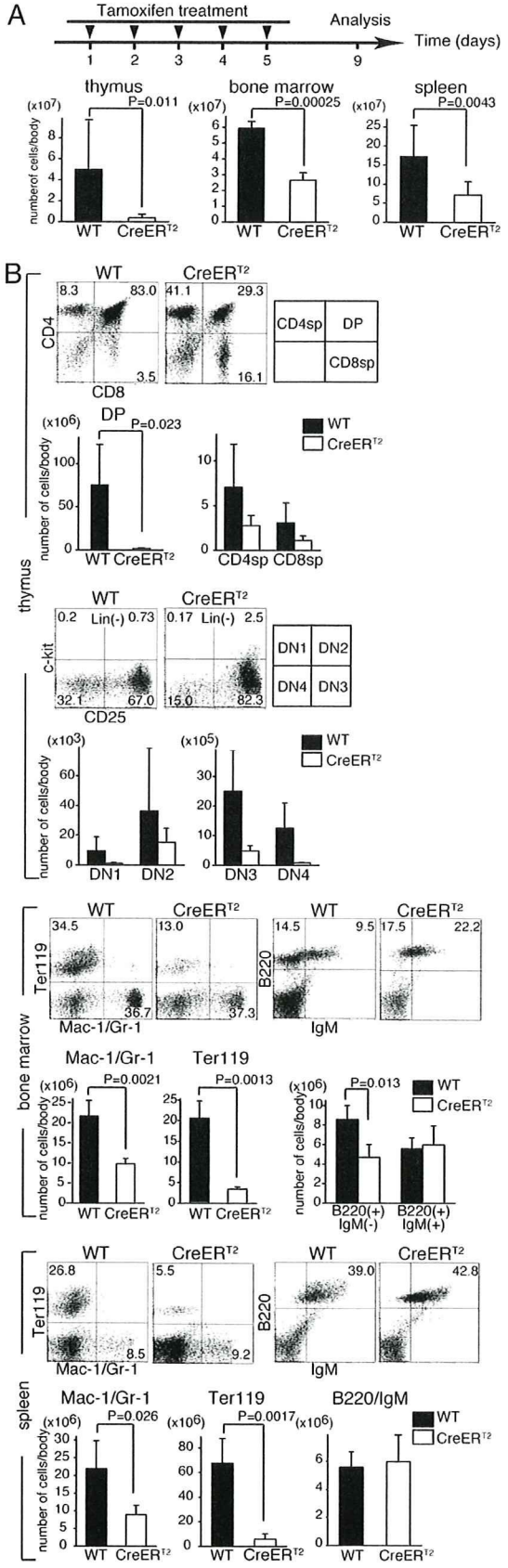


**FIGURE 4.** FACS analysis of hematopoietic tissues in R26CreER$^{T2}$ mice after the administration of TM. *A*, Mice were treated with 175 mg/kg of TM orally for 5 consecutive days and analyzed at day 9. Total numbers of cells of the thymus ($n$ = 7), bone marrow ($n$ = 3), and spleen ($n$ = 7) decreased in R26CreER$^{T2}$ mice after the administration of TM. *B*, Flow cytometric profiles of hematopoietic cells in the thymus, bone marrow, and spleen after the administration of TM. CD4$^+$CD8$^+$ double positive cells were significantly reduced in the thymus of R26CreER$^{T2}$ mice. In profiles of c-*kit*/CD25, Lin$^-$ fraction was subdivided into c-*kit*$^+$CD25$^-$, c-*kit*$^+$ CD25$^+$, c-*kit*$^-$ CD25$^+$, c-*kit*$^-$ CD25$^-$ subsets, which are designated as DN1, DN2, DN3, and DN4 subsets, respectively. Cell numbers of all subsets were decreased in R26CreER$^{T2}$ mice. The numbers of myeloid cells (Mac-1 or Gr-1 positive cells) and erythroblasts (Ter119$^+$ cells) in the bone marrow and spleen, as well as the number of immature B lymphocytes (B220$^+$/IgM$^-$ cells) in the bone marrow were significantly decreased of R26Cre-ER$^{T2}$ mice ($n$ = 3). The percentages of cells in each quadrant are indicated.

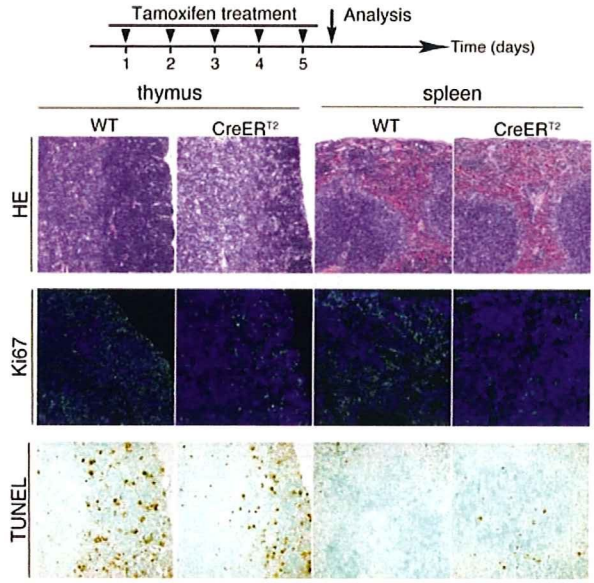


**FIGURE 5.** Increased apoptosis and attenuated proliferation in the thymus and spleen of R26CreER$^{T2}$ mice after the administration of TM. R26CreER$^{T2}$ mice were treated with 175 mg/kg of TM orally for 5 consecutive days and analyzed at the last day of the administration. The number of Ki67-positive cells was reduced both in the thymus and spleen of R26CreER$^{T2}$ mice, while the number of TUNEL-positive cells was increased in the spleen, but not in the thymus of R26CreER$^{T2}$ mice.

cells, for 14 days, and 4-OHT was administered to the culture at various time points (Fig. 6*B*). The generation of B cells, as examined by the expression of CD19, was significantly reduced by the administration of 4-OHT to the cells from R26CreER$^{T2}$ mice, but not the generation of myeloid cells determined by the expression of Mac-1 (Fig. 6, *B* and *C*). Finally, we analyzed the toxicity of CreER$^{T2}$ in already differentiated hematopoietic cells. We isolated Mac-1$^+$ cells and CD19$^+$ cells from bone marrow and cultured them on a monolayer of TSt-4cells in the presence or absence of 4-OHT. The number of CD19$^+$ cells was significantly reduced when 4-OHT was administered to the cells from R26Cre-ER$^{T2}$ mice, while the number of Mac-1$^+$ cells was not affected (Fig. 6*D*).

