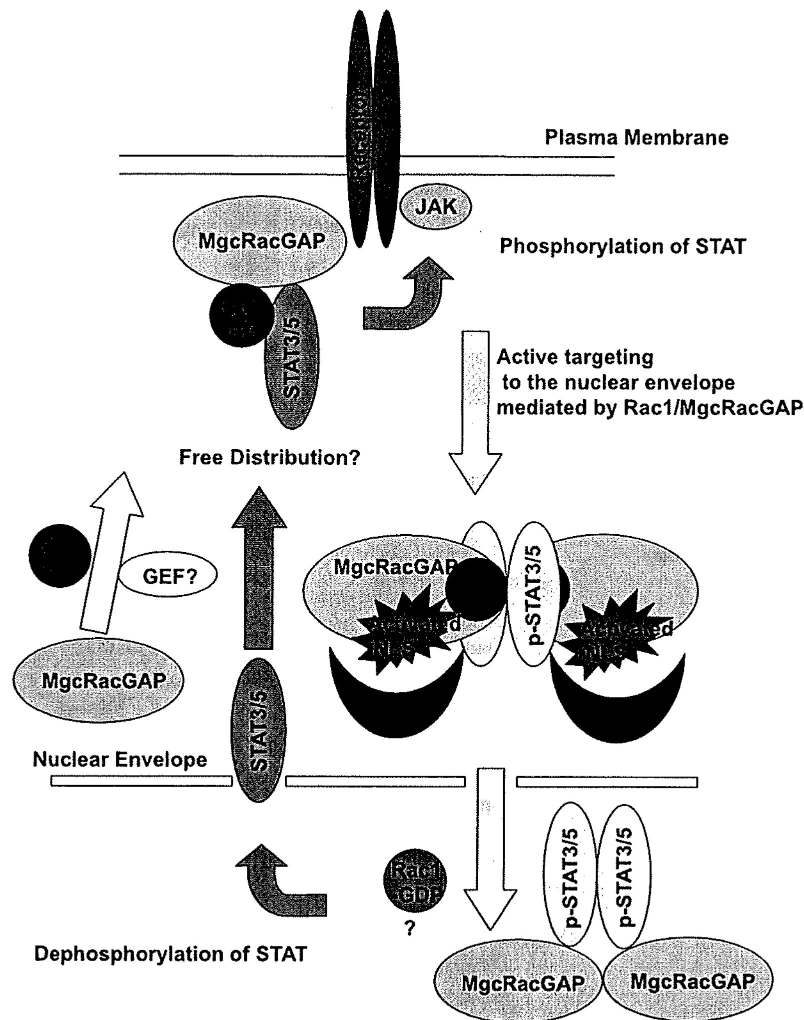

FIG. 8. A current model of nuclear import of p-STATs and a working hypothesis for membrane targeting and phosphorylation of STATs. In the present work, we demonstrated that the NLS of MgcRacGAP accompanied by GTP-bound Rac1 is essential for nuclear translocation of p-STATs via importin α/β. We also propose that binding of MgcRacGAP to STATs is required for their tyrosine phosphorylation after cytokine stimulation. Interestingly, the mutants that preferentially bind MgcRacGAP become constitutively active. Altogether, we conclude that MgcRacGAP critically functions both as a mediator of STAT's tyrosine phosphorylation and as an NLS-containing nuclear chaperone of p-STATs.

secondary result following the enhanced tyrosine phosphorylation of these mutants, and they imply a positive role for MgcRacGAP in facilitating STAT activation. In this context, it is interesting to note that V12Rac1 induced translocation of MgcRacGAP to the plasma membrane (see Fig. S5 in the supplemental material) and that MgcRacGAP bound JAK2 (17). In addition, the interaction of the STAT3-Y704F mutant, which does not undergo tyrosine phosphorylation, with MgcRacGAP was enhanced by IL-6 stimulation, similar to that of the WT STAT3 (data not shown). This indicates that the IL-6-induced MgcRacGAP interaction with STATs does not require tyrosine phosphorylation of STATs and occurs before their tyrosine phosphorylation. Based on these observations, we propose a model of STAT nucleo-cytoplasmic shuttling regulated by Rac1/MgcRacGAP (Fig. 8).

One important finding of the present paper is that the abilities of STAT mutants to bind MgcRacGAP correlated well with the activation of STATs (Fig. 5 and 6). To reveal the molecular mechanisms of MgcRacGAP-mediated regulation of STAT phosphorylation, we conducted an in vitro kinase reaction of purified STAT5 using purified JAK2 in the presence or absence of Rac1 and MgcRacGAP. However, STAT phosphorylation was not enhanced by the addition of Rac1/MgcRacGAP in this mixture (data not shown). Based on the result that MgcRacGAP binds JAK2, we speculate that MgcRacGAP regulates STAT phosphorylation by conveying STAT proteins to JAK2 or by serving as a scaffold for the interaction of JAK2 and STAT.

