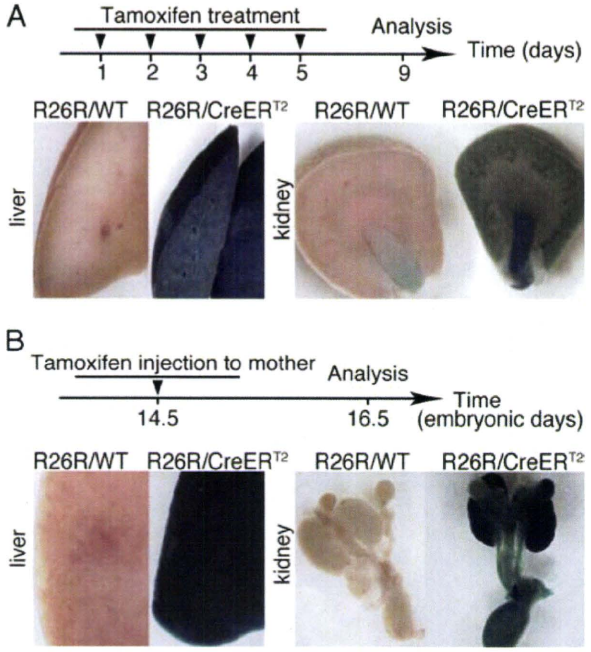

FIGURE 1. TM-inducible LacZ expression in R26R/R26CreER^{T2} mice. *A*, Whole-mount *LacZ* staining of liver and kidney of R26R/R26CreER^{T2} adult mice. R26R/R26CreER^{T2} mice and R26R/WT littermates (R26R/WT) were orally administered 175 mg/kg of TM for 5 consecutive days. Four days later, mice were 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.

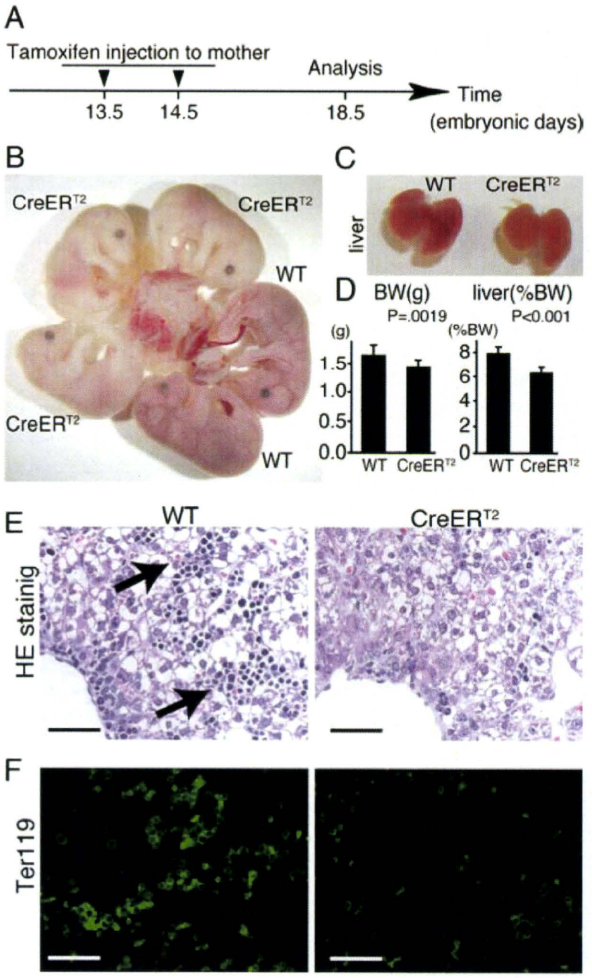

FIGURE 2. Severe anemia in R26CreER^{T2} embryos after the administration of TM. *A*, Pregnant female mice were administered 150 mg/kg TM i.p. at E13.5 and E14.5 and sacrificed at E18.5. *B*, R26CreER^{T2} embryos without floxed alleles were anemic compared with WT embryos. These embryos were treated 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 μM.

only the tissues from double transgenic mice exposed to TM showed β-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.