### *Chromosomal abnormalities in bone marrow cells caused by the activation of CreER$^{T2}$*

As the endonuclease activity of Cre is reported to cause chromosomal aberrations and growth arrest in MEF in vitro (18), we analyzed whether the chromosomal aberrations are caused in vivo in hematopoietic cells in R26CreER$^{T2}$ mice. R26CreER$^{T2}$ mice and

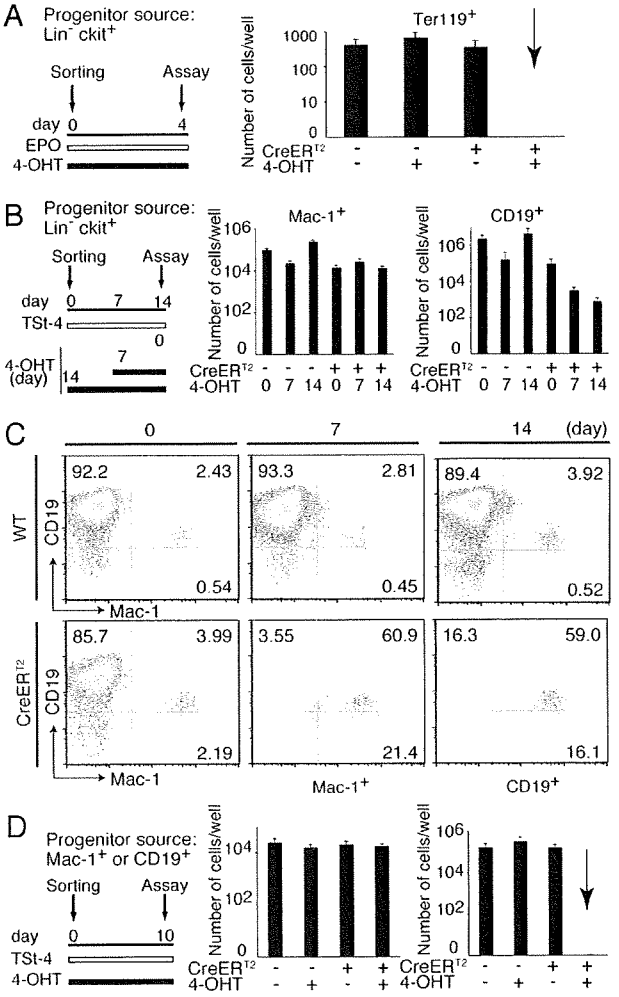


**FIGURE 6.** In vitro administration of 4-hydroxytamoxifen to the hematopoietic progenitor cells bearing CreER$^{T2}$ arrests proliferation and differentiation. *A*, Lin$^{-}$c-*kit*$^{+}$ cells (500 cells) collected from R26CreER$^{T2}$ mice and WT littermates were cultured with erythropoietin (EPO) to induce differentiation into the erythrocyte lineage in the presence or absence of 4-hydroxytamoxifen (4-OHT) at a concentration of 1 $\mu$M. Four days later, Ter119$^{+}$ cells were not generated in the culture where 4-OHT was administered to the cells from R26CreER$^{T2}$ mice. *B*, Lin$^{-}$c-*kit*$^{+}$ cells (300 cells) from bone marrow were cultured on a monolayer of stromal cell line TSt-4 for 14 days for myeloid and B lymphoid potentials. 4-OHT added from day 0 (14) or from day 7 (7), and the generation of the B cells examined by the expression of CD19 was significantly reduced in the cells from R26CreER$^{T2}$ mice treated with 4-OHT, but not the generation of myeloid cells determined by the expression of Mac-1. *C*, Representative FACS profiles of the experiment in Fig. 4*B* with their percentages in the respective quadrant. CD19$^{+}$ cells were eradicated by the administration of 4-OHT to the cells from R26CreER$^{T2}$ mice. *D*, Differentiated Mac-1$^{+}$ cells and CD19$^{+}$ cells ($10^4$ cells for each) were isolated from bone marrow and cultured on a monolayer of TSt-4cells in the presence or absence of 4-OHT. The number of CD19$^{+}$ cells was significantly reduced when 4-OHT was administered to the cells from R26CreER$^{T2}$ mice, while the number of Mac-1$^{+}$ cells did not.

WT littermates treated with vehicle or TM for 5 consecutive days were sacrificed 3 days after the last administration (Fig. 7*A*), and bone marrow cells were analyzed for chromosomal numbers and karyotype. In R26CreER$^{T2}$ mice treated with TM only 53% of the cells showed a normal diploid chromosome number of 40 (Fig. 7*B*), while 90% of the cells had 40 chromosomes in WT mouse

