Brief Definitive Report

Spred-2 Suppresses Aorta-Gonad-Mesonephros Hematopoiesis by Inhibiting MAP Kinase Activation

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Abstract

In midgestation mouse embryos, the aorta-gonad-mesonephros (AGM) region generates hematopoietic stem cells and definitive hematopoiesis is regulated by cell–cell interaction and signaling molecules. We showed that a Ras/mitogen-activated protein (MAP) kinase signaling-specific inhibitor and a dominant negative mutant Ras blocked the production of $CD45^+$ hematopoietic cells in embryonic day 11.5 AGM culture, indicating an essential role for the MAP kinase pathway in AGM hematopoiesis. Overexpression of the Ras/MAP kinase pathway regulator, Spred-2, in the AGM culture significantly reduced the number of $CD45^+$ cells. In contrast, production of $CD45^+$ cells from the AGM region of Spred-2–null mice was up-regulated as compared with wild-type littermates. Furthermore, Spred-2–deficient mice exhibited elevated hematopoietic colony formation from vascular endothelial-cadherin$^+$ cells. These data indicate that Spred-2 functions as a negative regulator of AGM hematopoiesis by inhibiting hematopoietic cytokine signaling.

Key words: hematopoiesis • differentiation • SCF • c-Kit • AGM

Introduction

Hematopoietic cells are derived from multipotent progenitors, hematopoietic stem cells (HSCs). During embryogenesis, development of the hematopoietic system is dependent on a hierarchical mechanism, which is influenced by environmental niches, having contacts with stromal cells and extracellular signals including cytokines, thereby inducing expression of unique transcription factors via an intracellular signaling pathway. In the midgestation mouse embryo, definitive hematopoiesis occurs in the aorta-gonad-mesonephros (AGM) region, which contains a population that is able to repopulate irradiated mice upon direct transplantation (1, 2). Within the AGM region, the first HSCs are generated in the dorsal aorta (3). More recently, endothelial cells that overlap with expression of HSCs markers (Sca-1 and c-Kit) in midgestation mouse aorta have long-term reconstitution activities (3–5). However, intracellular signals that regulate this hematopoiesis have not been clarified.

Recently, we cloned a family of novel membrane-bound molecules, Spred-1, Spred-2, and Spred-3 (6, 7), which are related to Sprouty family proteins that act as negative regulators during development (8, 9). Spred-1, Spred-2, and Spred-3 inhibit the Ras/mitogen-activated protein (MAP) kinase cascade mediated by fibroblast growth factor receptor and epidermal growth factor receptor, by binding to Ras and consequently inhibiting phosphorylation of Raf. Spred proteins have a negative effect on extracellular signal–regulated kinase (Erk)-dependent differentiation in PC12 pheochromocytoma cells and C2C12 myoblastic cells (7). The physiological functions of Spred proteins, however, remain to be elucidated. In this study, we demonstrated the importance of Spred-2 for hematopoiesis in the midgestation mouse embryo and, in part, in adult bone marrow.

Materials and Methods

Spred-2–null Mice. A targeting vector was constructed to delete exon 6 of Spred-2–encoding KBD and SPR domains. Targeting of embryonic stem cells and the generation of chimeric mice were performed according to a previously published procedure

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(10). Detailed analysis of embryonic stem clones and Spred-2–null mice will be published elsewhere.

AGM Cultures and Semisolid Colony-forming Assay. AGM cultures and semisolid colony-forming assay were performed as previously described (11).

RT-PCR. 5 μg total RNA isolated from E11.5 AGM was reverse transcribed with Superscript II (GIBCO BRL). PCRs were performed using rTaq (Takara Biotechnology Inc.) with the following settings: 95°C for 3 min, 26 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 1 min. The primer sets used were as follows: 5′-TGTGAGCACCGGAAGATTTATACC-3′, 5′-CGCGGCGGCTTTGTGCTT-3′ (for Spred-2); 5′-AGCACTGATTATATTCCTGG-3′, 5′-TGCTTGGCCAGTATTCTGCG-3′ (for CD45); 5′-ACCACCCGATACCCACCTAT-3′, 5′-GCCATGGCAGTCACCATGCT-3′ (for GATA-2); and 5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′ (for G3PDH).