We also observed that STAT3-d358L bound MgcRacGAP more strongly than WT STAT3 did in yeast (data not shown). In the crystal structure of tyrosine-phosphorylated STAT3β (2) (PDB ID 1BG1), the MgcRacGAP binding DB2 region includes the C terminus of the βa′ strand, the DNA-bound βa′-βb loop, the βb strand, the βb-βc loop, and the N terminus

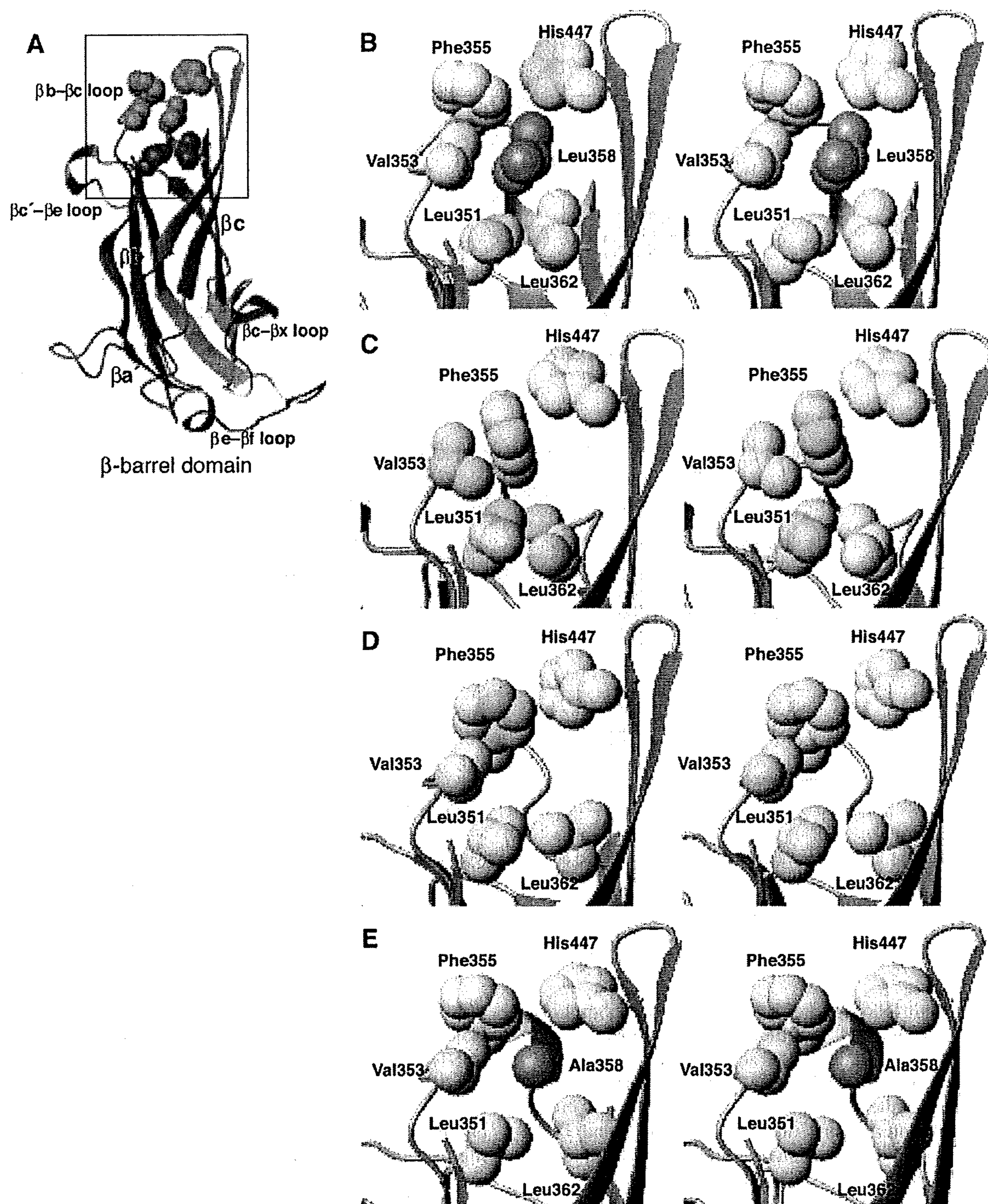

FIG. 9. Structure of the wild-type STAT3 and homology models of the hydrophobic core surrounding Leu358. The crystal structure of the β-barrel domain of the wild-type STAT3β (A) and its hydrophobic core region (B). In panel A, the B-factors are color coded, with lower values in blue and higher values in red, to show that the loop bearing L358 is well-ordered and rigid. (C to E) The hydrophobic core regions of the dD7, dL358, and L358A mutants, respectively. In panels B to E, only the residues corresponding to the boxed region of panel A are shown. The key residue, Leu358, is highlighted in orange, and the surrounding hydrophobic residues that form the core together with Leu358 are shown in yellow. The figures are in stereo view (wall eye) and were produced using MOLMOL (20). It should be noted that all of these modeled core structures are packed less tightly than the wild-type structure.

of the βc strand in the β-barrel domain, as shown in Fig. 5A and 9A. The tertiary structure of this domain appears to be rigid, as its B-factors (ca. 51 $Å^2$) are considerably lower than those of the other domains of the protein (ca. 66 $Å^2$). Deletion mutants of the βb strand (dD3 to dD5) resulted in loss of the MgcRacGAP binding ability of STAT3, suggesting that the βb strand is the MgcRacGAP binding site. Interestingly, the MgcRacGAP binding ability and the transcriptional activity of STAT3 were enhanced by deletion of the D6 to D9 region (Fig. 5C). These phenotypes may be explained by the flexibility around the βb strand. Within the β-barrel domain, the DNA-bound βa′-βb loop and the βb-βc loop region are as rigid as the β strands (the B-factors, ca. 40 $Å^2$), while the βc-βx, βc′-βe, and βe-βf loops are much more flexible (Fig. 9A). The rigidity of the βb-βc loop is probably because Leu358 in this loop is involved in the hydrophobic core formation. When a deletion mutation is introduced in the βb-βc loop (dD6-dD9, d356P, d357E, and d358L), the tertiary structure of the domain may be retained but become more flexible, as the hydrophobic core is packed less tightly than the wild type (see Fig. 9B, C, and D for representative structural models). These STAT3 mutants with more flexibility around the βb strand (binding site of MgcRacGAP) could bind more efficiently to MgcRacGAP, leading to their enhanced activation. In the L358A mutant, which behaves similarly to the d358L mutant (data not shown), the hydrophobic core of the domain is also loosened (Fig. 9E). Thus, all of these deletion and point mutations may destabilize the hydrophobic core of the domain around the βb strand (binding site of MgcRacGAP) and therefore seem to increase the binding ability of the βb strand to MgcRacGAP. The dD2 and dD10 mutants also strongly bind to MgcRacGAP, possibly because of the distortion of the domain structure, while hyperactive transcription was not observed, as they lacked the DNA binding activities. The loss of the MgcRacGAP binding ability in the dD1 mutant lacking the C terminus of the βa′ strand may be due to a secondary result of structural distortion of the βa′ strand and the DNA-bound βa′-βb loop. Our current hypothesis is that upon binding with MgcRacGAP, the β-barrel domain of STAT undergoes some conformational change so that the MgcRacGAP binding region (the βb strand) becomes more flexible and more exposed.