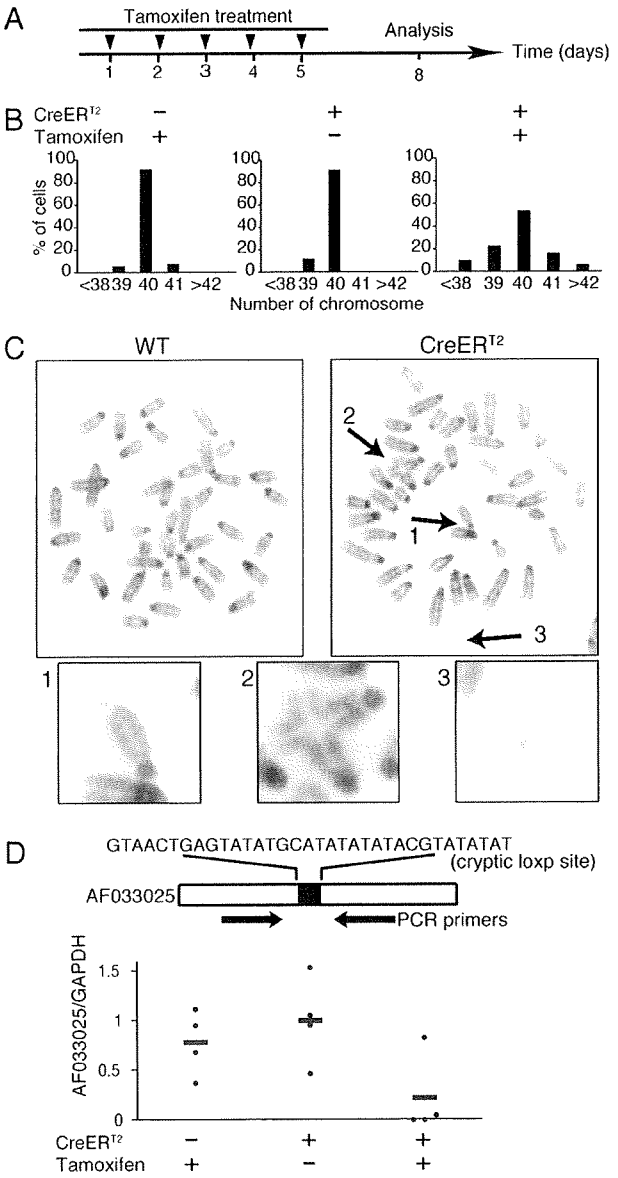


**FIGURE 7.** Chromosomal abnormalities in bone marrow cells caused by the activation of CreER$^{T2}$. *A*, Eight-week-old R26CreER$^{T2}$ mice and WT littermates were treated with 175 mg/kg of TM orally for 5 consecutive days, and bone marrow cells were analyzed for chromosomal numbers and karyotype 3 days after the last administration. *B*, In R26CreER$^{T2}$ mice treated with TM (*right*), only 53% of the cells showed a normal diploid chromosome number of 40, while 90% of the cells had 40 chromosomes in WT mouse treated with TM (*left*) as well as in R26CreER$^{T2}$ mouse treated with vehicle (*middle*). *C*, Various types of chromosome abnormalities such as chromosome exchanges (1), chromatic exchanges (2), and chromatid breaks (3) were observed only in R26CreER$^{T2}$ bone marrow cells after the administration of TM in karyotypic analysis. *D*, Cleavage at the cryptic/pseudo *loxP* site in R26CreER$^{T2}$ thymus genome after the administration of TM. We designed real-time PCR primer sets around the reported cryptic/pseudo *loxP* site in AF033025 locus to detect the amount of intact AF033025 locus (5-TGTTGGACGAGGCCACCT-3 and 5-TCCGGCCT TCTCTAGCCTAGA-3). The results were normalized to the amount of GAPDH gene without cryptic/pseudo *loxP* site using the following primers (5-CCAGAACATCATCCCTGCATC-3 and 5-CCTGCTTCACCACCT TCTTGA-3). In three of four R26CreER$^{T2}$ mice, the intact AF033025 locus was almost undetectable after the administration of TM, indicating illegitimate cleavage at the cryptic/pseudo *loxP* site due to the activation of CreER$^{T2}$.

treated with TM as well as in R26CreER$^{T2}$ mouse treated with vehicle. In karyotype analysis, 78% of bone marrow cells from R26CreER$^{T2}$ mouse treated with TM displayed chromosomal aberrations including chromosome exchanges (Fig. 7*C*, 1), chromatic exchanges (Fig. 7*C*, 2), and chromatid breaks (Fig. 7C, 3), while no chromosomal aberrations were observed in bone marrow cells from WT mice treated with TM (Fig. 7*C*) or in bone marrow cells from R26CreER$^{T2}$ mice treated with vehicle (data not shown).

Thyagarajan et al. (35) reported that mammalian genome contains several candidates for cryptic/pseudo *loxP* sites, and that one locus in mouse genome AF033025 (GenBank) serves as an active site for the Cre recombinase. To clarify whether inappropriate cleavage at cryptic *loxP* sites occurs after the activation of CreER$^{T2}$, we designed real-time PCR primer sets around the cryptic/pseudo *loxP* site in AF033025 locus to detect the amount of intact AF033025 locus (Fig. 7*D*). Intact AF033025 locus in the thymus of three of four R26CreER$^{T2}$ mice was almost undetectable after the administration of TM, indicating illegitimate cleavage at the cryptic/pseudo *loxP* site due to the activation of CreER$^{T2}$. The amount of intact AF033025 locus did not change until the administration of TM, excluding the possibility that the gene targeting procedure to generate R26CreER$^{T2}$ allele altered the locus.

## Discussion

In this study, we demonstrated that the administration of TM to R26CreER$^{T2}$ mice causes severe growth arrest, apoptosis, and illegitimate chromosomal rearrangement in hematopoietic cells, even in the absence of genes targeted by *loxP* sites. We tested two independent lines of R26CreER$^{T2}$ mice from different facilities, and the results were essentially the same. Furthermore, both strains recovered from the toxicity within a month. We also performed in vitro culturing of hematopoietic cells from these mice and demonstrated direct toxicity of CreER$^{T2}$ on growth and differentiation of certain cell types.

### *Hematological abnormalities in R26CreER$^{T2}$ mice is due to systemic activation of CreER$^{T2}$*

Previous reports regarding the adverse effects of Cre in vivo could not exclude the possibility that the unexpected phenotypes were due to the disruption of the genome loci where transgenes were integrated. On the contrary, the lines in this report are alleles introduced into the well-characterized R26 locus, and the disruption of the locus was proved not to cause adverse effects. In addition, no hematological abnormalities were detected until the administration of TM, indicating that an effect of the R26 locus is not likely to be the cause. Importantly, the hematological abnormalities was not due to the toxicity of TM, because the administration of TM to WT mice in vivo as well as to the hematopoietic cells from WT mice in vitro did not exert any effect. Therefore, we concluded that the hematological abnormalities observed in this report were due to the systemic activation of CreER$^{T2}$, which arrested cell proliferation and induced apoptosis (Fig. 5), and were the direct effect on hematopoietic cells (Fig. 6).

The cause of these hematological abnormalities after the systemic activation of CreER$^{T2}$ is likely to be Cre-mediated genomic rearrangements as observed in Fig. 7, perhaps at cryptic or pseudo-*loxP* sites within the mouse genome, which have recently been shown to serve as substrates for Cre recombinase (34, 35). Thygarajan et al. (35) reported that the sequences in mouse genomes considerably divergent from the consensus *loxP* sites serve as functional recognition sites for Cre mediated recombination, and the recombination efficiency of one locus (AF033025) was considerably high in bacterial assays. We further demonstrated that intact AF033025 locus in three of four R26CreER$^{T2}$ mice was almost undetectable after the administration of TM (Fig. 7*D*). Furthermore, recent bioinformatics analysis estimated the frequency of cryptic *loxP* sites in the mouse genome is 1.2 per megabase, and are homogeneously distributed throughout the genome.