Retrovirus Infection of Cells. We prepared mutant proteins of Spred-2 (see Fig. 2 B): ΔN, with the NH_2-terminal EVH-1 domain (residues 1–139) deletion; ΔC, with the COOH-terminal SPR domain (residues 289–410) deletion; and ΔKBD, with the c-Kit–binding domain (residues 197–287) deletion. Production of retroviruses and infection of AGM cultures were performed as previously described (11).

Flow Cytometry. The nonadherent cells, which were recovered from the AGM culture, were stained with PE-conjugated rat anti–mouse CD45 (30-F11; Becton Dickinson) for 30 min on ice. After washing, stained cells were analyzed by FACSCalibur™ (Becton Dickinson). The percentage of $CD45^+$ cells in the virus-infected green fluorescent protein $(GFP)^+$ cells was determined.

Cell Sorting and Coculture with OP9 Stroma Cells. Nonadherent cells derived from the mock-infected AGM culture were stained with PE-conjugated anti–mouse CD45 (30-F11) and allophycocyanin-conjugated anti–mouse c-Kit (2B8; Becton Dickinson). GFP^+ $CD45^-$ $c\text{-}Kit^-$, GFP^+ $CD45^{low}$ $c\text{-}Kit^{low/+}$, and GFP^+ $CD45^+$ $c\text{-}Kit^-$ cells were sorted by FACS Vantage™ (Becton Dickinson). Each of sorted cells (2×10^3) in each population were seeded on OP9 stromal cells in DMEM supplemented with 10% (vol/vol) fetal calf serum, 100 ng/ml IL-3, 100 ng/ml stem cell factor (SCF), and 4 U/ml erythropoietin (EPO).

$Spred\text{-}2^{+/+}$ and $Spred\text{-}2^{-/-}$ E10.5 embryonic trunk lower than the heart was incubated in dispase II (Roche) at 37°C for 20 min. After washing in PBS, cells were treated with cell dissociation buffer (Invitrogen) at 37°C for 20 min. Dissociated cells were stained with anti–mouse vascular endothelial-cadherin (VE-cad; 11D4.1; Becton Dickinson). 2×10^3 sorted $VE\text{-}cad^+$ cells were seeded on OP9 stromal cells in the medium as described above. After 3 d, cobble stone area–forming cells were counted.

Immunoblotting and Coimmunoprecipitation Analyses. 2×10^6 Plat-E cells (12) were transfected with expression constructs using a Trans-IT 293 (Mirus). On the next day, cells were starved for 16 h and then treated with 100 ng/ml SCF for 5 min. Cell lysates were immunoprecipitated with anti-GFP antibody (Medical & Biological Laboratories, Co.). Immunoprecipitates or cell lysates were separated by SDS-PAGE and immunoblotted with anti–phospho-Erk1/2 antibody (E10; Cell Signaling Technology), anti-Erk2 antibody (sc-145; Santa Cruz Biotechnology, Inc.), anti–c-Kit (M-14; Santa Cruz Biotechnology, Inc.), and anti-Flag (M2; Sigma-Aldrich) antibody.

Pharmacological Reagents. AGM cells cultured for 2 d were treated with 0, 1, 3, or 10 μM U0126 (Cell Signaling Technology) or SB203538 (Calbiochem). After 8 d of treatment, the nonadherent cells were counted and analyzed for determination of the percentage of $CD45^+$ cells.

Online Supplemental Material. Inhibition of SCF-induced Erk phosphorylation and Elk-1 activation by Spred-2 mutants is presented in Fig. S1. Fig. S1 and Supplemental Materials and Methods are available at http://www.jem.org/cgi/content/full/jem.20030830/DC1.