MgcRacGAP accompanied by GTP-bound Rac1 functions both as a mediator of the tyrosine phosphorylation of STATs and as an NLS-containing nuclear chaperone of p-STATs during interphase, while MgcRacGAP plays a critical role in cell division. We believe that MgcRacGAP is a molecule which functions in the nucleocytoplasmic transporting system during interphase and in the mitotic apparatus from metaphase to cytokinesis, as is the case with nucleocytoplasmic transporters, including importins and Ran, which are also involved in the formation of the mitotic spindle after the disassembly of the nuclear envelope (7). Although MgcRacGAP is not involved in nuclear translocation of NF-κB, whether it is involved in nuclear transport of other proteins is still open to question.

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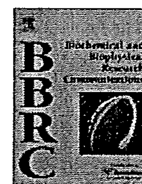

Wnt modulators, SFRP-1, and SFRP-2 are expressed in osteoblasts and differentially regulate hematopoietic stem cells

Hideaki Nakajima [a,b,*], Miyuki Ito [b], Yoshihiro Morikawa [c], Tadasuke Komori [c], Yumi Fukuchi [a,b], Fumi Shibata [d], Shinichiro Okamoto [a], Toshio Kitamura [d]

[a] *Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan*
[b] *Center of Excellence, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan*
[c] *Department of Anatomy and Neurobiology, Wakayama Medical College, Wakayama 641-8509, Japan*
[d] *Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan*

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ABSTRACT

Wnt signaling has been implicated in the self-renewal of hematopoietic stem cells (HSCs). Secreted frizzled-related proteins (SFRPs) are a family of soluble proteins containing a region homologous to a receptor for Wnt, Frizzled, and are thought to act as endogenous modulators for Wnt signaling. This study examined the role of SFRPs in HSC regulation. Among the four family members, SFRP-1 and SFRP-2 are specifically induced in the bone marrow in response to myelosuppression, and immunostaining revealed that both proteins were expressed in osteoblasts. Interestingly, SFRP-1 reduced the number of multipotent progenitors in *in vitro* culture of $CD34^{-}$KSL cells, while SFRP-2 did not. Furthermore, SFRP-1 compromised the long-term repopulating activity of HSCs, whereas SFRP-2 did not affect or even enhanced it in the same setting. These results indicate that although both SFRP-1 and SFRP-2 act as inhibitors for Wnt signaling *in vitro*, they differentially affect the homeostasis of HSCs.

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Introduction

Hematopoietic stem cells (HSC) are a rare population of cells that are capable of supporting life-long hematopoiesis [1]. They are characterized by the unique capacity to self-renew and differentiate into all blood cell lineages. The molecular mechanisms controlling self-renewal of HSCs have not yet been fully elucidated, but previous reports suggest that the Wnt family of proteins is implicated in this process [2].

Wnts are a family of secreted proteins that are involved in a variety of biological and pathological processes such as skeletal development, embryogenesis, organogenesis and tumor development [3,4]. Frizzled (Fz) family proteins serve as receptors for Wnt, and Wnt binding to Fz forms receptor complex with or without low-density lipoprotein receptor related protein (LRP)-5 or -6 to activate canonical or non-canonical signaling pathways. There are a number of endogenous Wnt regulators, such as secreted Frizzled-related protein (SFRP), WIF-1, Cerberus, and Dickkopf (Dkk)[5]. SFRPs contain a characteristic cysteine-rich domain (CRD) in the N-terminus which shares homology with CRD of Fz [5–7]. Since CRD of Fz serves as a binding surface for Wnt, SFRPs are speculated to modulate the Wnt-Fz interactions through its CRD; however, the physiological impact of SFRPs on Wnt signaling still remains to be elucidated. Previous studies have indicated that mammalian SFRPs were repressors of the canonical Wnt pathway when they are overexpressed *in vitro* [8–10], while other studies have shown the agonistic function of SFRP-1 for Wnt signaling [11], thus suggesting a context-dependent action of SFRPs. During embryonic development, SFRPs and Wnts are expressed in either distinct or sometimes overlapping areas, thereby coordinating overall Wnt activities critical for appropriate organogenesis [6,12–16]. Due to their putative Wnt-inhibitory activity, SFRPs have been postulated to serve as tumor suppressors. Indeed, some SFRPs have been reported to be inactivated by either promoter hypermethylation or chromosomal deletion in certain types of cancers [8,17,18].