High sensitivity of hematopoietic cells to the systemic activation of CreER$^{T2}$ might be due to their rapid proliferation rate, because the genome in rapidly proliferating cells are more easily accessible by CreER$^{T2}$ than the tightly packed genome in quiescent cells. FACS analysis also demonstrated that immature proliferating cells in each hematopoietic lineage tend be more sensitive to CreER$^{T2}$ toxicity (Fig. 4*B*). Positive correlation between Cre-induced toxicity and proliferation was previously reported in fibroblasts (18) as well as in transgenic flies (36). In addition to hematopoietic cells, intestinal epithelial cells also proliferate rapidly, and R26CreER$^{T2}$ mice occasionally demonstrated diarrhea and intestinal edema after the administration of TM, possibly due to the toxicity of CreER$^{T2}$ in rapidly proliferating intestinal epithelial cells (data not shown).

Sensitivity to the toxicity of CreER$^{T2}$ might also be influenced by the amount of CreER$^{T2}$ translocating to nuclei, which is defined by the level of CreER$^{T2}$ expression as well as dose and tissue distribution of TM. Seibler et al. (17) previously reported relatively high expression of CreER$^{T2}$ in thymus, where we observed severe toxicity.

### *Which is tolerated better, CreER$^{T2}$ or Cre?*

In previous reports demonstrating adverse effects in Cre transgenic mice, the authors suggested that the inducible form of Cre might be tolerated better because it stays outside the nucleus until induction (23). However, our results in this study indicated large amount of activated CreER$^{T2}$ was also able to cause cell toxicity. Although the growth arrest is prominent in CD19$^{+}$ cells from R26CreER$^{T2}$ mice after the administration of TM (Fig. 6), no hematological abnormalities have been reported in well-characterized CD19-Cre mice, in which Cre recombinase is highly expressed in B cells. One explanation for the discrepancy is that DNA damage in the cells bearing Cre recombinase induces the cells to develop DNA repairing system to counteract the damage, and such systems might be established in CD19-Cre mice, while R26CreER$^{T2}$ mice are not prepared when massive amounts of CreER$^{T2}$ would be suddenly activated and cause DNA damage.

### *To make good use of R26CreERT2 mice, which are still attractive*

Although the hematological abnormality in R26CreER$^{T2}$ mice might compromise the phenotypic analysis of the gene of interest, the strain is still of great value because of its efficient inducibility without leakage, and of ubiquitous expression of CreER$^{T2}$.

In this study, we suggest three points to take note of to make good use of this strain. One way to solve the problem is taking appropriate control for Cre toxicity: the use of the same mouse without floxed allele. In spite of the fact that Cre toxicity has been occasionally documented in the literatures, it seems still to be widely neglected. A recent study has systemically reviewed the use of RIP-Cre mice, which alone display glucose intolerance, and demonstrated that in more than half of the cases, the appropriate control was not included (23).

Second, it is better to postpone the analysis of the mice for at least 1 mo after the administration of TM. The hematological abnormalities will have diminished after 1 mo (Fig. 3*B*), possibly due to the proliferation of the surviving cells.

Third, it is better to minimize the dose of TM. The toxicity in R26CreER$^{T2}$ mice was dependent on the dose of TM, which regulates the inducibility of CreER$^{T2}$. The minimal dose of TM to

induce efficient recombination varies between target alleles, depending on the number of floxed alleles, the distance between *loxP* sites, the expression level of the target gene, and local chromatin structure. One should adjust the minimal dose of TM to induce efficient recombination in the gene of the interest (supplementary Fig. 3). In cell culture analysis, changing the medium after incubation for 6 h with 4-OHT minimizes the toxicity with efficient recombination (data not shown). The experiments where high recombination efficiency is not necessary or even desirable, such as lineage tracing and mosaic oncogene activation, might be ideal for R26CreER$^{T2}$ mice. The self-excising Cre vectors might be another option to reduce the toxicity (37–39).

*Therapeutic implications and possibility to be a disease model*

The result of the study warns of the potential consequences of Cre-mediated recombination between cryptic *loxP* sites in the genome in Cre/*loxP* based technologies in human gene therapy protocols. Paradoxically, however, immature and rapidly proliferating cells are more susceptible to the toxicity caused by the activation of CreER$^{T2}$, indicating the possible therapeutic implication of the technology for cancer treatment. Schimidt-Supprian et al. (40) reported that the activation of CreER$^{T2}$ transgene in c-Myc-driven primary B cell lymphoma leads to death of lymphoma at lower dose of TM compared with our experiment. Because the dose of TM they used in their experiment does not exert severe toxicity in healthy hematopoietic cells (data not shown), selective eradication of malignant cells might be possible. In addition, R26CreER$^{T2}$ mice might be useful as an inducible model for hematological abnormalities caused by aberrant chromosomal rearrangements.

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## Disclosures

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BioFactors

# BMP modulators regulate the function of BMP during body patterning and disease progression

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## Abstract.

Bone morphogenetic proteins (BMPs) are phylogenetically conserved signaling molecules that belong to the transforming growth factor (TGF)-β superfamily and are involved in the cascades of body patterning and morphogenesis. The activities of BMPs are precisely regulated at various stages, and extracellulary, mainly regulated by certain classes of molecules termed as BMP antagonists and pro-BMP factors. BMP antagonists inhibit BMP function by prohibiting them from binding their cognate receptors, whereas pro-BMP factors stimulate BMP function. In this review, the functions of these BMP regulators will be discussed.


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**Keywords:** BMP antagonist, pro-BMP factor, USAG-1

## 1. Bone morphogenetic protein signaling

Bone morphogenetic proteins (BMPs) are phylogenetically conserved signaling growth factors that belong to the transforming growth factor (TGF)-β superfamily [1–4]. BMPs are secreted dimeric protein with a single interchain disulfide bond, and the dimeric confirmation is an absolute requirement for the biological action of BMPs [5].

Although BMPs are originally identified by its ability to induce ectopic bone and cartilage formation *in vivo* [6–8], they are involved in the cascades of body patterning in vertebrates and invertebrates [9]. Furthermore, BMPs play important roles after birth in pathophysiology of several diseases including osteoporosis [10], arthritis [7], pulmonary hypertension [11,12], and kidney diseases [13–17].