Results and Discussion

First, to examine any possible role that the Ras/MAP kinase pathway has in AGM hematopoiesis at a midgestation stage, we examined the effect of U0126, a MEK inhibitor, on the appearance of $CD45^+$ hematopoietic lineage cells from E11.5 primary AGM culture. In the AGM culture with SCF, basic fibroblast growth factor (bFGF), and oncostatin M (OSM), endothelial-like cells are initially observed and then the nonadherent cells, which include lineage-committed cells (e.g., $CD45^+$), gradually emerge (13, 14). As shown in Fig. 1 A, U0126 dose dependently inhibited the production of $CD45^+$ hematopoietic cells, whereas SB203538, a p38 inhibitor, had no effect. Next, we tested the effect of a dominant negative Ras (Ras-17N) and a dominant negative mutant of Akt (K197A/T308A/S473A; Akt-DN; reference 15), which is known to function downstream of the PI3K pathway on the generation of $CD45^+$ cells in the AGM culture. We used bicistronic retrovirus vector pMY-IRES-EGFP so that the infected cells could be monitored by measuring expression of an enhanced GFP by flow cytometry.

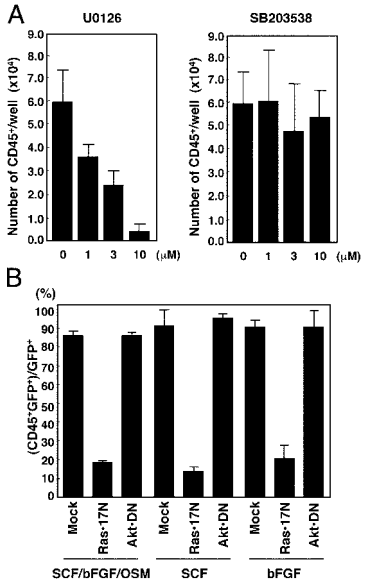

Figure 1. Role of the Ras/MAP kinase cascade in the AGM culture. (A) E11.5 AGM cells were cultured in DMEM with 15% (vol/vol) FCS, 100 ng/ml SCF, 1 ng/ml bFGF, and 10 ng/ml OSM. On the second day of culture, the cells were treated with various concentrations of U0126 and SB203538, respectively. After 5 d, the nonadherent cells were counted and then the number of $CD45^+$ hematopoietic cells per well was assessed by the percentage of $CD45^+$ cells in the nonadherent cells using flow cytometry ($n = 3$). (B) On the second day of the AGM culture with the indicated cytokines, the cells were infected with mock retrovirus vector, Ras-17N, or Akt-DN (K197A/T308A/S473A). After an additional 8 d of culture, the nonadherent cells were stained with an anti-CD45 antibody and analyzed by flow cytometry ($n = 3$).

