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Secreted Frizzled-Related Protein 1 Extrinsically Regulates Cycling Activity and Maintenance of Hematopoietic Stem Cells

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SUMMARY

Secreted frizzled-related protein 1 (*Sfrp1*) is highly expressed by stromal cells maintaining hematopoietic stem cells (HSCs). Sfrp1 loss in stromal cells increases production of hematopoietic progenitors, and in knockout mice, dysregulates hemostasis and increases Flk2− Cd34− Lin− Sca1+ Kit+ (LSK) cell numbers in bone marrow. Also, LSK and multipotent progenitors (MPPs) resided mainly in the G0/G1 phase of cell cycle, with an accompanying decrease in intracellular β-catenin levels. Gene-expression studies showed a concomitant decrease *Ccnd1* and *Dkk1* in Cd34− LSK cells and increased expression of *Pparg*, *Hes1*, and *Runx1* in MPP. Transplantation experiments showed no intrinsic effect of Sfrp1 loss on the number of HSCs or their ability to engraft irradiated recipients. In contrast, serial transplantations of wild-type HSCs into Sfrp1$^{-/-}$ mice show a progressive decrease of wild-type LSK and MPP numbers. Our results demonstrate that Sfrp1 is required to maintain HSC homeostasis through extrinsic regulation of β-catenin.

INTRODUCTION

Hematopoietic stem cells (HSCs) reside in a heterogeneous microenvironment (or niche) in which they generate all mature blood cells. The niche is made up of several different cell types: hematopoietic cells at different stages of differentiation, vascular cells, bone-lining cells, as well as neural cells that tightly regulate the self-renewal capacity and multipotency of HSCs (Morrison and Spradling, 2008). The microenvironment permits the maintenance of HSCs for the entire lifetime of an organism. Moreover, the microenvironment is an efficient sensor of cellular stress, which allows flexible responses to maintain the steady state of the blood cell system. The precise mechanisms of HSC maintenance are still not fully understood.

Prominent players in the regulation of self-renewal and differentiation are the lipid-modified Wnt-signaling members. The Wnt family members bind frizzled (Fzd) receptors to activate downstream signaling in catenin-dependent canonical and noncanonical pathways. In the canonical pathway, Fzd associates with the Lrp5/6 receptor, and signals are propagated through catenins to activate Tcf/Lef transcription complexes. The level of catenins is regulated through serine and threonine phosphorylation and marked for degradation by the Skp1/Cul1/beta-TrCP ubiquitin ligase complex. Activation of catenin-dependent signaling regulates genes involved in cell-cycle regulation and proliferation such as *Ccnd1* and *Myc*, but also *Spp1*, *Socs2*, *P2ry14*, and many others (Nygren et al., 2007). In noncanonical signaling, Fzds associate with either Ryk or Ror receptor tyrosine kinases to activate calmodulin/Ca^{2+}-, or Rho-dependent responses, which, in turn, activate Nfat or the Jun-dependent AP-1 complex, respectively. These pathways regulate a different set of Wnt targets, such as Pparg and Pcdh8 (Takada et al., 2007). Also, there is crosstalk between noncanonical and canonical pathways, as Ca^{2+}-dependent signals inhibits catenin stability through Camk2-mediated activation of Nemo-like kinase (Nlk) and subsequent phosphorylation of catenin (Ishitani et al., 2003).

Knowledge about specificity of Fzds and Wnts for canonical or noncanonical pathways is limited. To complicate matters, there is a range of different Wnt-signaling inhibitors, such as Dkk, Wif or Sfrp, or other Wnt antagonists, such as Kremen, Ctgf, Cyr61, Sost, and Sostdc1. Paradoxically, some of these directly stimulate certain Fzds independent of Wnt factors. For instance, Sfrp1 directly activates Fzd2 (Rodriguez et al., 2005), as well as Fzd4 and Fzd7 (Dufourcq et al., 2008). Also, Wnt factors have been suggested to initiate Fzd-independent signaling events. For instance, Wnt5a activates Fzd-independent signaling through both Ror (Fukuda et al., 2008) and Ryk (Keeble and Cooper, 2006).