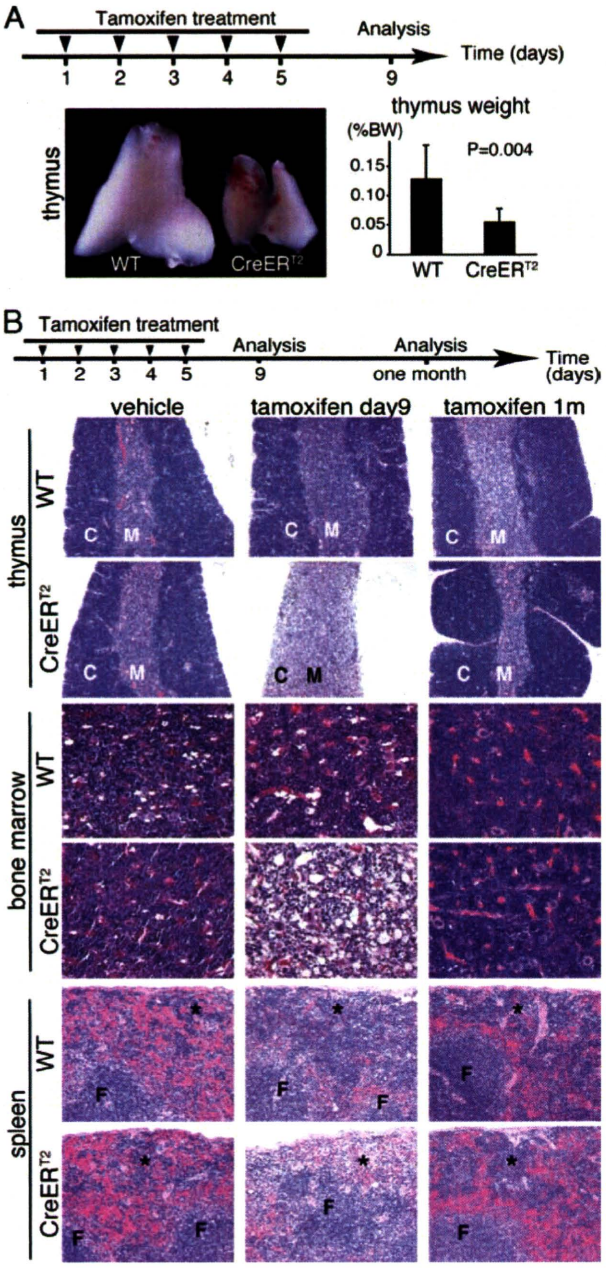

FIGURE 3. Thymus atrophy and hematological abnormality in R26CreER^{T2} adults after the administration of TM. *A*, Eight-week-old R26CreER^{T2} mice and WT littermates were treated with 175 mg/kg of TM orally for 5 consecutive days. Animals were analyzed 4 days after the administration. Representative 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

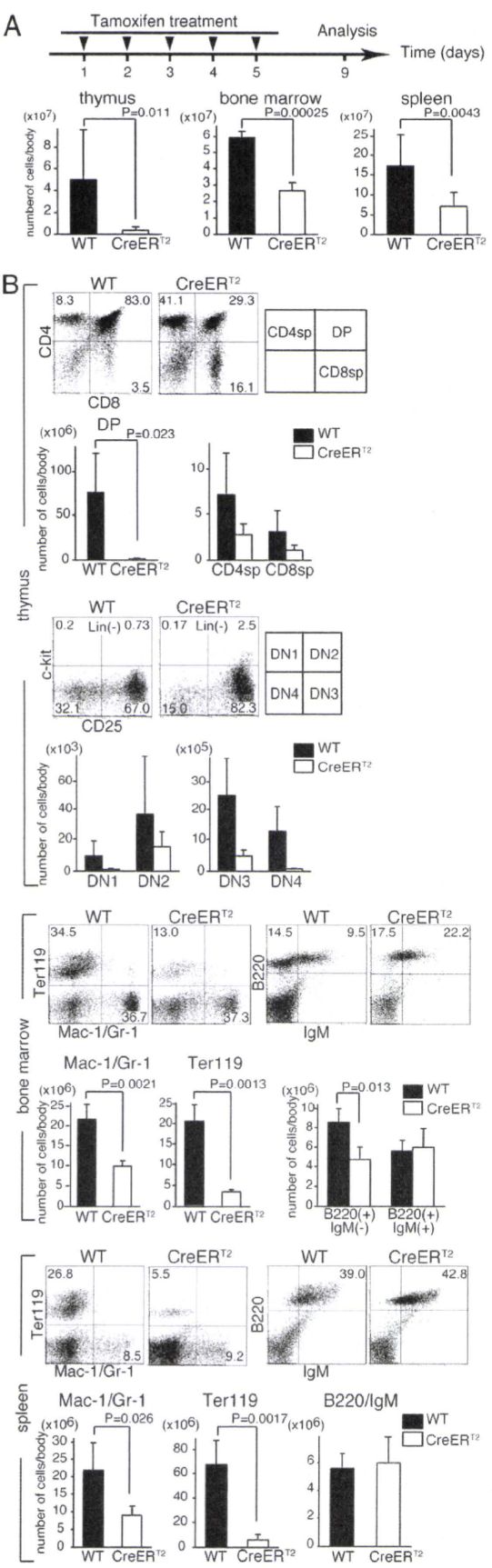

FIGURE 4. FACS analysis of hematopoietic tissues in R26CreER^{T2} mice after the administration of TM. *A*, Mice were treated with 175 mg/kg of TM orally for 5 consecutive days and analyzed at day 9. Total numbers of cells of the thymus (n = 7), bone marrow (n = 3), and spleen (n = 7) decreased in R26CreER^{T2} 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.

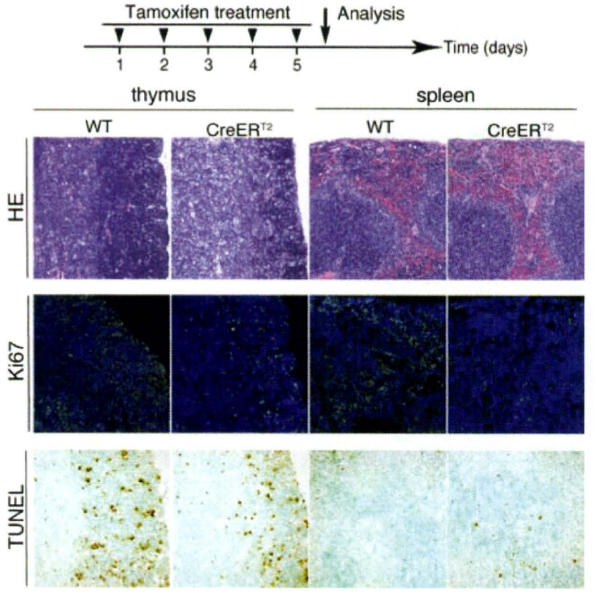

FIGURE 5. Increased apoptosis and attenuated proliferation in the thymus and spleen of R26CreER^{T2} mice after the administration of TM. R26CreER^{T2} mice were treated with 175 mg/kg of TM orally for 5 consecutive days and analyzed at the last day of the administration. The number of Ki67-positive 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