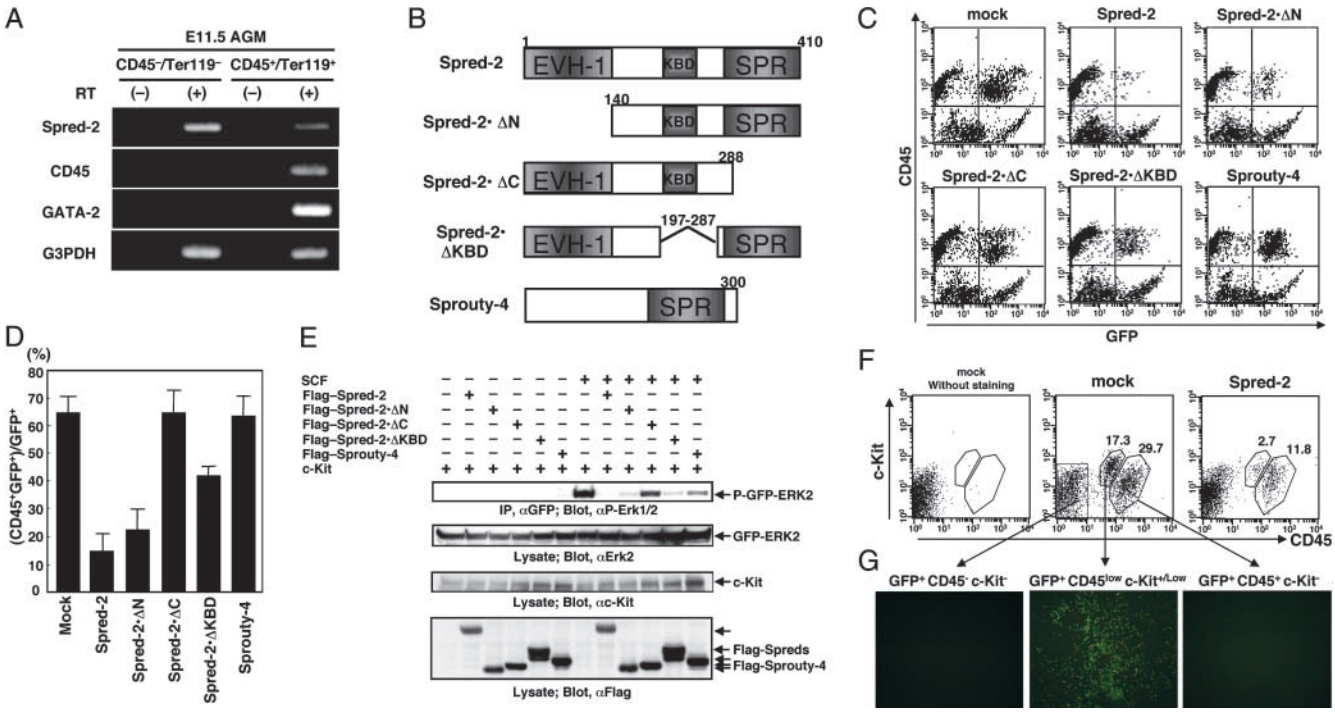

Figure 2. Inhibition of the hematopoietic differentiation by Spred-2 in cultured AGM cells. (A) Expression of Spred-2 in the AGM region. Total RNAs were extracted from CD45$^+$ Ter119$^+$ or CD45$^-$ Ter119$^-$ cells and subjected to RT-PCR using specific primers for Spred-2, CD45, GATA-2, and G3PDH. (B) Schematic representation of the structures of Spred-2 mutants. (C) On the second day of the AGM culture, the cells were infected with GFP and/or a Spred-2 mutant retrovirus. After an additional 5 d of culture, the nonadherent cells were stained with an anti-CD45 antibody and analyzed by flow cytometry. (D) The percentage of CD45$^+$ cells in the GFP$^+$ cells was determined ($n = 4$). (E) Plat-E cells were transfected with vectors encoding GFP-Erk-2, c-Kit, and Flag-tagged Spred-2 mutants or Sprouty-4. The cells were treated with or without 100 ng/ml SCF for 5 min. Cell lysates were subjected to immunoprecipitation with anti-GFP antibody. Precipitates or lysates were separated by SDS-PAGE and analyzed by immunoblotting with indicated antibody. (F) The AGM culture was infected with either mock viruses or Spred-2 viruses, and the nonadherent cells were stained with anti-CD45 and c-Kit antibodies. In the flow cytometric analysis, only the GFP$^+$ (i.e., virus-infected) cells were analyzed for the expression of CD45 and c-Kit. (G) Sorted populations of GFP$^+$ CD45$^-$ c-Kit$^-$, GFP$^+$ CD45^{low} c-Kit$^{low/+}$, and GFP$^+$ CD45$^+$ c-Kit$^-$ were cultured with OP9 stromal cells in the presence of IL-3 and SCF.

As shown in Fig. 1 B, overexpression of Ras-17N reduced the production of CD45$^+$ cells, whereas Akt-DN did not strongly affect the production of CD45$^+$ cells in the culture containing SCF, bFGF, and OSM (Fig. 1 B). Similar results were observed for stimulation with SCF or bFGF alone (Fig. 1 B). These data indicate that the Ras/MAP kinase cascade plays an essential role in AGM hematopoiesis.