Definitive HSCs emerge from the AGM region between E10 and E11. During this midgestational transition, a significant

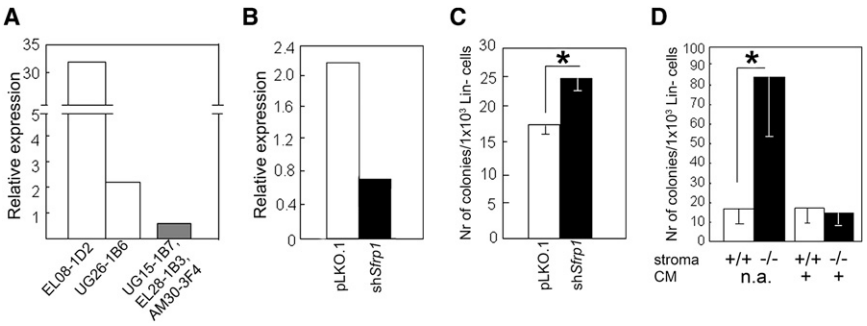


Figure 1. Increased Number of Wild-Type Progenitors after Being Cocultured on Sfrp1 Knockdown and Sfrp1 Knockout Stroma

(A) The expression of *Sfrp1* in EL08-1D2 and UG26-1B6 cell lines relative to nonsupportive stroma (UG15-1B7, EL28-1B3, and AM30-3F4) as measured by real-time PCR. Values were calculated using $2^{\Delta Ct} \times 100\%$ relative to housekeeping gene Rpl13a.

(B) The relative expression (calculated on $2^{\Delta Ct} \times 100\%$ values) of *Sfrp1* in the knockdown clone of UG26-1B6 (sh*Sfrp1*) and in control clone of UG26-1B6 (pLKO.1) relative to Rpl13a.

(C) The number of colonies formed from wild-type (WT) Lin− cells after 2 weeks of coculture on UG26-1B6 sh*Sfrp1* and UG26-1B6 expressing the negative control pLKO1 vector.

(D) The number of colonies formed from WT Lin− cells cultured with primary WT and *Sfrp1*$^{-/-}$ stroma, with and without addition of cell-free conditioned medium (CM) from Sfrp1$^{+/+}$ (control) primary stroma. Data shown are the mean number ± SD of colonies of three to five independent experiments. *$p < 0.05$.

upregulation of β-catenin (Ctnnb1) is observed (Orelio and Dzierzak, 2003). In addition, overexpression of Ctnnb1 results in expansion of the HSC pool, but, at the same time, to loss of myelopoiesis (Kirstetter et al., 2006; Scheller et al., 2006), suggesting that Ctnnb1 regulates self-renewal and inhibits differentiation. Surprisingly, conditional deletion of Ctnnb1 or both Ctnnb1 and plakoglobin does not affect the repopulating ability of HSCs (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). Taken together, these results suggest that canonical Wnt signaling may be important in definitive hematopoietic specification, and that catenin stabilization inhibits differentiation and/or lineage commitment.

Noncanonical Wnt signaling has also been shown to play a role in hematopoiesis. Current evidence suggests that this pathway is important in the establishment of the niche by promoting trabecular bone formation and enlarging endosteal surfaces (reviewed by Yin and Li, 2006), as well as regulating cell cycle of HSCs and more mature progenitors. Wnt5a, the principal noncanonical stimulator, was shown to maintain HSCs (Murdoch et al., 2003; Nemeth et al., 2007). Also, the noncanonical intermediate Camk2 is essential for myeloid proliferation (Si and Collins, 2008). These results show that noncanonical signaling may be important in the control of HSC behavior. Furthermore, the decrease of Ctnnb1 after Wnt5a stimulation suggests that noncanonical signaling is a natural downregulator of canonical signaling.