Although Wnts, especially Wnt3a, have been reported to play critical roles in the self-renewal of HSCs, little is known about the roles of Wnt modulators in the HSC physiology. This study investigated the effects of SFRP-1 and SFRP-2 on the differentiation, proliferation, and self-renewal of HSCs. The results suggest SFRP-1 and SFRP-2 are differentially involved in the homeostasis of HSCs.

Materials and methods

Mice. C57BL/6 (B6) mice were from Japan CLEA Inc. (Tokyo, Japan), and B6-Ly5.1 mice were from Sankyo Lab Service Co.

* Corresponding author. Address: Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81 3 3353 3515.
E-mail address: hnakajim@sc.itc.keio.ac.jp (H. Nakajima).

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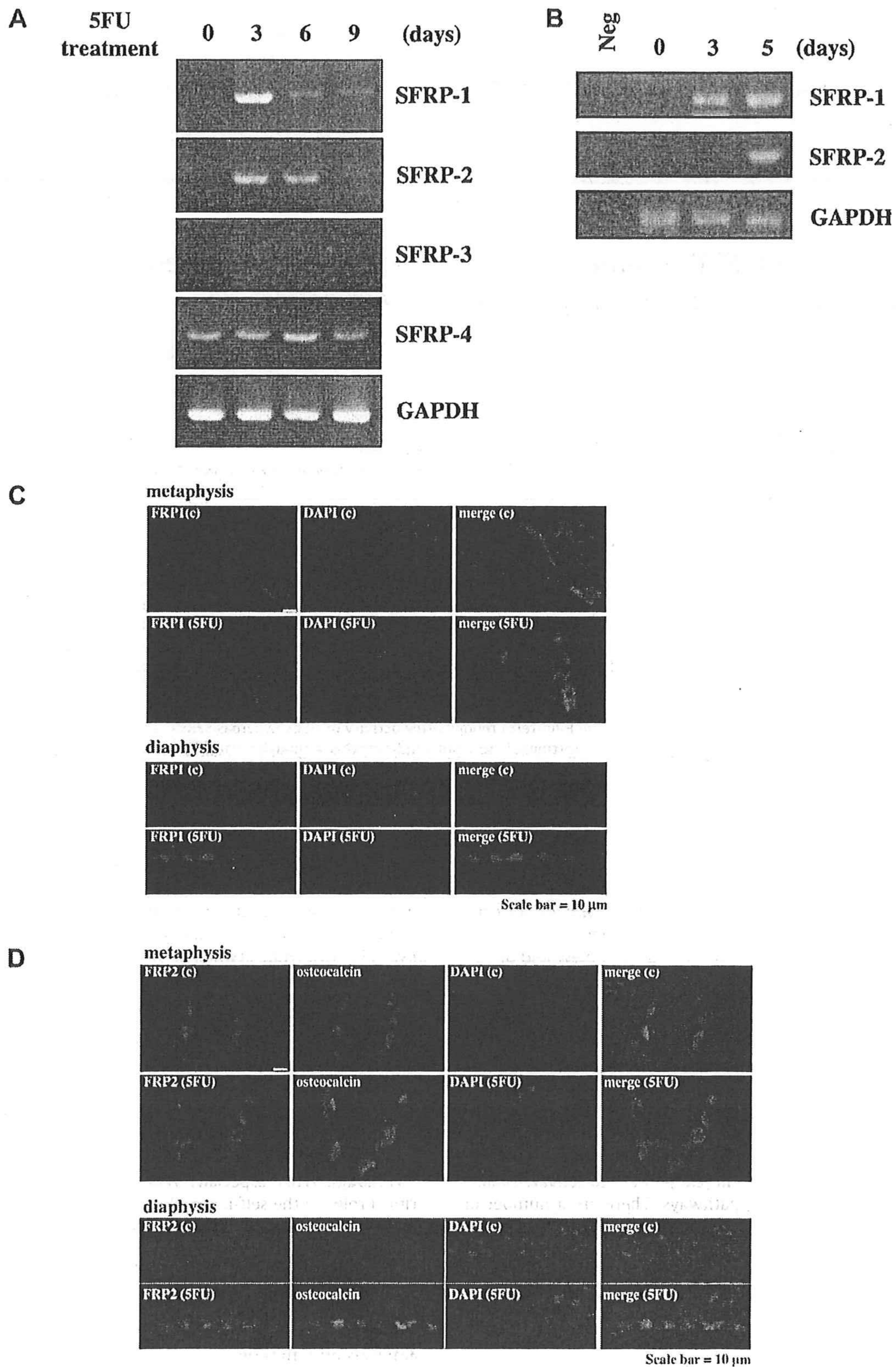

Fig. 1. Expression of SFRP-1 and SFRP-2 in osteoblasts. (A,B) Expression of the SFRP family genes in the BM and their regulation by myelosuppression. The mice were treated with 5-FU (150 mg/kg, i.p.) (A) or irradiation (950 rads) (B), and the BM cells were harvested at the indicated time points. The expressions of SFRP genes in the whole BM cells were analyzed by RT-PCR. PCR was run for 35 cycles. (C,D) The sections of femurs prepared from either control non-treated mice (C) or mice treated with 5-FU for 3 days (5-FU) were immunostained with anti-SFRP-1 (FRP1) (C) or SFRP-2 (FRP2) (D) antibodies. SFRP-2 was stained together with an anti-osteocalcin antibody. In (C), double staining of SFRP-1 and osteocalcin was not possible since both antibodies were from the same species. The nuclei were counterstained with DAPI.

(Tsukuba, Japan). Mice from 8 to 12 weeks old were used in all experiments. All animal experiments were reviewed and approved by the Internal Review Board of the Institute of Medical Science, the University of Tokyo and by the Committee of Animal Use and Care of Keio University School of Medicine.