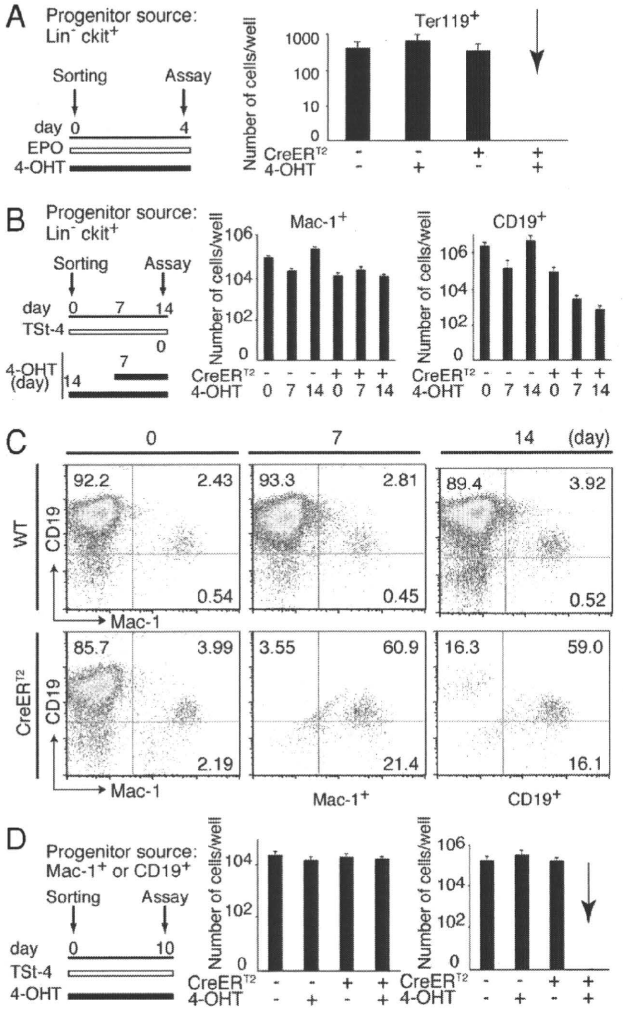

FIGURE 6. In vitro administration of 4-hydroxytamoxifen to the hematopoietic progenitor cells bearing CreER^{T2} arrests proliferation and differentiation. *A*, Lin$^-$c-*kit*$^+$ cells (500 cells) collected from R26CreER^{T2} mice and WT littermates were cultured with erythropoietin (EPO) to induce differentiation into the erythrocyte lineage in the presence or absence of 4-hydroxytamoxifen (4-OHT) at a concentration of 1 μM. 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

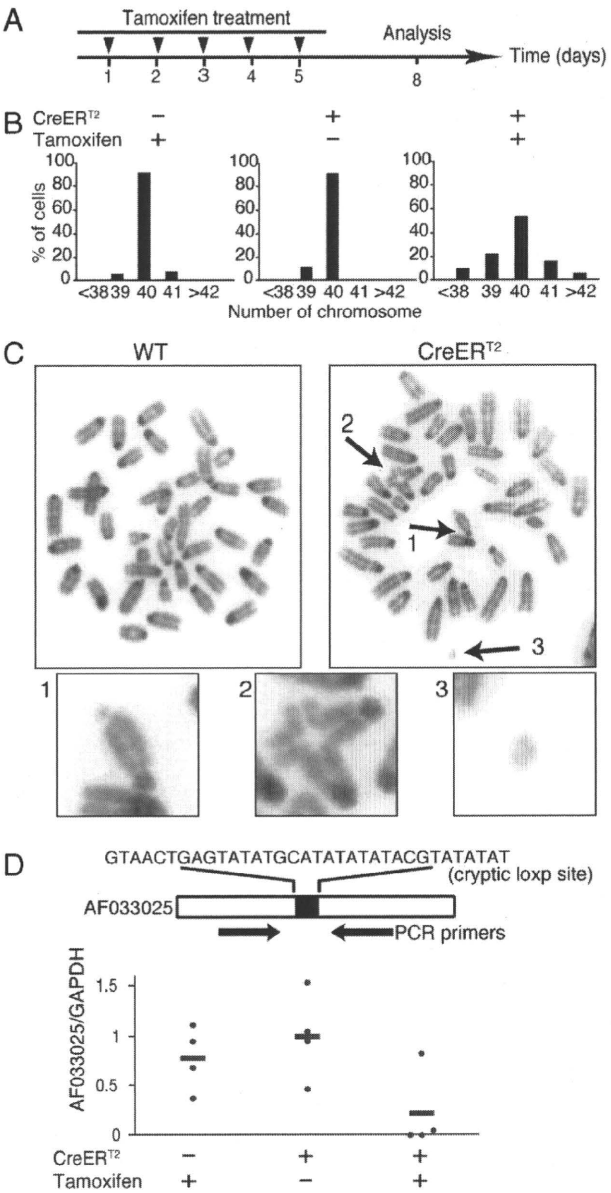

FIGURE 7. Chromosomal abnormalities in bone marrow cells caused by the activation of CreER^{T2}. *A*, Eight-week-old R26CreER^{T2} mice and WT littermates were treated with 175 mg/kg of TM orally for 5 consecutive days, and bone marrow cells were analyzed for chromosomal numbers and karyotype 3 days 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

The authors have no financial conflict of interest.

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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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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.

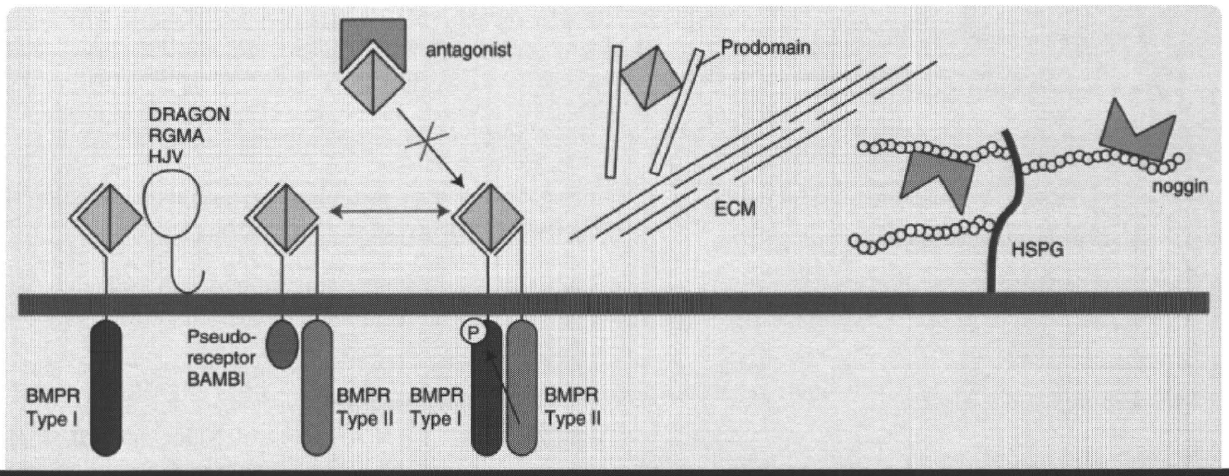

Fig. 1. Extracellular modulation of BMP signaling. Modified from Ref. 20. ECM, extracellular matrix.