In addition to this intrinsic role of Wnt signaling, there is also a role for extrinsic Wnt regulation. For instance, Wnt3a stimulates proliferation and inhibits differentiation in cocultures of marrow cells with stromal cells (Yamane et al., 2001). More interestingly, recently, it was shown that wild-type HSCs show increased cell cycling when transplanted into mice transgenic for the canonical inhibitor Dkk1, while at the same time, the HSC pool is gradually lost (Fleming et al., 2008). Further extrinsic effects were demonstrated in experiments where stromal cells deficient in Nlk were shown to be defective in maintaining hematopoietic progenitors (Kortenjann et al., 2001).

We have isolated a large panel of stromal cell clones from which two cell lines, EL08-1D2 and UG26-1B6, maintain both fetal and adult HSCs (Oostendorp et al., 2002, 2005). Comparison of gene expression in these two cell lines with a panel of nonsupporting stromal cell lines revealed a number of overrepresented secreted molecules, including Secreted frizzled-related protein 2 (Sfrp2) (Oostendorp et al., 2005). Here we additionally identify Secreted frizzled-related protein 1 (Sfrp1), a secreted 37kDa protein that inhibits canonical Wnt signaling (Kawano and Kypta, 2003) and interacts with Wnts 1, -2, -3a, and -7b, as a factor upregulated in both supportive stromal cell lines. Our study shows that Sfrp1 regulates production of clonogenic cells and long-term HSC maintenance through an extrinsic signaling mechanism.

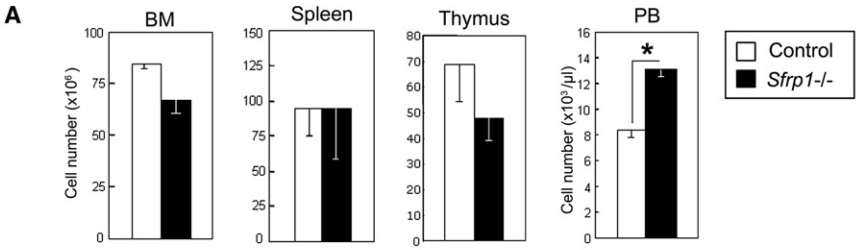
RESULTS

Sfrp1 Is Involved in the Maintenance of Hematopoiesis In Vitro

Sfrp1, one of the secreted-type of Wnt signaling modulators, was shown to be expressed by bone-lining osteoblast-like cells (Yokota et al., 2008), to regulate skeletogenesis (Häusler et al., 2004) and to inhibit trabecular bone formation in adult mice (Bodine et al., 2004). In hematopoietic malignancies, Sfrp1 acts as a putative tumor suppressor molecule (Huang et al., 2007). We found that *Sfrp1* is upregulated in embryo-derived stromal cells UG26-1B6 (2.2% of Rpl13a) and EL08-1D2 (31.8% of Rpl13a), known to support HSCs in non-contact cultures when compared to cell lines not capable of HSC maintenance (0.6% of Rpl13a in a 1:1:1 mixture of UG15-1B7, EL28-1B3, and AM30-3F4), (Oostendorp et al., 2002) (Figure 1A). In order to investigate the role of Sfrp1 in the maintenance of HSCs, we established a stable knockdown of *Sfrp1* of 80% (0.4% of Rpl13a) in the HSC-supportive UG26-1B6 stromal cell line (Figure 1B), which correlates well to the level expressed by the mix of nonsupportive stroma cell lines. Irradiated knockdown stromal cells (*shSfrp1*) or control cells (pLKO.1) were cocultured with the lineage negative (Lin−) fraction of total bone marrow (BM) cells from control mice in a direct-contact manner. Interestingly, Lin− cells cultured on the sh*Sfrp1* UG26-1B6 generated more colonies compared to the Lin− cells cultured on the control clone (Figure 1C), suggesting that lack of microenvironmental Sfrp1 promotes hematopoietic progenitor activity in vitro.