BMPs are synthesized as large precursor proteins. Upon dimerization, the molecules are proteolytically cleaved within the cells to yield carboxy-terminal mature proteins. After secretion, mature BMP dimers initiate signaling by binding to serine/threonine kinase receptor type I and II. Upon ligand binding, type II receptor transphosphorylates type I receptor, and the latter phosphorylate members of Smad family of transcription factors. These Smads are subsequently translocated to nucleus, where they activate transcription of target genes [18]. In addition to Smad pathway, other signaling pathways like MAP kinases are activated by BMPs in certain cell types [19].

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## 2. Extracellular modification of BMP activity

Precisely regulated signaling is necessary to coordinate all aspects of development to homeostasis of adult tissues [20]. For this reason, the local activity of endogenous BMP is precisely regulated intracellulary and extracellulary (including the membrane site) (Fig. 1). In this review, we focused on the extracellular modification of BMP signaling.

### 2.1. Modification of BMP signaling at the membrane site

At the membrane, the transmembrane protein BAMBI (BMP and Activin membrane-bound inhibitor) functions as a pseudoreceptor to interfere with BMP, Activin, and TGF-b signling in *Xenopus* [21,22]. BAMBI and its mammalian homologue Nma are structurally related to type I serine/threonine kinase receptors in the extracellular domain, but lack the intracellular serine/threonine kinase domain. BAMBI/Nma stably associate with type II receptors, thus preventing the formation of active receptor complex.

Recently, repulsive guidance molecule (RGMA) [23], DRAGON (RGMB) [24,25], and hemojuvelin [26] are reported to act as BMP activating coreceptors. These are glycosyl phosphatidyl inositol (GPI) anchored proteins, which form a complex with BMP type I receptors and enhance receptor binding to BMP-2 and BMP-4, potentiating their biological effects.

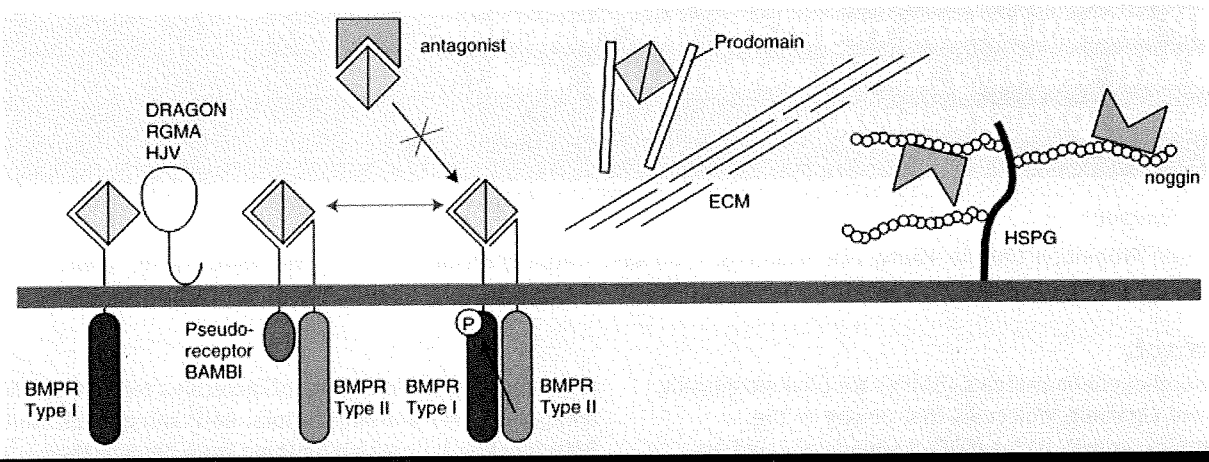


**Fig. 1. Extracellular modulation of BMP signaling. Modified from Ref. 20. ECM, extracellular matrix.**

### 2.2. Modification of BMP signaling in the extracellular space

In the extracellular space, BMP signaling is precisely regulated by certain classes of molecules termed as BMP antagonists [20,27]. BMP antagonists function through direct association with BMPs, thus prohibiting BMPs from binding their cognate receptors. The interplay between BMP and their antagonists fine-tunes the level of available BMPs and governs developmental and cellular processes as diverse as establishment of the embryonic dorsal–ventral axis [28], induction of neural tissue [29], formation of joints in the skeletal system [7], and neurogenesis in the adult brain [30]. In some cases, the synthesis of BMP antagonists is induced by BMPs, suggesting the existence of a protective mechanism to prevent cells from excessive exposure to BMP signaling.

In addition to the modulation by BMP antagonists, high affinity binding of BMP to extracellular matrix modifies the local activity of BMP. Vukicevic et al. previously showed that BMP-7 binds to basement membrane components including type IV collagen [31]. In addition, Gregory et al. recently demonstrated that the prodomain of BMP-7 targets BMP-7 complex to the extracellular matrix [32]. In most tissues *bmp* mRNA expression and BMP protein are found colocalized. Restricted diffusion of BMP proteins is considered to increase its local concentration.

Heparin sulfate proteoglycans (HSPGs) is also reported to shape BMP gradient at cell surface. Jiao et al. recently reported that HSPGs mediate BMP2 internalization and modulate BMP2 osteogenic activity [33], whereas other groups reported that BMP antagonists such as chordin and noggin are retained at cell surface and regulated diffusion by binding to HSPGs [34].

## 3. BMP antagonists

BMP antagonists have a secretory signal peptide and cysteine arrangement consistent with the formation of the cystine knot structure and represent a subfamily of cystine knot superfamily, which comprises of TGF-β, growth differentiation factors (GDFs), gonadotropins, and platelet-derived growth factors, and BMPs [35]. Previously, Avsian-Kretchmer et al. classified BMP antagonists into three subfamilies based on the size of the cystine knot: the differential screening-selected gene aberrative in Neuroblastoma (Dan) family (eight-membered ring), twisted gastrulation (Tsg) (nine-membered ring), and chordin and noggin (10-membered ring) [36]. They further divided the DAN family into four subgroups based on a conserved arrangement of additional cysteine residues outside of the cystine knots: (1) PRDC (protein related to DAN and cerberus) and gremlin, (2) coco and Cer1 homologue of *Xenopus* Cerberus, (3) Dan, and (4) uterine sensitization-associated gene-1 (USAG-1)/wise/ectodin and sclerostin. This subdivision is almost consistent with the phylogenic tree based on the overall amino acid sequence similarity shown in Fig. 2.