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

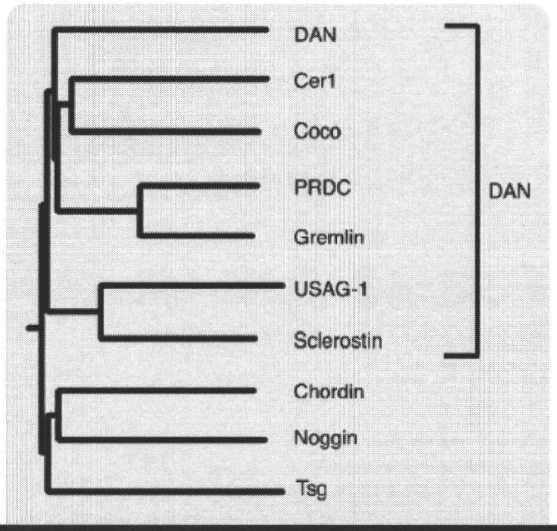

Fig. 2. Phylogenetic tree of human BMP antagonists based on the overall amino acid sequence similarity of representative members from each subfamily. The GenomeNet server at http://www.genome.jp/ was used for phylogenetic tree construction.

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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症 例

腎生検にて血栓性微小血管障害を認めた若年性加速型高血圧の1例

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A young case of kidney failure with thrombotic microangiopathy lesions in renal biopsy caused by accelerated hypertension

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要 旨

症例は19歳，男性。新生児期に髄膜炎とその後の脳波異常の既往があり，16歳で高血圧(200/100 mmHg)を指摘された。当時，腎動脈狭窄や内分泌異常を認めず，本態性高血圧と診断されたが，その後通院を自己中断していた。19歳で高血圧(210/140 mmHg)と血清クレアチニン 2.3 mg/dL, BUN 31 mg/dL の腎機能低下を指摘された。心肥大，高血圧性網膜症(Keith-Wagener III度)を認め，加速型高血圧(accelerated hypertension)と診断された。腎生検では縞状の間質の線維化があり，小動脈に内膜・中膜の肥厚と壊死を認め，細動脈領域にフィブリノイド変性，内皮細胞増殖を伴った多数の血栓閉塞像を認めた。電子顕微鏡にて糸球体に係蹄内皮細胞の膨化と血栓形成，基底膜の二重化や mesangial interposition を認めた。以上の所見から血栓性微小血管障害(thrombotic microangiopathy)と診断した。血小板減少，破砕赤血球や神経症状は認めず，抗リン脂質抗体は陰性であった。降圧治療と抗血小板薬投与で腎機能は改善を示した。この症例においては加速型の重症高血圧による血管内皮障害から多数の細動脈に血栓形成を生じ，腎機能が低下したと考えられた。

A 19-year-old male was admitted to our hospital for the treatment of severe hypertension with renal dysfunction. Two years before admission, his hypertension had been diagnosed as essential hypertension based on a series of examinations when his renal function was not impaired. Visits to his primary physician ended when he developed severe hypertension of 210/140 mmHg, at which time renal dysfunction and serum creatinine of 2.25 mg/dL were discovered. Renin and antidiuretic hormone were slightly elevated, but renal artery stenosis or other abnormalities were not detected by magnetic resonance imaging and computer tomography. After the hypertension was controlled by medication, a renal biopsy was performed to assess renal impairment. Histology demonstrated lesions compatible with thrombotic microangiopathy (TMA) and ischemic lesions, including fibrinoid necrosis, intimal thickening, occlusion in the small arteries, wrinkling and duplication of the glomerular basement membrane with microthrombi, and focal interstitial fibrosis. Renal function ameliorated after the hypertension was controlled. This case suggests that severe and accelerated hypertension can cause TMA with renal impairment even in young people.

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Key words：thrombotic microangipathy, accelerated hypertension, renal biopsy

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緒　言

従来，悪性高血圧は脳症，うっ血性心不全，あるいは腎不全が急速に進行し予後不良の疾患であるとされていた[1,2]。組織学的には，細動脈のフィブリノイド壊死や小動脈の内皮細胞増殖と内膜の線維化による肥厚，それによる内腔狭窄や閉塞を特徴とし，しばしば onion-skin lesion と呼ばれる病変を形成する。今回われわれは，古典的な悪性高血圧の基準に達する臨床症状は満たさなかったものの，腎生検にてフィブリノイド壊死や細動脈・糸球体毛細血管における血栓性微小血管障害所見を呈した加速型高血圧の若年発症例を経験したのでここに報告する。

症　例

患　者：19歳，男性

主　訴：高血圧と腎機能低下の精査希望

既往歴：生後直後に細菌性髄膜炎に罹患し，3歳まで脳波異常を認められ抗てんかん薬を投与されていた。6歳まで熱性痙攣を生じていた。14歳から夜尿症を自覚し，imipramine 25 mg が処方されていたが，16歳から通院を中断していた。