Reagents. Recombinant human SFRP-1, mouse SFRP-2, and mouse Wnt3a proteins were purchased from R&D systems.

Flow cytometry. The following monoclonal antibodies were used for flow cytometric analysis: c-Kit (ACK2), Sca-1 (E13-161.7), and CD34 (RAM34). All antibodies were purchased from BD Pharmingen. Purification of $CD34^-$KSL cells was done as previously described [19]. Briefly, BM cells were harvested from 8- to 12-week-old mice, and mononuclear cells were separated by density-gradient centrifugation using Lymphoprep (Nycomed). Lineage-positive cells were depleted with Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's protocol. Lineage-negative cells were stained with anti-CD34-FITC, anti-Sca-1-PE, anti-c-Kit-APC, and an anti-lineage antibody cocktail in the Lineage Cell Depletion Kit, followed by staining with streptoavidin-PE-Cy7. FACS Calibur or FACS Aria was used for analysis and cell sorting.

Culture of hematopoietic stem cells and colony-forming assays. $CD34^-$KSL cells were sorted into 96-well plates and cultured in S-clone SF-03 (Sanko Junyaku) + 0.5% bovine serum albumin (BSA) + 50 ng/ml SCF + 50 ng/ml TPO with or without 1 μg/ml of recombinant SFRP-1 or SFRP-2 (R&D systems). Colony-forming assays were performed as previously described [20]. Cells were recovered, cytospun onto glass slides, and then subjected to May-Giemsa staining for morphological examination.

Bone marrow transplantation. Cultured $CD34^-$KSL cells from C57BL/6-Ly5.1 mice were transplanted into lethally irradiated (950 rad) C57BL/6-Ly5.2 recipients in competition with 2×10^5 BM mononuclear cells from Ly5.2 mice. Reconstitution of donor cells in peripheral blood was monitored by staining cells with anti-Ly5.1 antibody. Secondary transplantation was performed by injecting 2×10^6 whole BM cells taken from primary recipient mice at 24-weeks after the first transplant into lethally irradiated C57BL/6-Ly5.2 secondary recipients.

RT-PCR. $PolyA^+$ mRNA was extracted from BM cells using a Micro-FastTrack 2.0 Kit (Invitrogen). cDNAs were reverse-transcribed by SuperScript II reverse transcriptase (Invitrogen), and PCR was performed with Ex Taq-HS (Takara). Primer sequences are shown in Supplementary Table 1.

Immunostaining. Mice treated with 5-FU (150 mg/kg, i.p.) for 3 days were fixed by perfusing Zamboni's fixative. Femurs were then dissected and decalcified in 10% EDTA at 4 °C. The bones were embedded in OCT compound (Sakura Fine Technical, Tokyo, Japan), frozen, and sectioned in 5 μm thick by Cryostat (Leica). Sections were blocked with biotin and normal donkey serum, and stained with rabbit anti-FRP-1 (H-90, Santa Cruz), goat anti-FRP-2 (C-18, Santa Cruz), and anti-osteocalcin (Takara) antibodies, which were then subjected to secondary staining with anti-rabbit or goat IgG-biotin and anti-mouse IgG-Cy3, and tertiary staining with streptoavidin-Cy2. Sections were washed three times for 5 min in PBS between each step and finally mounted in Vectashield anti-fading medium (Vector Labs; Burlingame, CA) containing DAPI (Sigma Chemical; St. Louis, MO) for nuclear labeling. Fluorescent images were examined and captured by laser confocal microscope (Olympus).

Plasmids. Retroviral plasmid expressing mouse SFRP-1 (pMXs-IG/mSFRP-1) was described previously [21]. For pMXs-IG/mSFRP-2, cDNA was amplified by PCR with Pfu polymerase (Stratagene) using murine bone marrow cDNA as a template, and was subcloned into pMXs-IG vector. Integrity of the amplified sequence was confirmed by DNA sequencing.

Retrovirus production and infection. Production of retrovirus was performed as previously described [22]. Mice were treated with single dose of 5-FU (150 mg/kg, i.p.) and their BM cells were harvested four days after the treatment. BM mononuclear cells were separated and used for retrovirus infection. Infection of retrovirus was performed using RetroNectin (Takara) according to the manufacturer's protocol.

Statistical analysis. All statistical analyses were performed by unpaired Student's *t*-test using PRIZM software.

Results

Induction of SFRP-1 and SFRP-2 in the bone marrow by myelosuppression

The expression of SFRPs in response to myelosuppressive stress was examined to determine the role of SFRPs in the regulation of