More recently, growing number of additional proteins such as Crim1 and the members of CCN family are demonstrated to have the activity of BMP antagonist.

In the following section, we will briefly review the role of these BMP modulators.

### 3.1. The Dan family

**3.1.1. Gremlin: Essential for kidney and limb development.** Gremlin was identified from a *Xenopus* ovarian library for activities inducing secondary axis [37]. Gremlin is a 28-kDa protein, and it binds to BMP-2/4 and inhibits their binding to the receptors. *Gremlin*-null mice are neonatally lethal because of the lack of kidneys and septation defects in lung [38]. In early limb buds, mesenchymal gremlin is required to establish a functional apical ectodermal ridge and the epithelial-mesenchymal feedback signaling that propagates the sonic hedgehog morphogen [39]. In the gremlin *null* embryos, metanephric development is disrupted at the stage of intiating ureteric bud outgrowth and genetic lowering of BMP4 levels in *gremlin* null embryos completely

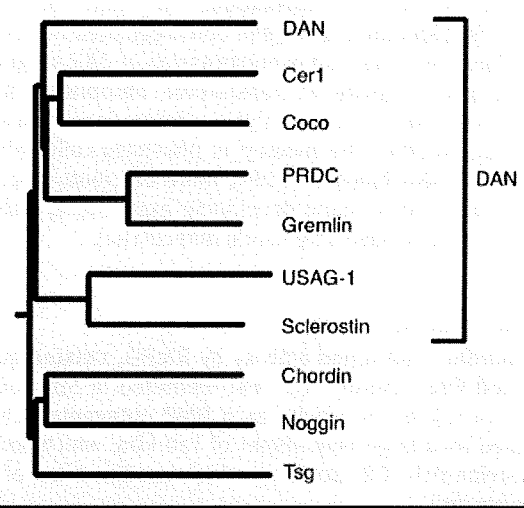


**Fig. 2. Phylogenetic tree of human BMP antagonists based on the overall amino acid sequence similarity of representative members from each subfamily. The GenomeNet server at http://www.genome.jp/ was used for phylogenetic tree construction.**

restores ureteric bud outgrowth and branching morphogenesis, indicating that initiation of metanephric kidney development requires the reduction of BMP4 activity by the antagonist gremlin in the mesenchyme, which in turn enables ureteric bud outgrowth and establishment of autoregulatory GDNF/WNT11 feedback signaling [40].

Gremlin is also known as DRM (downregulated by v-*mos*) because it was identified as a gene that downregulated in *mos*-transformed cells [41,42]. Another name for gremlin is IHG-2 (induced in high glucose 2) because its expression in cultured kidney mesangial cells is induced by high ambient glucose, mechanical strain, and TGF-β [43]. The expression of gremlin is not detected in adult healthy kidney, but is increased in kidney diseases associated with tubulointerstitial fibrosis ([44], Dolan, 2005 #145).

Recently, Sun et al. reported a novel intracellular regulatory mechanism by which gremlin interacts with BMP4 precursor, prevents secretion of mature BMP4, and therefore inhibits BMP4 activity more efficiently. This result implies that the level of BMP4 mRNA expression does not truly reflect BMP4 activity when gremlin and BMP4 are coexpressed within the same cell. Similar regulatory mechanisms may be utilized by other DAN family proteins [45].

**3.1.2. USAG-1: The most abundant BMP antagonist in the kidney and essential in kidney disease progression.** USAG-1 was first identified as a gene preferentially expressed in the sensitized endometrium of rat uterus [46] and recently demonstrated to be a BMP antagonist abundantly expressed in the kidney [47].

USAG-1 protein is a heavily glycosylated 28–30 kDa secretory protein [47], and it behaves as a monomer, in spite that a number of BMP antagonists form disulfide-bridged dimers. Recombinant USAG-1 protein physically interacts with BMP-2, -4, -6, and -7, leading to the inhibition of alkaline phosphatase activities (ALP) induced by each BMP in C2C12 cells and MC3T3-E1 cells dose-dependently [47,48].

In adult tissues, the expression of USAG-1 was by far the most abundant in the kidney, and its localization in the kidney overlaps with that of BMP-7 in distal convoluted tubules. Although BMP-7 is known to prevent renal injury, *USAG-1* null mice exhibited prolonged survival and preserved renal function in acute and chronic renal injuries [49]. Renal BMP signaling, assessed by phosphorylation of Smad proteins, was significantly enhanced in *USAG-1* null mice during renal injury, indicating that the preservation of renal function was attributed to enhancement of endogenous BMP signaling. Furthermore, the administration of neutralizing antibody against BMP-7 abolished renoprotection in *USAG-1* null mice, indicating that USAG-1 plays a critical role in the modulation of renoprotective action of BMP and that inhibition of USAG-1 is a promising means of development of novel treatment for renal diseases.

In addition to the possibility to be a therapeutic target for kidney diseases, USAG-1 might be also useful as a biomarker for the renal prognosis. The expression of USAG-1 in the kidney biopsy in mouse model of renal injury correlated with the renal function in the future [50].

USAG-1 also plays important role in the developing teeth. *USAG-1* null mice have enlarged enamel knots, highly altered cusp patterns, and supernumerary teeth. Excess BMP signaling accelerates patterning in *USAG-1*-deficient teeth and inhibits the apoptosis of the rudimentary upper incisor tooth [51–53].

In addition to its funtion as a BMP antagonist, Itasaki et al. reported that *wise*, *Xenopus* orthologue of USAG-1 functions as a context-dependent activator and inhibitor of Wnt signaling in *Xenopus* embryogenesis [54]. They showed physical interaction between wise/USAG-1 and Wnt coreceptor LRP6, and that Wise/USAG-1 can compete with Wnt8 for binding to LRP6.

**3.1.3. Sclerostin, a product of SOST gene responsible for sclerosteosis.** Using positional cloning method to identify the gene responsible for sclerosteosis, a sclerosing bone dysplasia characterized by syndactily and overgrowth of the skeleton, SOST gene was identified, which encodes a protein named sclerostin. Sclerostin is expressed in osteoblasts, osteocytes, and hypertrophic chondrocytes. In scleosteosis, loss of sclerostin might prolong the active bone-forming phase of osteoblasts, resulting in the increased bone mass. Winkler et al. reported that transgenic mice overexpressing SOST/sclerostin exhibited low bone mass and decreased bone strength as the result of a significant reduction in osteoblast activity and subsequently, bone formation [55]. Based on its suppressive role in bone formation and relatively specific localization of sclerostin in osteoblasts and osteocytes, sclerostin could be a therapeutic target for the treatment of osteoporosis.