家族歴：祖父が高血圧

生活歴：喫煙歴 10 本/day 3 年間，飲酒歴なし，卵アレルギーあり，現在無職

現病歴：16歳時に近医で高血圧(170～200/100 mmHg)を指摘され精査を受けた。レニン活性 9.9 mg/mL/hr と上昇を認めたが，antidiuretic hormone(ADH)，コルチゾール，カテコラミン系の異常は認めず，レノグラムでも異常所見は認めなかった。当時血清クレアチニン(Cr)0.7 mg/dL，blood urea nitrogen(BUN)12.0 mg/dL と腎機能は正常範囲内であった。Ca 拮抗薬による治療が開始されたが，その後通院を自己中断した。その後は医療機関を受診しておらず，19歳時に感冒様症状をきっかけに近医を受診し，高血圧(210/140 mmHg)と腎機能低下(Cr 2.25 mg/dL，BUN 23 mg/dL)を指摘され，amlodipine 10 mg の内服が開始された。夜尿症に対し sodium valproate も処方された。その後，精査のため紹介され入院となった。

日常生活において，食事については塩分の摂取過多の傾向があった。運動については犬と散歩をする程度であった。もともと肥満があり，最近の体重変化はない。入院の数日前より腹痛と食欲不振を認めていた。

入院時現症：身長 164.5 cm，体重 80 kg，BMI 29.8，血圧 196/141 mmHg，体温 36.2℃，脈拍 102/分整，貧血・黄疸・浮腫なし。心音はⅢ音とⅣ音を聴取，雑音なし。肺音は清。腹部は右下腹部に圧痛あり。

検査所見(Table)：尿検査では 0.7 g/day の蛋白尿を認めた。K 2.8 mEq/L と低下を認め，BUN 31.1 mg/dL，Cr 2.3 mg/dL，UA 8.0 mg/dL，Creatinine clearance(CCr)43.0 mL/min と腎機能低下を認めた。肝・胆道系酵素の異常は認めなかった。WBC 11,400/μL，CRP 0.82 mg/dL と炎症反応の軽度増加を認めた。甲状腺機能，下垂体ホルモンの異常はなかったが，カテコラミンとアルドステロンが軽度上昇しており，レニン活性も 9.9 mg/mL/hr と上昇していた。hap-

Table.　Laboratory data

WBC	11,400/μL(3,300～9,000)	Na	139 mEq/L(135～147)
Neu	75.0 %	K	2.8 mEq/L(3.5～5.1)
Eos	3.5 %	Cl	99 mEq/L(98～108)
Bas	0.3 %	Ca	9.3 mg/dL(8.4～10.3)
Lym	15.3 %	P	3.0 mg/dL(2.5～4.5)
Mon	5.9 %	TP	7.0 g/dL(6.5～8.2)
Hb	13.8 g/dL(11.4～15.5)	Alb	4.2 g/dL(3.7～5.2)
Plt	299×10³/μL(140～360)	BUN	31.1 mg/dL(8.0～20.0)
		Cr	2.3 mg/dL(0.5～0.8)
		UA	8.0 mg/dL(<7.0)
PT	10.5 sec(11.0～14.0)	GOT	14 IU/L(10～40)
APTT	34.5 sec(30.0～45.0)	GPT	16 IU/L(5～45)
FBG	406 mg/dL(200～400)	LDH	310 IU/L(115～245)
D-dimer	3.9 μg/mL(<1.0)	ALP	211 IU/L(110～350)
		T. Bil	0.72 mg/dL(0.2～1.2)
CRP	0.82 mg/dL(<0.1)	CK	57 IU/L(33～150)
haptoglobin	165 mg/dL(50～300)	T. Chol	68 mg/dL(130～219)
		HDL-C	40 mg/dL(40～96)
抗 CL-β_2GPI	1.2 U/mL(<3.5)	TG	238 mg/dL(35～149)
		HbA_{1c}	3.5 %(4.3～5.8)
lupus anticoagulant	1.12(<1.3)	sOsm	286 mOsm/L(284～294)

Endocrine data		Urinalysis	
TSH	1.73 μU/mL(0.35～3.73)	比重	1.019
fT3	2.49 pg/mL(2.2～4.1)	pH	6.0
fT4	1.21 ng/dL(0.88～1.81)	OB	(−)
BNP	9.8 pg/mL(<18.4)	Pro	(2+), 0.7 g/day(<0.13)
PRA	9.9 mg/mL/hr(0.2～3.9)	Glu	(+), 0.6 g/day(<0.1)
aldosterone	220 pg/mL(3～21)	Uro	(±)
adrenalin	0.01 ng/mL(<0.10)	Bil	(−)
noradrenalin	0.88 ng/mL(≦0.50)	Ket	(−)
dopamine	0.04 ng/mL(<0.30)	RBC	<1/5F
ACTH	17.7 pg/mL(7.0～56.0)	WBC	1～4/F
cortisol	5.9 μg/mL(4.0～23.3)	NAG	15.5 IU/L(<7.0)
ADH	8.4 pg/mL(0.3～3.5)	β_2MG	13,814 μg/L(<230)
		Osm	500 mOsm/L