Absence of *Sfrp1* Alters Hematopoiesis In Vivo

To find out how Sfrp1 affects hematopoiesis, we decided to study the mice deficient in Sfrp1 (Satoh et al., 2006). The



B

	Control (n = 32)	*Sfrp1 -/-* (n = 30)	P Value
WBC (10^3/µl)	8.38 ± 0.55	13.12 ± 0.50	<0.0001
Lymph(10^3/µl)	6.80 ± 0.48	10.59 ± 0.39	<0.0001
Mono (10^3/µl)	0.25 ± 0.02	0.50 ± 0.03	<0.0001
Gran (10^3/µl)	1.31 ± 0.09	2.04 ± 0.13	<0.0001
Eos (10^3/µl)	0.04 ± 0.01	0.05 ± 0.01	NS
RBC (10^6/µl)	10.10 ± 0.19	9.63 ± 0.11	NS
HCT (%)	54.30 ± 0.94	51.91 ± 0.56	NS
PLT (10^3/µl)	1156 ± 100	1104 ± 52.8	NS

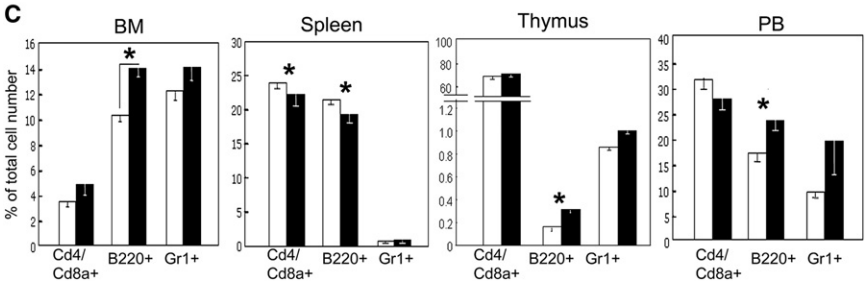


Figure 2. Alterations in Steady-State Hematopoiesis of *Sfrp1*$^{-/-}$ Mice

(A) Total cell numbers from bone marrow (BM, four long bones: two femurs and two tibia per mouse), spleen, thymus, and peripheral blood (PB, 10^3 cells/µl).

(B) Blood cell counts in the PB of control (n = 32) and *Sfrp1*$^{-/-}$ (n = 30).

(C) Total number of Cd4/Cd8a+, B220+ and Gr1+ cells in the BM, spleen, thymus, and PB. White bars represent control animals (n = 7), and black bars represent the *Sfrp1*$^{-/-}$ mice (n = 5). Eos, eosinophiles; Gran, granulocyte; HCT, hematocrit; Lymph, lymphocyte; Mono, monocyte; PLT, platelets; RBC, red blood cell WBC, white blood cell; NS, not significant. All values are shown as mean ± SEM *p < 0.05.

knockout mice were originally generated on the 129 background and were paired at least 5 generations with 129B6 background. In contrast to the old Sfrp-deficient mice studied by others (Bodine et al., 2004), the present study revealed no gross differences in the extension of the growth plates, but a slight increase in chondrocytes of 10-week-old Sfrp1$^{-/-}$ mice compared to their controls (Figure S1). As a first experiment, we investigated the capacity of primary Sfrp1$^{-/-}$ stroma to maintain hematopoiesis using cocultures with Lin- negative cells; the results were in full accordance with the above findings with the knockdown stromal cell line UG26-1B6 (Figures 1B and 1C). Interestingly, the increased production of colony-forming cells was completely abolished when the cultures were supplemented by cell-free conditioned medium (CM) from wild-type (Sfrp1$^{+/+}$) stroma, indicating that an Sfrp1-dependent soluble factor was the cause of the increase in hematopoietic progenitors (Figure 1D). However, addition of recombinant Sfrp1, in a concentration of 1 µg/ml every 3 days, to cocultures of WT Lin− cells and primary marrow stroma did not affect the generation of hematopoietic progenitors (Figure S2). This suggests that either recombinant Sfrp1 is not biologically active or that Sfrp1 does not directly act on hematopoietic progenitors. Because of the above results, we decided to characterize the hematopoiesis of *Sfrp1*$^{-/-}$ mice in more detail.