Next, we attempted to define the role of Spreds in AGM hematopoiesis. Among Spred family proteins, we focused on Spred-2 because it has been shown to negatively regulate c-Kit signaling (16). We first examined Spred-2 expression in the AGM region. Hematopoietic (positive for either CD45 or Ter119) and nonhematopoietic (negative for both CD45 and Ter119) cells were prepared from E11.5 AGM, and RNA from each population was subjected to RT-PCR. As shown in Fig. 2 A, transcripts for Spred-2 were detectable in both populations, whereas those for hematopoietic transcription factor GATA-2 were detected only in the former population. Spred-2 protein expressed in the AGM region may play a role in the hematopoiesis in midgestation mouse embryo.

To examine whether the production of hematopoietic cells in the AGM culture was regulated by Spred-2, wild-type and truncated forms of Spred-2 and related molecules (Fig. 2 B) were overexpressed in cultured AGM cells by retrovirus gene transfer. As shown in Fig. 2 C, full-length Spred-2 exhibited a significant inhibitory effect on the appearance of CD45$^+$ nonadherent cells in the AGM cultures. In contrast, neither Sprouty-4, which is related to the Spred proteins, nor STAP-1, which interacts with c-Kit (17), was able to inhibit the appearance of CD45$^+$ cells from cultured AGM cells (Fig. 2, C and D, and unpublished data). It has been shown that Sprouty-4 has the inhibitory effect on bFGF-induced Erk phosphorylation, but not epidermal growth factor– and vascular endothelial growth factor (VEGF)-induced Erk phosphorylation (7, 18, 19).

Next, we examined the function of each domain in the Spred-2 protein by using deletion mutants. ΔC could not inhibit the production of CD45$^+$ cells in AGM cultures, whereas ΔN and ΔKBD exhibited inhibitory effect though the latter showed weaker effect (Fig. 2, C and D). These results suggest that the SPR domain of Spred-2 plays a critical role in the suppression of hematopoietic cell development in AGM cultures. We then examined the role of each domain of Spred-2 in the SCF/c-Kit signaling pathway. Full-length Spred-2 and ΔN interacted with c-Kit upon

SCF stimulation of AGM cells. ΔC and ΔKBD showed no detectable binding to c-Kit (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20030830/DC1). SCF-induced Erk phosphorylation was suppressed by Spred-2, ΔN, and ΔKBD, though the suppression by the latter two was incomplete. By contrast, ΔC and Sprouty-4 show little or almost no inhibition of Erk activation (Fig. 2 E). Similar results were obtained by SCF-induced Elk-1 activation in 293 cells (Fig. S1 B). These results indicated that the COOH-terminal region of Spred-2 is required for the interaction with c-Kit and for efficient suppression of SCF-induced Erk-2 phosphorylation. In a previous study, we found that the COOH-terminal region of Spred-2 or Sprouty alone was sufficient for the suppression of Erk activation induced by VEGF in 293 cells (18). Therefore, VEGF and SCF may have a similar activation mechanism for MAP kinase. It is interesting to note that Spred-2•ΔN does not inhibit Erk activation induced by epidermal growth factor or nerve growth factor (16).

Next, we sought to examine the role of Spred-2 in the development of hematopoietic lineage cells expressing CD45. Nonadherent cells from the AGM culture, which had been infected with mock viruses, were gated for GFP expression and then analyzed for CD45 and c-Kit expression. As shown in Fig. 2 F, middle, the cells could be divided into three populations: $CD45^{-}$ c-Kit^{-}, $CD45^{low}$ c-$Kit^{low/+}$, and $CD45^{+}$ c-Kit^{-}. In Spred-2 virus–infected AGM cells (Fig. 2 F, right), the size of the $CD45^{low}$ c-$Kit^{low/+}$ population was dramatically reduced (6.4-fold) and that of the $CD45^{+}$ c-Kit^{-} population was moderately reduced (2.5-fold). To characterize the nature of the $CD45^{low}$ c-$Kit^{low/+}$ population, which was dramatically affected by Spred-2 expression, the above mentioned three populations of cells were cultured on the monolayer of a stromal cell line, OP9. As shown in Fig. 2 G, only the $CD45^{low}$ c-$Kit^{low/+}$ population produced nonadherent hematopoietic cells, indicating that the $CD45^{low}$ c-$Kit^{low/+}$ population could be in a more immature stage and that the other two populations could not produce hematopoietic cells on OP9. These results suggest that Spred-2 is a negative regulator of hematopoiesis in AGM.