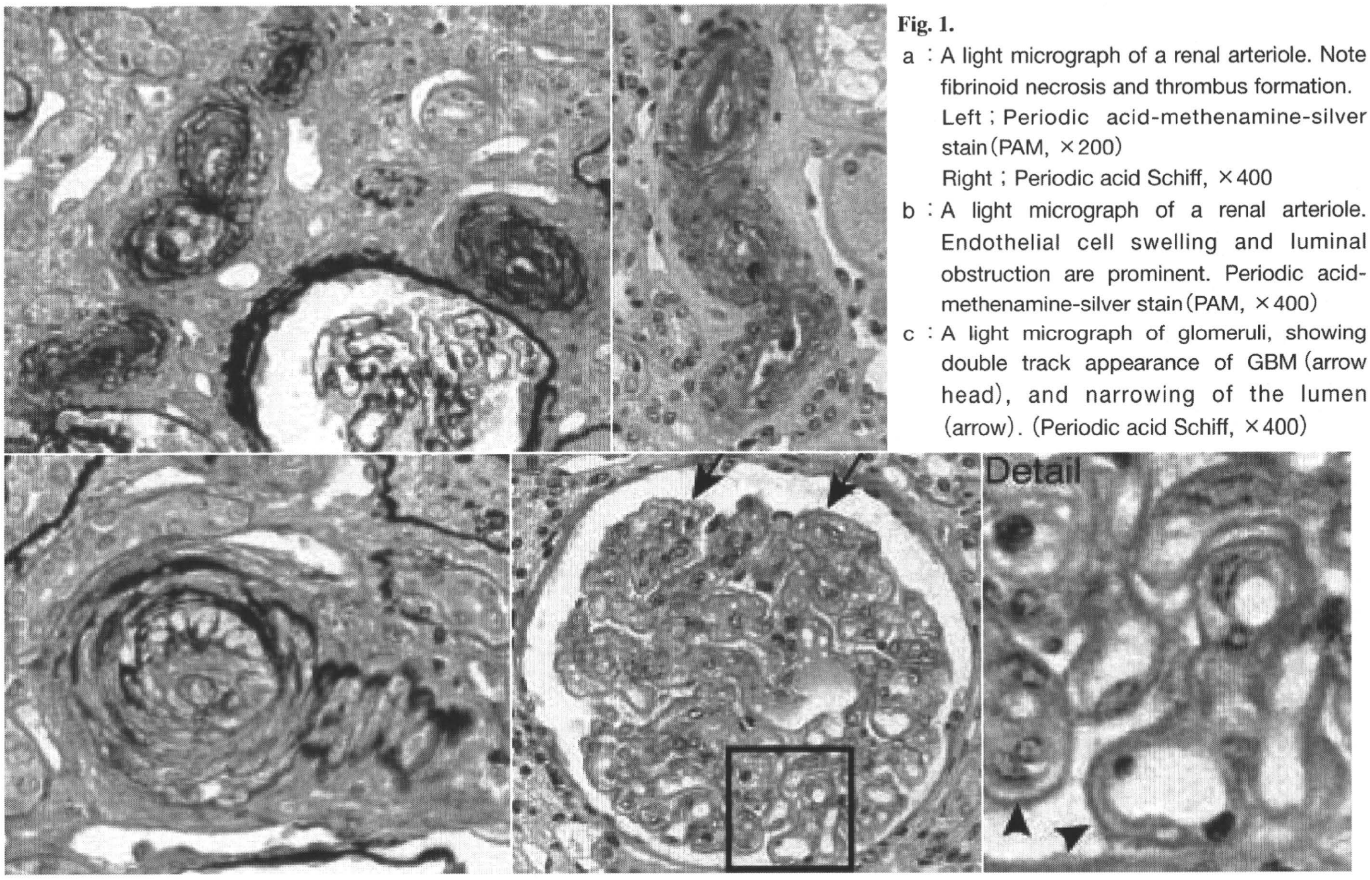

Fig. 1.
a：A light micrograph of a renal arteriole. Note fibrinoid necrosis and thrombus formation. Left；Periodic acid-methenamine-silver stain (PAM, ×200)
Right；Periodic acid Schiff, ×400
b：A light micrograph of a renal arteriole. Endothelial cell swelling and luminal obstruction are prominent. Periodic acid-methenamine-silver stain (PAM, ×400)
c：A light micrograph of glomeruli, showing double track appearance of GBM (arrow head), and narrowing of the lumen (arrow). (Periodic acid Schiff, ×400)

toglobin，抗 CL-β_2 GPI，lupus anticoagulant はいずれも異常を認めなかった。

胸部 X 線写真では心胸比 53 %，左第 3・4 弓が突出しており，心陰影の拡大を認めたがうっ血や胸水は認めなかった。心電図でも Ⅰ，Ⅱ，aVL，aVF，V4-6 で ST の低下を伴う左室肥大の所見を認めた。心エコーでは拡張期左房内径 43 mm，拡張期左室内径 51 mm と拡張しており，さらに求心性心肥大(心室中隔厚 15 mm，後壁厚 15 mm)を認め，駆出率 74 %と収縮能は保たれていたが，拡張能障害を認めた。眼底検査では Keith-Wagener(K-W)Ⅲ度の高血圧性網膜症を認めた。腎エコーでは左 101×58×64 mm，右 94×40×52 mm と左右差を認め，ともに軽度の表面不整を伴う萎縮所見を認めた。レノグラムでは両側腎機能低下のパターンを示し，右腎機能は左の 2/3 程度であった。血管相の立ち上がりの低下は認めなかった。腹部 computer tomography (CT)では副腎に腫瘤性病変は認めなかった。腸管リンパ節の軽度腫脹を認め，腸管リンパ節炎と考えられた。magnetic resonance(MR)angiography では腎動脈に狭窄は認めなかった。頭部 magnetic resonance imaging(MRI)でも下垂体に異常所見を認めなかった。

低カリウム血症と尿糖，β_2ミクログロブリンの異常高値を認めたが，入院時に認めた腸間膜リンパ節炎の改善とともに血清カリウム値は改善し尿糖は消失した。しかし蛋白尿は改善せず，腎機能低下や高血圧に関して慢性糸球体腎炎による腎実質性高血圧の可能性も考えられ，降圧を行った後に腎生検を施行した。

腎生検所見は，光顕では散在性に間質の線維化があり，小動脈に内膜・中膜の肥厚や壊死を認め(Fig. 1a)，細動脈領域に血管膜の肥厚を伴った閉塞を認めた(Fig. 1b)。糸球体は 4 個含まれ，1 個の糸球体は全硬化を認めた。それ以外の糸球体は毛細血管係蹄の二重化や内腔の狭小化(Fig. 1c)を認めた。一方，毛細血管係蹄の wrinkling が主体である糸球体もあり，糸球体によって所見が一様ではなかった。半月体は認めなかった。電顕では基底膜の蛇行，mesangial interposition と基底膜の新生，内皮細胞の膨化と(Fig. 2a)血栓による糸球体係蹄内腔の閉塞を認めた(Fig. 2b)。electron