The BM, spleen, and thymus cellularity was unchanged (Figure 2A), while the peripheral blood (PB) cell number was significantly increased in *Sfrp1*$^{-/-}$ mice (Figures 2A and 2B). The numbers of eosinophiles (Eos), red blood cells (RBC), hematocrit (HCT), and platelets (PLT) were within normal range (Figure 2B). The main contribution to the increased cell number in peripheral blood comes from increased B220+ B cell populations (1.4-fold) and Gr1+ cells (2.1-fold) (Figure 2C). Also notable was a slight, but significant, increase of B220+ cells in the BM and thymus, as well as a minor, but significant, decrease of both T and B lymphocytes in the spleen (Figure 2C). Taken together, these findings suggest an altered homeostasis of lymphocytes and leukocytes in the absence of Sfrp1.

To find out whether these changes result from alterations in early hematopoiesis, we analyzed the primitive cell populations in multiparameter flowcytometry (Figure 3A). Numbers of Lin−, Sca1+, and Kit+ (LSK) cells were unchanged in *Sfrp1*$^{-/-}$ mice (Figure 3B). However, the number of as the more primitive Flk2 and Cd34 double-negative population of LSKs (Cd34-Flk2-LSK) (Osawa et al., 1996, Christensen and Weissman, 2001; 1.5-fold, p = 0.013 [n = 7]; Figures 3A and 3B) as well as Cd150+ Cd48- Cd34-Flk2-LSK (Wilson et al., 2008; 1.8-fold, p = 0.044 [n = 3]; Figure S3), were significantly increased in *Sfrp1*$^{-/-}$ mice compared to controls. Further down the hematopoietic hierarchy, we found that multipotent progenitors (Lin−, Sca1−, and Kit+ MPP) were significantly reduced. This, in turn, suggested that the progenitors that make up the MPP population (common myeloid progenitors [CMPs], granulocytic progenitors [GMPs], and megakaryocytic/erythroid progenitors [MEPs]) should be reduced as well; however, we only observed a significant reduction (1.4-fold) in the MEP population (Figures 3C and 3D). Additionally, the number of common lymphoid progenitors (CLPs) in these mice also seemed reduced (1.4-fold), but due to the intraanimal variance, this difference never reached statistical significance (p = 0.12, n = 8 for both control and *Sfrp1*$^{-/-}$ groups) (Figures 3C and 3D). Functionally, the number of colony-forming cells, such as CFU-GEMM, was unchanged, but the number of CFU-GM, particularly the large CFU-GM, as