To define the role of endogenous Spred-2 in hematopoiesis, we generated mice having Spred-2 gene mutation by homologous recombination, in which exons encoding the KBD and SPR domains were deleted. Offspring were born within the Mendelian expectation ratio from intercrosses of heterozygotes as well as incrosses of homozygotes. This indicates that Spred-2 is not necessary for fertility and development. Adult Spred-$2^{-/-}$ mice appeared to be healthy and showed no apparent abnormalities in most organs (unpublished data).

To examine the effect of Spred-2 deficiency on AGM hematopoiesis at the midgestation stage, we compared the colony-forming activity in methylcellulose of E11.5 aortic cells from Spred-$2^{-/-}$ with that from wild-type littermates (Fig. 3 A, +/+). The number of granulocyte and macrophage colonies formed from Spred-$2^{-/-}$ aortic cells was significantly higher than that from Spred-$2^{+/+}$ littermate aortic cells, indicating that Spred-2 has an inhibitory effect on colony-forming potential of hematopoietic progenitors.

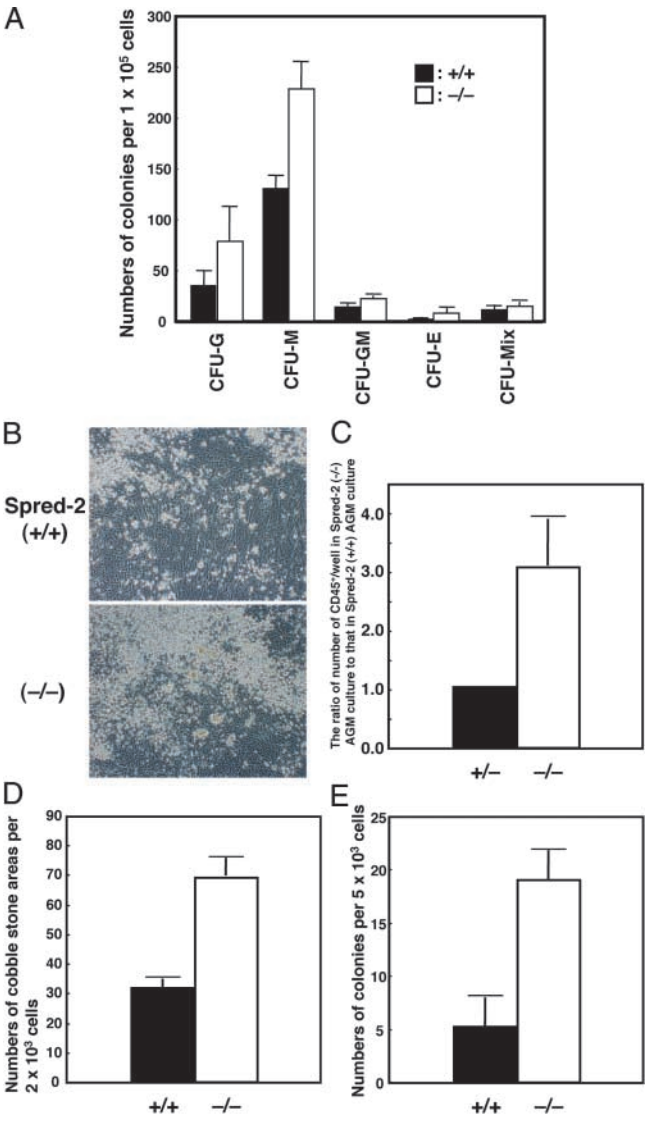

Figure 3. Induction of AGM hematopoietic differentiation in Spred-$2^{-/-}$ cells. (A) Colony-forming activities in primary E11.5 aorta cells in Spred-2–deficient mice. Primary E11.5 aorta cells from Spred-$2^{+/+}$ and Spred-$2^{-/-}$ littermates were inoculated in methylcellulose media containing 100 ng/ml SCF, 20 ng/ml IL-3, and 4 U/ml EPO. Colonies were scored after 7 d of culture ($n = 4$). Colony types: G, granulocytes; M, macrophages; GM, granulocytes and