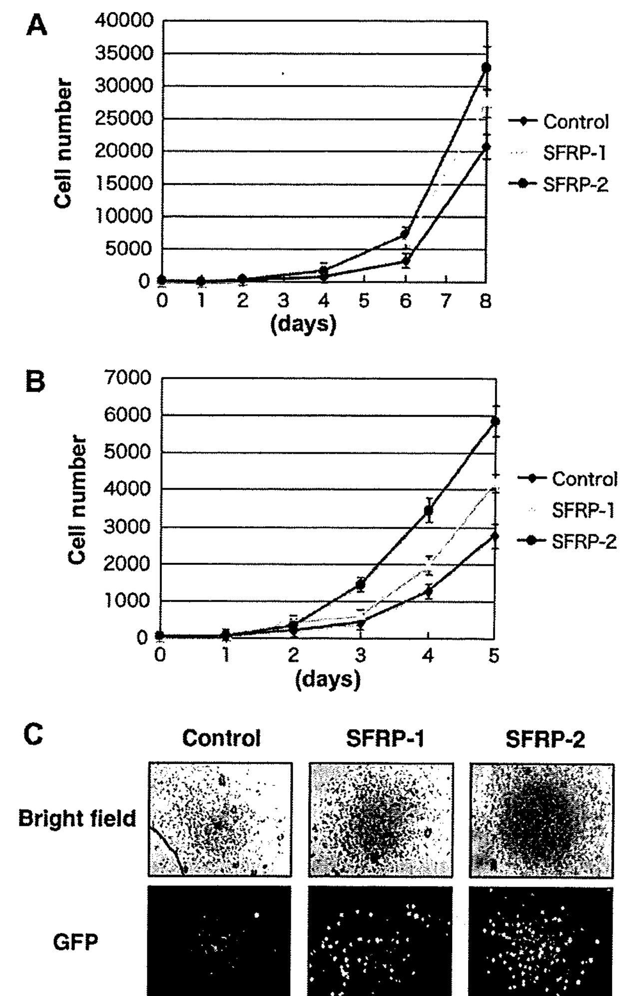

Fig. 2. The effects of SFRP-1 and SFRP-2 on the proliferation of $CD34^-$KSL cells. (A) One hundred $CD34^-$KSL cells/well were sorted into a 96-well plate and cultured in S-Clone SF-03 supplemented with 0.5% BSA, 50 μM 2-ME, stem cell factor (SCF, 50 ng/ml) and thrombopoietin (TPO, 50 ng/ml) with SFRP-1 (1 μg/ml) or SFRP-2 (1 μg/ml). Cell numbers were counted at each time points under a microscope ($n = 3$, mean ± S.D.). (B) One hundred $CD34^-$KSL cells were sorted into a 96-well plate coated with RetroNectin, and prestimulated in αMEM supplemented with 1% FBS, 50 ng/ml SCF, 50 ng/ml TPO and 50 μM 2-mercaptoethanol (2-ME). Control-, SFRP-1- or SFRP-2-retrovirus was added after 24 h of prestimulation; the cells were further cultured in S-clone SF-03 containing 0.5% BSA, 50 ng/ml SCF, 50 ng/ml TPO and 50 μM 2-ME. GFP^+ cell numbers were counted at the indicated time points (upper panel). Data are means ± S.D. ($n = 3$). Representative pictures of cells cultured for five days were also shown (lower panel, original magnification, 100×).

hematopoietic stem cells (HSCs). Interestingly, among the four SFRP members, SFRP-1 and SFRP-2 were specifically induced in the bone marrow (BM) in response to myelosuppressive stimuli such as 5-fluorouracil (5-FU) or irradiation (Fig. 1A and B). In contrast, SFRP-3 and SFRP-4 were not expressed or constitutively expressed in the BM, and their expressions were not influenced by myelosuppression. These results suggest that SFRP-1 and SFRP-2 might be involved in the homeostasis of HSCs during myelosuppression.

SFRP-1 and SFRP-2 are expressed in osteoblasts

To elucidate where SFRP-1 and SFRP-2 are expressed in the BM, we performed immunostaining of the BM during myelosuppression. Interestingly, both proteins were constitutively expressed in osteoblasts (OBs), but not in hematopoietic cells (Fig. 1C and D). Under steady state conditions, OBs lined the surface of trabecular bones in metaphysis, but they were scarcely found in diaphysis (Fig. 1C and D). In contrast, 5-FU treatment increased the number of OBs in the diaphysis, whereas that in the metaphysis was unchanged. The expression of SFRP-1 or SFRP-2 in each osteoblast did not differ in the control or 5-FU-treated BM. These results suggest the increase of the number of OBs contributed to the up-regulation of SFRP-1 and SFRP-2 in the BM by 5-FU. Since OBs are one of the major components of the HSC niche, we speculated that SFRP-1 and SFRP-2 might regulate the HSC physiology under myelosuppression.

SFRP-1 and SFRP-2 stimulate proliferation of hematopoietic stem/progenitor cells, while they differentially affect their multilineage differentiation potential

Next, the effect of SFRP-1 and SFRP-2 on the growth and differentiation of HSCs was examined. CD34$^-$c-Kit$^+$Sca-1$^+$lineage$^-$ (CD34$^-$KSL) cells [23] were sorted from murine BM by flow cytometry (FACS) and subjected to an *in vitro* growth assay with or without SFRPs. As shown in Fig. 2A, both SFRP-1 and SFRP-2 stimulated the proliferation of CD34$^-$KSL cells approximately 1.3–1.5-fold in comparison to the control-treated cells. This stimulatory effect for growth was more evident when SFRP-1 and SFRP-2 were overexpressed in CD34$^-$KSL cells allowing autocrine production of the proteins (Fig. 2B). In this case, cellular proliferation was stimulated by approximately 2-fold by SFRP-2 and by 1.5-fold by SFRP-1.

Interestingly, colony assay revealed that the number of multipotent progenitors (MPPs; GEMM, and GEM in Fig. 3) generated from CD34$^-$KSL cells decreased by approximately half by the treatment with SFRP-1 for 2-weeks. This indicates that SFRP-1 facilitates differentiation of CD34$^-$KSL cells and many of the cells lose clonogenic potential during 2-weeks' culture. In sharp contrast, SFRP-2 slightly increased the number of MPPs (about 1.2-fold) in comparison to the controls by 2 weeks of culture (Fig. 3), showing that SFRP-2 preserves, or slightly enhances multilineage differentiation potential of CD34$^-$KSL cells.

Taken together, while both SFRP-1 and SFRP-2 stimulate proliferation of CD34$^-$KSL cells *in vitro*, they clearly differ in their ability to sustain multipotency of hematopoietic stem/progenitor cells.