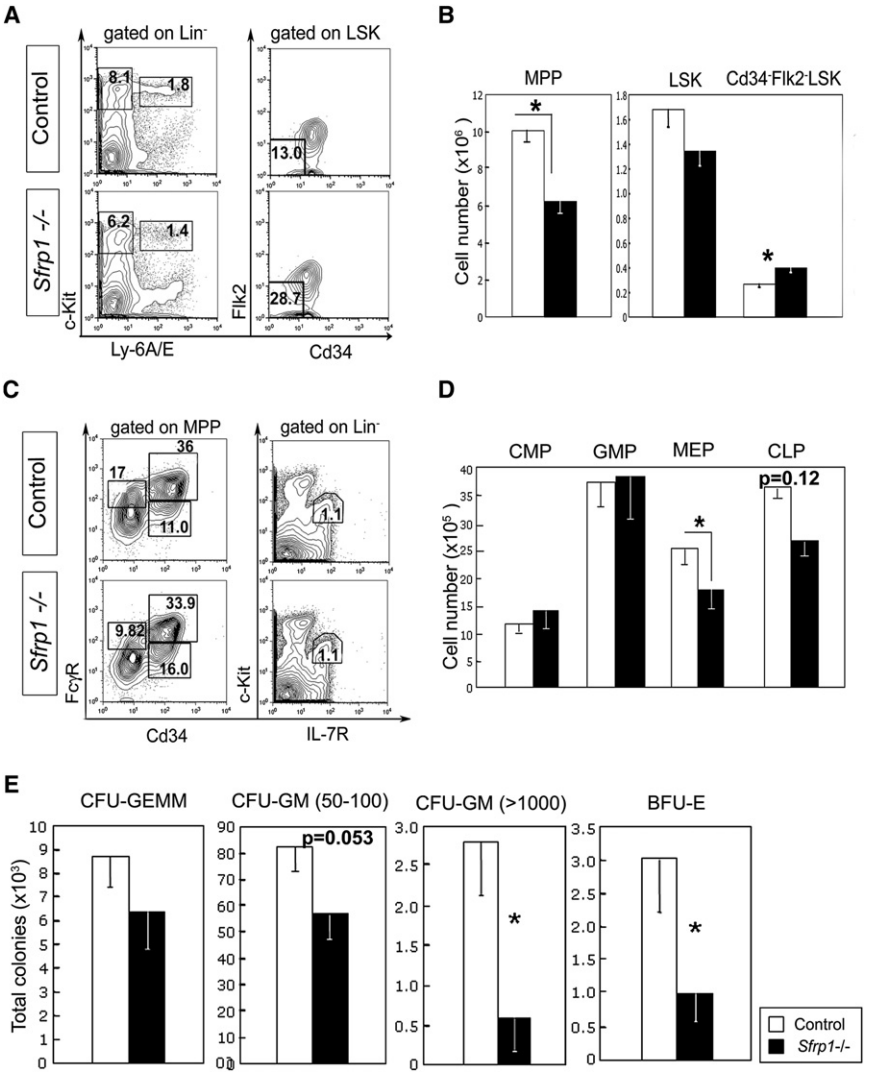


Figure 3. Alterations in Early Hematopoiesis in *Sfrp1*$^{-/-}$ Mice

(A) Representative FACS plots of BM cells isolated from control and *Sfrp1*$^{-/-}$ mice with gates for primitive HSCs.

(B) MPPs, LSKs, and Cd34-Flk2-LSKs are presented in absolute numbers (n = 7).

(C) Representative FACS plots of BM cells isolated from control and *Sfrp1*$^{-/-}$ mice with gates for committed progenitors.

(D) CMPs, GMPs, MEPs, and CLPs in absolute numbers. (n = 8 for control animals and n = 6 for *Sfrp1*$^{-/-}$).

(E) Total number of colonies formed from four long bones (2 femurs and 2 tibia) (n = 15 for control and n = 11 for *Sfrp1*$^{-/-}$ animals). Open bars represent control and closed bars *Sfrp1*$^{-/-}$. All values are shown as mean ± SEM. *p < 0.05.

well as the BFU-E, were significantly decreased (Figure 3E). Thus, loss of Sfrp1 results in a decreased progenitor cell activity.

Altered Cell-Cycle Regulation in the Absence of Sfrp1

Entry of HSC into the cell cycle is regulated by the microenvironment as well as modified by intrinsic factors. Our results suggest that absence of Sfrp1 affects the production of both progenitors and mature hematopoietic cells. In order to understand which mechanisms underlie these alterations, we studied the cell-cycle status of LSKs, MPPs, and mature cells in vivo. Animals were injected with BrdU and were sacrificed 3 hours later. Bone marrow and blood cells were analyzed for hematopoietic subpopulations (Figure 4A). We found that in the bone marrow, the G0/G1 population is increased in both LSK and MPP cells from *Sfrp1*$^{-/-}$ mice. Correspondingly, a decrease of the S phase cells was observed, indicating that in *Sfrp1*$^{-/-}$ mice, LSKs ($p = 0.063$) and MPPs ($p = 0.012$) cycle less (Figure 4B). However, this cycling behavior changes from early to late hematopoiesis, as both mature myeloid Gr1+ cells as well as B B220+ cells show an expanded S phase population in the marrow (Figure 4C). The increase in S phase was not noticed in Cd4+ or Cd8a+ T cells, indicating that *Sfrp1* does not affect cell cycle of T cells in the marrow (Figure 4C).