Although sclerostin was initially considered as a new member of BMP antagonist [55–58], its mechanism of action on BMP signaling is controversial [59]. Sclerostin has a weak homology to USAG-1, a BMP antagonist that can also act as a Wnt modulator (see the previous section). Similar to USAG-1, Sclerostin binds to Wnt coreceptors and inhibits the Wnt canonical pathway [60,61]. High bone mass diseases are also caused by gain-of-function mutations in LRP5 [62], a coreceptor for Wnt proteins, and the clinical features are quite similar to those of sclerosteosis.

Furthermore, high affinity binding between noggin and sclerostin was reported, and that noggin-sclerostin complex was competitive with BMP binding and mutually attenuated the activity of each BMP antagonist [58]. These data demonstrate the possibility that function of noggin and sclerostin are pleiotrophic. They affect BMP signaling pathway negatively as BMP antagonists, but when their expression overlaps, they might form a complex, and BMP are available to bind to their receptors and induce downstream signaling pathways.

**3.1.4. Cerberus/Cer1: A multifunctional antagonist for BMP, Wnt, and nodal signaling.** Cerberus was identified in a search for transcripts that are abundantly expressed in Spemann organizer of *Xenopus* embryos [63] and injection of Cerberus mRNA into *Xenopus* embryos causes unique phenotype of inducing an ectopic head without trunk formation [64]. Trunk formation requires Nodal and Wnt signaling, whereas head induction requires inhibition of Wnt and BMP signaling. Cerberus, as a multifunctional antagonist, inhibits all three signaling pathways, which leads to simultaneous head formation and trunk inhibition [64]. Cerberus binds to BMP-4 and inhibits the activities, while it binds to Wnt and nodal by separate sites and inhibits these signaling pathways as well. Recently, it is further demonstrated that Cerberus is a feedback inhibitor of Nodal asymmetric signaling in the chick embryo [65].

**3.1.5. Coco regulates cell fate specification.** Another closely related protein, named Coco was discovered in a screen for Smad7-induced genes [66]. Coco blocks BMP/TGF-β signaling in the ectoderm and regulates cell fate specification and competence prior to the onset of neural induction. In addition, Coco acts as a neural inducer and induces ectopic headlike structures in neurula staged embryos. This gene is expressed maternally in an animal to vegetal gradient, and its expression levels decline rapidly following gastrulation. Coco also inhibits Wnt signaling.

**3.1.6. PRDC: A role in the ovary?** PRDC was identified by gene trapping in embryonic stem cells [67]. PRDC binds to BMP-2 and BMP-4 and blocks the activities. PRDC transcripts are widely expressed showing higher levels in ovary, brain, and spleen. PRDC is expressed in granulosa cells of the ovaries and might play some roles in follicular development by antagonizing the actions of BMPs.

**3.1.7. Dan: A role in proliferation?** Dan gene (also called NO3) was first identified as a gene downregulated in *src*-transformed fibroblasts [68] and overexpression of DAN suppresses the tumorigenic activity of transformed fibroblasts [69–71]. Dan can bind BMP-2 and -4 at high concentrations; however, it is not clear whether the binding is physiologically relevant, because Dan also binds to GDF-5 more efficiently [72]. Dan mRNA is localized in many developing axon tracts, although Dan deficient mice have only subtle defects [72].

## 3.2. The chordin family

**3.2.1. Chordin: Fine tuned activity by BMP-1, twisted gastrulation, and Cv2.** Chordin was first identified in Spemann organizer [73,74] for its activity as a BMP antagonist. Chordin is secreted as a large homodimer of 120-kDa, which contains four cysteine-rich (CR) domains which are the sites of BMP binding [73–75].

Chordin binds to BMP-2, BMP-4, and BMP-7 and prevents their interaction with BMP receptors [73]. The activities of chordin are precisely regulated by a metalloprotease BMP-1/tolloid [76] and twisted gastrulation (Tsg). Chordin-BMP complex is a substrate for the BMP-1/tolloid, which cleaves chordin and inactivating its activity and releasing free BMP [77]. Cleavage of chordin by BMP-1 is regulated by Tsg, a BMP binding protein, which can bind to BMP/chordin/BMP-1 complex and enhance the cleavage of chordin, resulting in the release of BMP [78]. More recently, it is reported that crossveinless-2 (Cv2), a concentration-dependent biphasic modulator of BMP signaling, binds to chordin and Tsg as well as BMP-4 [79,80]: Cv2 and chordin synergistically inhibit BMP signaling when chordin levels are low, whereas Cv2 limit the activity of chordin as a BMP antagonist when chordin is overexpressed.

*Chordin* null mice demonstrate, at low penetrance, early lethality, and a ventralized gastrulation phenotype [81]. The mutant embryos that survive, die perinatally, displaying an extensive array of malformations in pharyngeal and cardiovascular organization that encompass most features of DiGeorge and Velo-Cardio-Facial syndromes in humans [82]. *Noggin/chordin* double mutant mice display severe defects in the development of head and facial structures and demonstrate disrupted mesoderm development and abnormal left to right patterning [81].

Recently, growing number of proteins with chordin-like CR domains are demonstrated to be involved in BMP signaling [27]. This group of proteins include Kielin, Crossveinless-2 (Cv2), Crim1, Kielin/chordin-like protein (KCP), and the members of CCN family including cystein-rich 61 (Cyr-61), connective tissue growth factors, nephroblastoma overexpressed (Nov) [83]. CCN family members associate with extracellular matrix, mediate cell adhesion and migration, and can modulate BMP signaling.

**3.2.2. Crim1: A membrane-bound antagonist and a role in renal glomerular development.** Crim1 is a transmembrane protein possessing CR domains and plays a role in the

tethering of growth factors at the cell surface [84]. Crim1 binds to BMP-4 and -7 via the CR domains and modulates BMP activity by affecting its processing and delivery to the cell surface.