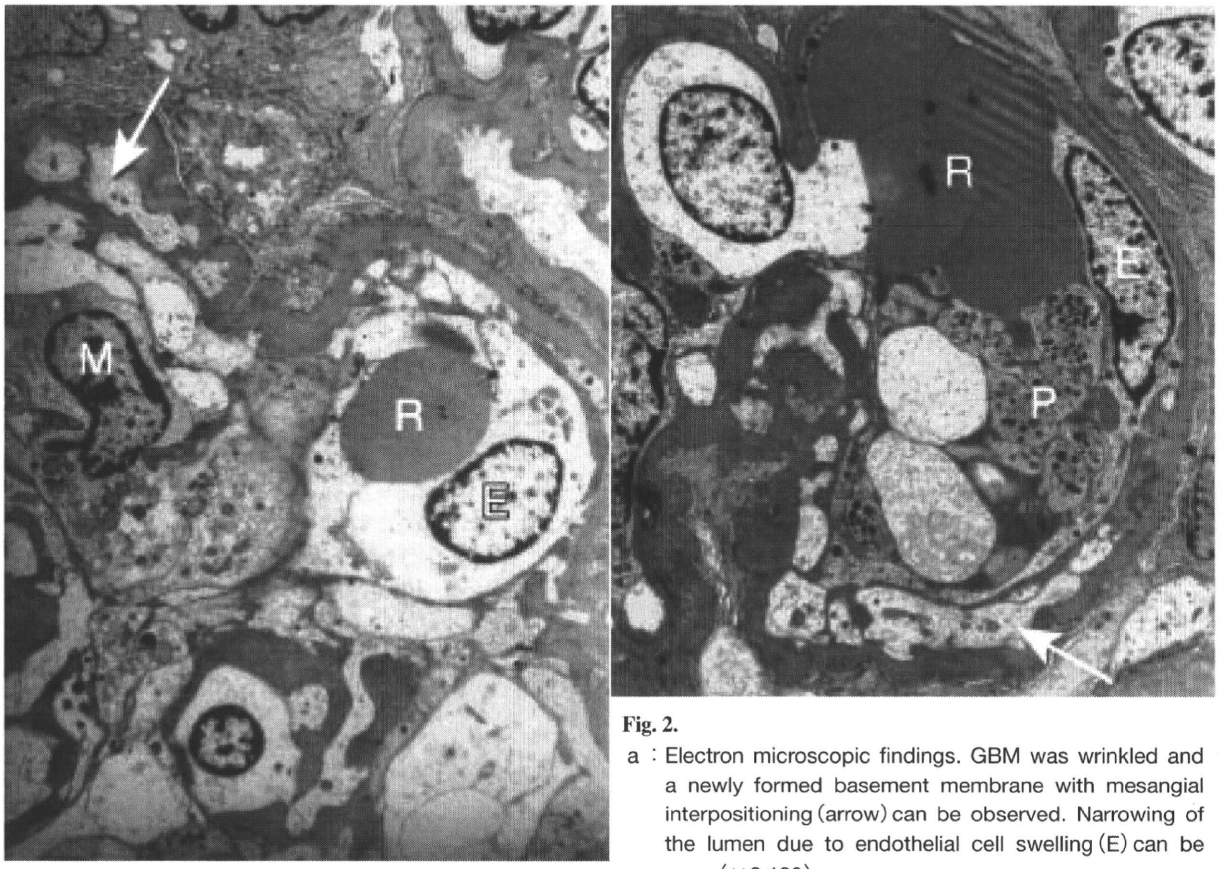

Fig. 2.
a：Electron microscopic findings. GBM was wrinkled and a newly formed basement membrane with mesangial interpositioning (arrow) can be observed. Narrowing of the lumen due to endothelial cell swelling (E) can be seen. (×6,180)
b：Thrombus in a glomerular capillary (×10,300)
arrow：mesangial interpositioning, E：endothelial cell, M：mesangial cell, P：platelets, R：red blood cell

dense deposit は認めなかった。蛍光染色のための新鮮凍結切片が小さく，そこに含まれている糸球体，細動脈には IgG, IgA, IgM, C3, C4, C1q, fibrinogen の有意な沈着は認めなかった。

臨床経過：上記の腎生検の結果より，著明な進行性の高血圧による血栓性微小血管障害(thrombotic microangiopathy：TMA)と診断した。安静，塩分制限，低たんぱく質食と Ca 拮抗薬の amlodipine，ACE 阻害薬(ACEI)の temocapril，アンジオテンシン受容体拮抗薬(ARB)の valsartan，抗アルドステロン薬の spironolactone を含む内服治療で血圧は 130〜140/80 mmHg に低下した。入院前より使用していた sodium valproate は入院後も続行した。なお，ACEI, ARB, 抗アルドステロン薬は一連の内分泌検査終了後に開始した。抗アルドステロン薬により，経過中血中 K の上昇を認めたため trichlormethiazide へ変更した。たんぱく質制限食(50 g)，塩分制限(7 g)，運動，禁煙を指導し退院となった。退院後一時内服を自己中断しており，そのときは血圧が 182/132 mmHg と再上昇したが，腎機能の更なる低下は認めなかった。その後通院と内服を徹底することで血圧は 130/70 mmHg 程度を維持しており，腎機能も Cr 1.5〜1.7 mg/dL と安定している。

考　察

本症例では過去と今回の 2 度にわたり高血圧の検索が行われたが，二次性高血圧の所見を認めず，若年発症の本態性高血圧と考えられた。今回の入院時，カテコラミン系，ADH などが上昇していたが一次性のものではないと考えられた。

悪性高血圧(malignant hypertension)は重篤な高血圧によって血管障害と末梢組織の虚血が急速に進行する病態であり[1,2]，強皮症の crisis などがその典型例である[3]。(旧)厚生省の診断基準では A 群：1) 治療前の拡張期血圧が 130 mmHg 以上，2) 高血圧性網膜症 K-W IV度の乳頭浮腫，3) 急速に進行し腎不全に至る腎機能障害，4) 体重減少，脳症状，心不全などを伴う急速な全身症状の悪化。B 群：1) 治