Lack of Sfrp1 Alters the Expression of Signaling Molecules in Early Hematopoietic Cells

In order to identify the molecular mechanisms underlying altered hematopoiesis in the absence of Sfrp1, we analyzed the expression level of several molecules that could possibly be involved. Intracellular flowcytometric analyses demonstrated that the number of cells expressing high levels of the central canonical Wnt signaling intermediate Ctnnb1 was significantly decreased in LSKs, MPPs, and CLPs of *Sfrp1*$^{-/-}$ mice compared to the control animals (Figure 5A and Figure S4). At the same time, P-Ctnnb1 was significantly decreased only in LSK cells of *Sfrp1*$^{-/-}$ compared to the wild-type controls (Figure 5A and Figure S4). In contrast, the number of cells expressing Jnk1 (Mapk8), one of the mediators of noncanonical Wnt signaling, was not altered in Sfrp1 knockout animals (Figure 5B and Figure S4). To address the more critical question of whether Ctnnb1 accumulates in the nucleus, we performed single-cell stains. These stains independently confirmed that Ctnnb1 is present at a lower level in *Sfrp1*$^{-/-}$ LSKs, MPPs, and CLPs (Figures 5C and 5D). In LSK and CLP control cells, Ctnnb1 was present in clearly visible membrane-bound forms (Figure 5C), whereas in cells derived from *Sfrp1*$^{-/-}$ bone marrow, membrane-bound Ctnnb1 appeared to be absent, resulting in a relatively high nuclear to cytoplasmic Ctnnb1 ratio, particularly in *Sfrp1*$^{-/-}$ LSK cells (Figure 5E). Thus, the difference in nuclear, cytoplasmic Ctnnb1 distribution was clearest in LSK cells (Figure 5E).

To find out how the level and altered distribution of Ctnnb1 affects gene expression, we studied expression levels of "classical" downstream transcriptional targets of the catenin/Tcf/Lef