A gene-trap mouse line with an insertion of β-Geo casette into intron 1 of the *Crim1* gene (*Crim1*$^{KST264/KST264}$) is a Crim1 hypomorph and displayed perinatal lethality with defects in multiple organ systems [85]. In the kidney, *Crim1*$^{KST264/KST264}$ mice displayed abnormal glomerular development, including enlarged capillary loops, podocyte effacement, and mesangiolysis [86]. When outbred, homozygotes that reached birth displayed marked albuminuria. The podocytic coexpression of Crim1 with vascular endothelial growth factor-A (VEGF-A) suggested a role for Crim1 in the regulation of VEGF-A action. Crim1 and VEGF-A were shown to interact directly, providing evidence that CR-containing proteins can bind to non-TGF-β superfamily ligands.

In addition, a homologue of Crim1, crm-1 is reported to facilitate BMP signaling to control body size in *Caenorhabditis elegans* [87].

### 3.3. Twisted gastrulation: A biphasic molecule controlling BMP signaling

Twisted gastrulation (Tsg) is a secreted protein that regulates BMP signaling in the extracellular space through its direct interaction with BMP and chordin [88–90], and the ternary complex of BMP/Chordin/Tsg is cleaved by the metalloprotease BMP-1/tolloid [76]. Tsg can display both BMP antagonist and agonist functions. As a BMP antagonist, Tsg binds directly with BMP-2 or BMP-4 or with a BMP-chordin preformed complex, which is more efficient in inhibiting BMP signaling. As a BMP agonist, Tsg can enhance chordin cleavage by BMP-1/tolloid as indicated above.

*Tsg* null mice were born healthy, but more than half of the neonatal pups showed severe growth retardation shortly after birth and displayed dwarfism with delayed endochondral ossification and lymphopenia, followed by death within a month [91]. *Tsg* null thymus was atrophic, and phosphorylation of Smad1 was augmented in the thymocytes, suggesting enhanced BMP-4 signaling in the thymus. Since BMP-4 promotes skeletogenesis and inhibits thymus development [92], these findings suggest that Tsg acts as both a BMP-4 agonist in skeletogenesis and a BMP-4 antagonist in T-cell development.

### 3.4. Noggin: Effective tool to inhibit BMP signaling

Noggin is a 32-kDa glycoprotein secreted by Spemann organizer of *Xenopus* embryos and is found to rescue dorsal development in the UV-induced ventralized embryos [93]. Noggin antagonizes the action of BMPs and induces neural tissues and dorsalizes ventral mesoderm [94]. Noggin binds to BMP-2 and BMP-4 with high affinity and to BMP-7 with low affinity, and prevents BMPs from binding to its receptors. Groppe et al. reported the crystal structure of Noggin bound to BMP-7, which shows that Noggin inhibits BMP signaling by blocking the molecular interfaces of the binding epitopes for both type I and type II receptors [95]. The BMP-7-binding affinity of site-specific variants of Noggin is correlated with alterations in bone formation and apoptosis in chick limb development, showing that Noggin functions by sequestering its ligand in an inactive complex. The scaffold of Noggin contains a cystine knot topology similar to that of BMPs; thus, ligand and antagonist seem to have evolved from a common ancestral gene.

In mice, noggin is expressed in the node, notochord, dorsal somite, condensing cartilage, and immature chondrocytes, and null mutation of *noggin* results in serious developmental abnormalities including failure of neural tube formation, and dismorphogenesis of the axial skeleton and joint lesions [96–98]. Noggin is often used as a tool to block BMP activity since it is a specific BMP inhibitor. Systemic gene transfer of *noggin* in mouse models of ankylosing enthesitis and spondyloatrhopathy antagonized BMP signaling, preventing the initiation and progression of anlylosis [99].

## 4. Pro-BMP factors related to Kielin

### 4.1. Kielin/chordin-like protein: BMP agonist with a role in kidney injury

Lin et al. recently identified a cDNA clone from an embryonic kidney library that contained multiple CR domains [100]. The entire coding lesion was similar to the *Xenopus* kielin protein, thus was named kielin/chordin-like protein (KCP). KCP is a secretory protein with 18 CR domains and increases the binding of BMP-7 to its receptor and enhances downstream signaling pathways. The expression of KCP was detected in develping nephrons, but not in adult healthy kidneys. *KCP* null mice developed normally. When introduced with kidney injury model, *KCP* null mice showed reduced levels of phosphorylated Smad1 and are susceptible to developing renal interstitial fibrosis, and more sensitive to tubular injury.

In contrast to the enhancing effect on BMPs, KCP inhibits both activin A- and TGF-β1- mediated signaling through the Smad2/3 pathway. KCP binds directly to TGF-β1 and blocks the interactions with its receptors. Consistent with this inhibitory effect, primary renal epithelial cells from *KCP* null cells are hypersensitive to TGFb1 [101].

### 4.2. Crossveinless 2: Biophasic modulator of BMP signaling related to kielin

Crossveinless 2 (Cv2) is also closely related to kielin and is first identified in the fly mutant study as a gene required for the formation of cross-vein in the fly wings [102]. Genetic studies in flies showed that the formation of these veins requires high Bmp signaling activity, and that Cv2 is essential for enhancing the local Bmp signal near the receiving cells. By contrast, the *in vivo* role of the vertebrate counterpart of Cv2 remains to be elucidated, because some reports indicate that Cv2 is an anti-BMP factor [103], whereas the others reported its pro-BMP activity [104]. As mentioned above, Cv2 is shown to be a biphasic, concentration dependent BMP modulator in *Xenopus* embryogenesis [79,80].

In mammalian embryogenesis, Cv2 seems to function as a pro-BMP factor [105]. In *Cv2* null mouse, gastrulation occurs normally, but a number of defects are found in Cv2-expressing tissues such as the skeleton. The defects of the

vertebral column and eyes in the *Cv2* null mouse are substantially enhanced by deleting one copy of the *Bmp4* gene, suggesting a pro-Bmp role of Cv2 in the development of these organs. In addition, *Cv2* null mice exhibit kidney hypoplasia, and the phenotype is synergistically enhanced by the additional deletion of *Kcp*, that encodes a pro-Bmp protein structurally related to Cv2.

## 5. Conclusions

Negative and positive modulators of BMP signaling regulate and define the boundaries of BMP signaling in embryogenesis as well as adult tissue homeostasis. Further understanding would provide greater insights into the pathophysiological functions of these modulators, and provide a rationale for a therapeutic approach against these proteins.

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