macrophages; E, erythrocytes; Mix, granulocytes, macrophages, and erythrocytes. (B) Expansion of nonadherent cells from the Spred-$2^{-/-}$ AGM culture. E11.5 AGM cells from Spred-$2^{+/+}$ and Spred-$2^{-/-}$ littermates were plated on gelatin-coated 24-well plates at a density of 1.5×10^5 per well with cytokines as described above. On the seventh day of culture, the nonadherent cells generated were counted. (C) The numbers of $CD45^{+}$ nonadherent cells per well were determined by the percentage of $CD45^{+}$ cells in the nonadherent cells ($n = 3$). (D) 2×10^3 sorted VE-cad^{+} cells from E10.5 Spred-$2^{+/+}$ or Spred-$2^{-/-}$ littermates were cultured on OP9 stromal cells in the presence of IL-3, SCF, and EPO. The number of cobble stone areas was counted by phase contrast microscopy ($n = 3$). (E) 5×10^3 sorted VE-cad^{+} cells from Spred-$2^{+/+}$ or Spred-$2^{-/-}$ littermates were inoculated in the methylcellulose medium as described above. The number of colonies was scored after 7 d of culture ($n = 3$).

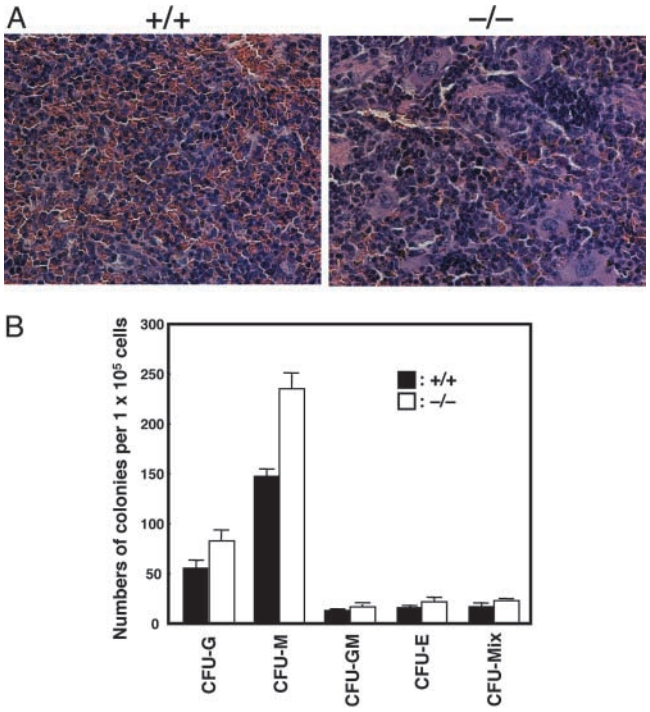

Figure 4. Impairment of hematopoiesis in Spred-2–deficient adult mice. (A) Morphology of the spleen of Spred-2–deficient mice. Paraffin sections of Spred-$2^{+/+}$ and Spred-$2^{-/-}$ spleens were stained with hematoxylin and eosin and viewed at ×400. (B) Colony-forming activities in adult bone marrow cells in Spred-2–deficient mice. Adult bone marrow cells from Spred-$2^{+/+}$ and Spred-$2^{-/-}$ were inoculated in methylcellulose media containing SCF, IL-3, and EPO. Colonies were scored after 7 d of culture ($n = 5$).