療前の拡張期血圧が120～130 mmHg，2）K-W Ⅲ度の眼底所見，3）腎不全に至らない腎機能障害，があげられ，そのうち，A群で3条件以上，B群では1)と3)のいずれかを満たすものを悪性高血圧と定めている。一方，歴史的にはK-W Ⅳ度を満たすものを悪性高血圧と定義し[1]，悪性高血圧と同様の重篤な臓器障害を呈し，眼底所見でK-W Ⅲ度を呈するものを加速型高血圧(accelerated hypertension)として区別してきた。降圧治療が発達した今日においてはK-W Ⅲ，Ⅳ度でも重症度や予後には差がないといわれている[4]。本症例では経過中に脳症や心不全は認めなかったが，高血圧(210/140 mmHg)と短期間に高血圧性網膜症(K-W Ⅲ度)，腎機能低下の進行を認め，加速型高血圧と診断した。

悪性高血圧のメカニズムは血管内皮障害によると考えられている[3,5~8]。血圧上昇によるshear stressの増加により力学的に血管内皮細胞に障害が生じるが[9,10]，同じ血圧でも悪性高血圧で認めるような血管障害を呈さないこともあり[3,6]，血圧以外にレニン・アンジオテンシン系の活性化[11,12]，プロスタグランジン[11,13]，カテコラミン[6]，グルココルチコイド[14,15]，免疫機序[16]，凝固異常[17]，ADH[6,18]などの関与も考えられている。内皮障害が細動脈のフィブリノイド壊死や葉間動脈の内皮細胞増生と内腔狭窄をきたすと同時に，血管内凝固を亢進させ，TMAの病態を引き起こすこともある[6]。しかしながら，今までこういった血管内皮障害は悪性高血圧と臨床診断され，いずれも急速な腎機能低下をきたした症例の腎生検の病理診断で報告されていることが多い。

しかし本症例では，神経症状などの臨床症状を認めず，検査所見でも血小板減少，破砕赤血球の出現やhaptoglobin値低下を認めなかったにもかかわらず，腎組織上ではTMA所見を認めた。具体的には，細・小動脈壁のフィブリノイド変性と血管内皮細胞の増殖・膨化による内腔閉塞，糸球体では毛細血管の内皮細胞の膨化と血栓形成，mesangial interpositionと新生基底膜などである。糸球体によって所見が一様でないことは，途中の小・細動脈の通過障害の違いによって高血圧による糸球体障害が糸球体ごとに異なるためと考えられた。またこのことから，糸球体障害は原発性糸球体疾患によるものではないと考えられた。同時に硬化糸球体や間質の線維化も進行しており，急性と慢性の混在した所見を認めた。

悪性高血圧以外にTMAを呈する病態として，溶血性尿毒症症候群(hemolytic uremic syndrome：HUS)，血栓性血小板減少性紫斑病(thrombotic thrombocytopenic purpura：TTP)，cyclosporinなど血管内皮障害を起こす免疫抑制薬やVEGF阻害薬の使用，臓器移植後，DIC，HELLP症候群，SLEなどの全身性血管炎が知られている[19]。本症例は薬剤の使用歴はなかった。入院後の精査では抗リン脂質抗体を含め自己抗体は陰性であり，自己免疫疾患は否定的であった。入院時は腸管リンパ節炎を起こしていたが，発熱下痢などは認めず，短期間に軽快したため，入院直前の消化管感染症によるHUSを合併したとは考えにくく，数年間続いた高血圧以外に血管内皮障害を起こす要因は認めなかった。高血圧や血管内皮障害に関与しうるその他の要因として肥満や喫煙の影響が考えられた。

近年，TTPの患者で血中ADAMTS13活性が減少しており，ADAMTS13活性が低位の患者では血漿交換が奏効することが報告されている[19]。本症例の入院当時はADAMTS13の測定は一般的でなかったため測定できなかった。前述のように病歴と検査所見より，重症高血圧による血管内皮障害が長期に続き，緩徐にTMAが進行し慢性腎不全に至ったと考え，ACEI・ARBを含めた降圧治療と抗血小板薬投与をまず開始した。その後の経過において腎機能は改善を示した。

結　語

悪性高血圧にみられる臨床症状や検査所見を認めなかったものの，腎生検組織で血管内皮細胞障害によるTMA所見を呈した若年性重症高血圧の1例を経験した。本例においては高血圧による腎障害のメカニズムとしてTMAの関与が考えられた。またこの症例の経験より，原因不明の腎機能低下患者では可能な限り積極的に腎生検を行い，病理診断すべきであると考えられた。

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文　献

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CASE REPORT

Angio-Embolization of Renal Artery Pseudoaneurysm after Renal Biopsy: A Case Report

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Renal artery pseudoaneurysm is a rare clinical entity that has been reported after renal biopsy, percutaneous renal surgery, penetrating trauma, and rarely blunt renal trauma. We present the case of a 37-year-old man with ruptured renal artery pseudoaneurysm accompanied by massive gross hematuria, urinary clot retention, and bladder tamponade, which were the presenting signs seven hours after renal biopsy. Abdominal CT scan showed a large perinephric, intracapsular hematoma of left kidney. His angiogram revealed a left renal segmental artery pseudoaneurysm that measured 1 cm × 1 cm. He was successfully treated by selective embolization of the arterial branch supplying the pseudoaneurysm.

Keywords pseudoaneurysm, renal artery, renal biopsy, tamponade, coiling

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INTRODUCTION

Renal artery pseudoaneurysm is a rare clinical entity; however, it has great clinical significance when encountered because of its propensity for rupture.[4] It is a rare complication of renal biopsy percutaneous renal procedures, penetrating trauma and, rarely, blunt renal trauma.