SFRP-1 and SFRP-2 differentially regulate long-term repopulating potential of hematopoietic stem cells

Given that SFRP-1 and SFRP-2 affect the proliferation and differentiation potential of HSCs, they may also regulate the self-renewal and long-term repopulating (LTR) activities of HSCs. CD34$^-$KSL cells were sorted by FACS, cultured *in vitro* with SFRP-1 or SFRP-2 for 2-weeks, and transplanted into lethally irradiated congenic recipient mice to assess their LTR activity. Surprisingly, HSCs treated with SFRP-1 almost completely lost their LTR activity, whereas those treated with SFRP-2 sustained that to a degree comparable to control-treated cells (Fig. 4A). Furthermore, the cells treated with SFRP-2 presented enhanced LTR potential in comparison to the controls in the secondary recipients (Fig. 4B), thus suggesting that SFRP-2 enhances self-renewal capacity of HSCs. These data show a clear difference between SFRP-1 and SFRP-2 in the regulation of LTR and self-renewal capacity of HSCs.

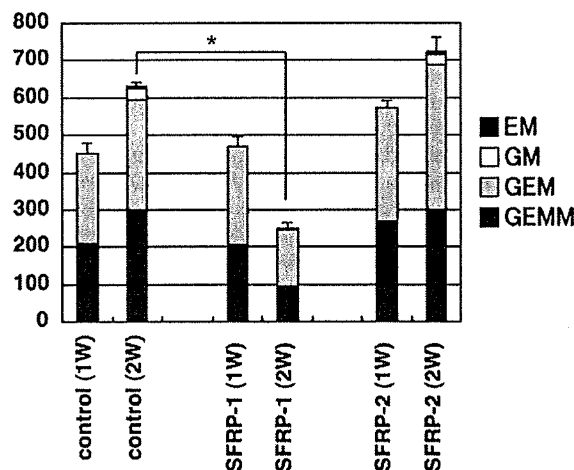

Fig. 3. Differential effects of SFRP-1 and SFRP-2 on the progenitor production by CD34$^-$KSL cells. Fifty CD34$^-$KSL cells were cultured in S-Clone SF-03 supplemented with 0.5% BSA, 50 μM 2-ME, SCF (50 ng/ml) and TPO (50 ng/ml) with SFRP-1 (1 μg/ml) or SFRP-2 (1 μg/ml) for 1 or 2 weeks, after which cells were subjected to colony assays. GEMM, granulocyte/erythroid/macrophage/megakaryocyte; GEM, granulocyte/erythroid/macrophage; GM, granulocyte/macrophage; EM, erythroid/megakaryocyte. Data are means ± S.D. ($n = 3$). $^*p < 0.05$.

Discussion

Although Wnt family proteins are well-known regulators of HSCs [2], the regulation of their activities remains obscure. This study showed that endogenous Wnt modulators, SFRP-1 and SFRP-2 are expressed in osteoblasts and they differentially affect the HSC activities. These findings raise a possibility that SFRP-1 and SFRP-2 play critical roles in regulating Wnt activities in the osteoblastic niche in the BM.

A previous study using drosophila Wnt and human SFRP-1 suggested that SFRPs act either positively or negatively on Wnt signaling depending on their concentration [11]. However, we observed that both human SFRP-1 and mouse SFRP-2 equally repressed mouse Wnt3a activity *in vitro* regardless of their concentration (Supplementary Fig. 1). Based on this observation, we speculated that both SFRP-1 and SFRP-2 act as negative regulators for Wnt signaling. However, our study revealed that, while both SFRP-1 and SFRP-2 enhanced the proliferation of CD34$^-$KSL cells, they affected their clonogenicity and the LTR activity in a completely different manner at least *in vitro*.

In the colony assays, SFRP-1 reduced the number of mix colonies (GEMM and GEM) derived from CD34$^-$KSL cells after 2-weeks of culture, while SFRP-2 enhanced it by approximately 1.2-fold. These data indicate that SFRP-1 facilitated differentiation of CD34$^-$KSL cells so that many of the cells passed the stage of MPPs, leading to the decrease of mix colonies. In contrast, SFRP-2 enhanced the proliferation of CD34$^-$KSL cells and increased the number of MPPs, suggesting that SFRP-2 stimulates proliferation of hematopoietic stem/progenitor cells with their multipotency being preserved.

The differential effect of SFRP-1 and SFRP-2 on the LTR capacity of HSCs was even more striking. By the treatment of CD34$^-$KSL