Next, we examined the effect of Spred-2 deficiency on embryonic hematopoiesis using the AGM culture. As shown in Fig. 3 B, the number of nonadherent cells produced in the culture of AGM from Spred-$2^{-/-}$ was greater than that from Spred-$2^{+/+}$ littermates, and the ability of the AGM region of Spred-$2^{-/-}$ to generate $CD45^+$ hematopoietic cells in this culture was threefold higher than that of Spred-$2^{+/+}$ (Fig. 3 C). These results indicate that loss of Spred-2 function leads to an increase in hematopoiesis in the AGM region of midgestation mouse.

It has been reported that hematopoietic cells develop from VE-cad^+ cells (20). When VE-cad^+ cells in E10.5 embryo were cultured on a monolayer of OP9 stromal cells, we observed cobble stone area–forming cells, which are suggested to produce HSCs (21). The number of cobble stone areas found in the cultured VE-cad^+ cells from E10.5 Spred-$2^{-/-}$ embryo was 2.2-fold larger than that from wild-type littermates (Fig. 3 D, +/+). Colony-forming ability of VE-cad^+ cells sorted from E10.5 Spred-$2^{-/-}$ and Spred-$2^{+/+}$ littermates was assayed in methylcellulose media. As shown in Fig. 3 E, VE-cad^+ cells from Spred-$2^{-/-}$ formed a 3.6-fold larger number of colonies. These results suggest that Spred-2 negatively regulates hematopoiesis in AGM.

Finally, we examined whether a deficiency of Spred-2 causes hematopoietic abnormality in adult mice. As shown in Fig. 4 A, marked increase in the number of megakaryocytes was observed in the spleen of Spred-2–deficient mice, although no qualitative or quantitative abnormalities in peripheral blood hematopoietic cells were observed. Colony-forming assay of adult bone marrow cells in methylcellulose revealed that approximately twofold more hematopoietic cell colonies were produced from bone marrow cells of Spred-$2^{-/-}$ mice (381.9 ± 33.1 colonies/3×10^4 cells) as compared with those from wild-type littermates (250.7 ± 18.9 colonies/3×10^4 cells; Fig. 4 B). These data suggest that Spred-2 is at least in part involved in the regulation of hematopoiesis in vivo.

In this study, Spred-2–deficient mice displayed an enhanced ability of their AGM cells to differentiate into hematopoietic cells at ontogeny. The phenotype in Spred-2–deficient mice resembles that in Lnk-deficient mice whose E11.5 AGM exhibited more potent capacity of hematopoietic differentiation in the culture and in the colony-forming assay (11). It has been reported that a stable transformant of mast cell line MC9 with Lnk cDNA showed a marked attenuation of the SCF-mediated phosphorylation of Gab-2 and the subsequent phosphorylation of Erk-1/2 using the mast cell line MC9 (22). Thus, these proteins having a negative effect on the Ras/MAP kinase cascade demonstrate inhibitory activity on AGM hematopoiesis. On the other hand, Spred-2 deficiency caused a significant increase in the number of megakaryocytes in the adult spleen (Fig. 4 A). This phenotype is very similar to that observed in mice with homozygous deletion of Lnk (23). It should be noted that the maturation of progenitor cells to megakaryocytes is induced by activation of Erk MAP kinase, which is consistent with our finding (24, 25). Therefore, elevation of megakaryopoiesis in the spleen of Spred-$2^{-/-}$ mice supports our idea that Spred-2 is a negative regulator of the Ras/MAP kinase pathway in hematopoiesis.

In conclusion, here we have shown that Spred-2 plays an inhibitory role in AGM hematopoiesis. Our results suggest that Spred-2 has negative effects on differentiation of vascular endothelial cells into hematopoietic cells (Fig. 3, D and E). To further support our idea, time lapse analysis or single cell fate analysis of VE-cad^+ cells may need to be undertaken.

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