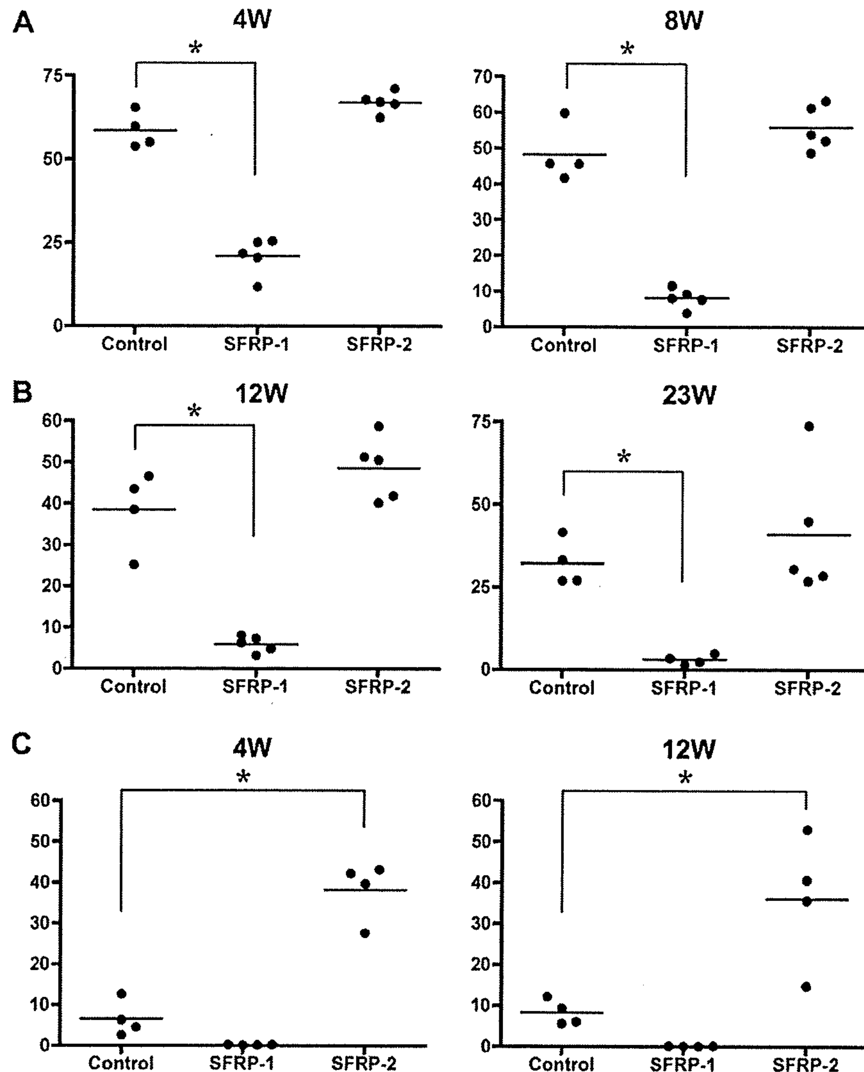

Fig. 4. Differential effects of SFRP-1 and SFRP-2 on the long-term repopulating capacity of CD34$^-$KSL cells. (A) Long-term repopulating activity of CD34$^-$KSL cells cultured with SFRP-1 or SFRP-2. Twenty CD34$^-$KSL cells (Ly5.1) were cultured for 2 weeks in S-Clone SF-03 supplemented with 0.5% BSA, 50 μM 2-ME, 50 ng/ml SCF and 50 ng/ml TPO with SFRP-1 (1 μg/ml) or SFRP-2 (1 μg/ml). After the culture, cells were harvested and transplanted into lethally irradiated congenic hosts (Ly5.2) with 2×10^5 competitors (Ly5.2). Percentages of donor cells in peripheral blood were analyzed at the indicated time points. The data were not statistically different between the control- ($n = 4$) and SFRP-2-treated ($n = 5$) groups at any time points. In contrast, the control- and SFRP-1-treated groups were statistically different at all time points ($^*p < 0.05$). (B) Secondary transplantation. Bone marrow cells taken from the primary recipient mice shown in (A) were pooled and used for the secondary transplantation as described in the Materials and methods. The percentages of donor cells in peripheral blood were analyzed at the indicated time points ($n = 4$, $^*p < 0.05$).

cells with SFRP-1 for 2 weeks completely impaired their LTR capacity. In clear contrast, SFRP-2 enhanced the LTR activity of HSCs as revealed by the secondary transplantation. These observations are compatible with the adverse or positive effect of SFRP-1 or SFRP-2, respectively, on the multipotency of HSCs in the colony assays. Considering a role of Wnts in the self-renewal of HSCs, this study suggests that SFRP-1 and SFRP-2 may act as an antagonist and an agonist for Wnt signaling, respectively. Differential effects of SFRP-1 and SFRP-2 like this case have actually been reported previously in the apoptotic process of cancers and developmental patterning [24–26]. In addition, Yoshino et al. have suggested SFRP-2 did not inhibit Wnt signaling during metanephric kidney development, supporting our hypothesis of agonistic function of SFRP-2 on Wnt activity [27]. It is therefore plausible that SFRP proteins are context-dependent modulators for Wnt signaling and different SFRPs have distinct functions. Molecular basis for these differences is not clear yet, however, it is suggested that some SFRPs inhibit other SFRP function, or they may activate Fz receptors through direct binding [28].

One puzzling finding is SFRP-1 and SFRP-2 are simultaneously expressed in the osteoblasts in the BM. In this respect, different time courses of induction for both proteins (Fig. 1A) may give us a clue how these proteins play roles during myelosuppression. SFRP-1 is strongly induced in response to 5-FU on day 3, and its expression is sustained until day 9. In contrast, SFRP-2 is being turned on from day 3 to day 6, and is abruptly shut down on day 9. These different patterns of expression predict that SFRP-1 facilitates progenitor differentiation throughout the period of myelosuppression to supply mature hematopoietic cells, while SFRP-2 transiently stimulates self-renewing cell division of HSCs to provide MPPs for further differentiation and expansion. Irrespective, precise roles of SFRP-1 and SFRP-2 *in vivo* remain to be determined in the future study.

Conclusion

In conclusion, we revealed that SFRP-1 and SFRP-2 are expressed in the BM osteoblasts and differentially regulate HSC/progenitor functions *in vitro*. In addition to SFRPs, there are a number of endogenous modulators for Wnt activities such as WIF-1, Cerberus, and Dkk [5], and we still do not know how these modulators are integrated *in vivo* to regulate Wnt activities in the BM. Revealing the molecular network of Wnt modulators in the BM niche will enable us to better understand HSC regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.09.067.

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