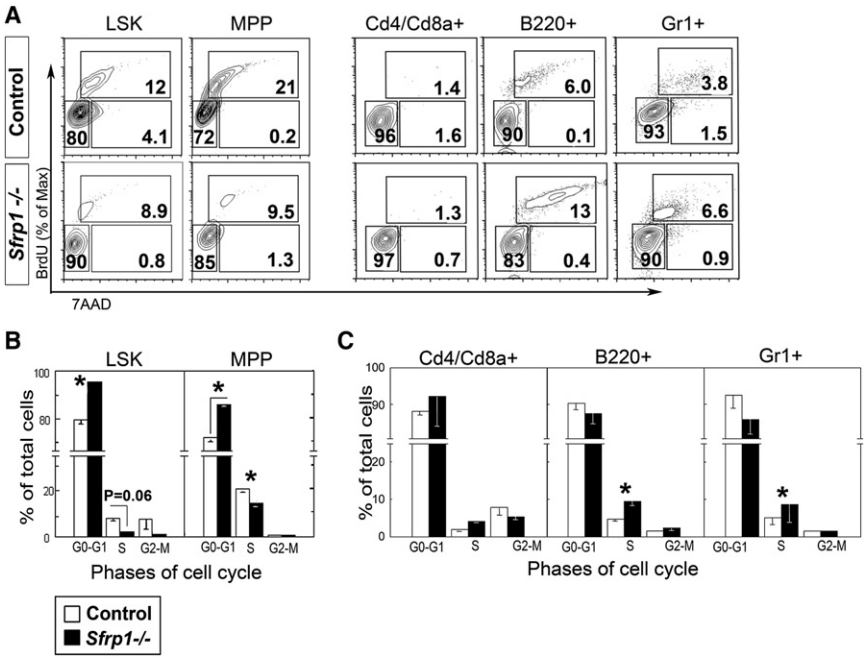


Figure 4. Detection of Alterations in Cell-Cycle Behavior in Hematopoietic Cells from *Sfrp1*$^{-/-}$ Mice Using In Vivo BrdU Treatment

(A) Representative FACS plots showing BrdU incorporation in primitive and mature hematopoietic cells isolated from control and *Sfrp1*$^{-/-}$ BM.

(B) Percentage of BrdU-positive cells in G0-G1-, S-, and G2-M phases of cell cycle in LSK and MPP of control and *Sfrp1*$^{-/-}$ mice.

(C) Percentage of BrdU-positive cells in G0-G1-, S-, and G2-M phases of cell cycle in T−, B−, and myeloid cells of control and *Sfrp1*$^{-/-}$ mice (n = 6). All values are shown as mean ± SEM.

pathway as well as other molecules mediating Wnt signaling: *Lef1*, *Fzd4*, and *Pparg*. The decreased catenin levels resulted in a significant reduced expression of both catenin targets: cyclin D1 (*Ccnd1*) and the Wnt inhibitor dickkopf 1 (*Dkk1*) in Cd34-LSK, but not MPP cells (Figure 5F and Figure S5). The expression of transcription factor *Lef1*, which activates target genes in complex with the nuclear Ctnnb1, was unchanged in Cd34-LSKs and MPPs of *Sfrp1*$^{-/-}$ mice (Figure 5F and Figure S5). In contrast, the expression of *Pparg*, which is a repressor of Wnt signaling, was significantly upregulated in MPP (Figure 5F; Figure S5). On the other hand, *Fzd4* expression, which binds Sfrp1 (Dufourcq et al., 2008) and was previously shown to be expressed by HSC (Yokota et al., 2008), was unchanged in Cd34-LSKs, but was significantly decreased in MPPs of *Sfrp1*$^{-/-}$ mice compared to the controls (Figure 5F; Figure S5).

Wnt signaling was shown to affect Notch pathway in HSCs (Duncan et al., 2005), and we found a significant upregulation of the Notch target gene *Hes1* in Cd34-LSKs and MPPs of *Sfrp1*$^{-/-}$ mice compared to the controls (Figure 5F and Figure S5). This finding is in line with previously published results from transgenic mice overexpressing Dkk1 (Fleming et al., 2008), another repressor molecule in Wnt signaling. Also, the expression of *Runx1*, a downstream mediator of Notch signaling (Burns et al., 2005), was significantly upregulated in MPPs and not altered in LSKs of *Sfrp1*$^{-/-}$ mice (Figure 5F and Figure S5). The NF-κB modulator *Ikbkg* was slightly lower expressed in Cd34-LSK cells from *Sfrp1*$^{-/-}$ mice, but unchanged in their MPPs (Figure 5F and Figure S5), suggesting possible crosstalk of NF-κB and Sfrp1 signals in the earliest hematopoietic cells. The expression levels of the transcription factor *Lmo2* was unchanged in both populations of Sfrp1 knockout mice (Figure 5F and Figure S5). Taken together, these results show that the absence of Sfrp1 alters the expression of both Wnt and Notch pathway mediators and targets in both MPP and Cd34-LSK.

The Sfrp1-Deficient Microenvironment Shows a Defect in HSC Maintenance

In order to separate intrinsic from extrinsic effects caused by Sfrp1 loss, we first analyzed engraftment of *Sfrp1*$^{-/-}$ HSCs transplanted into 129Ly5.1 recipients. In addition, we performed this experiment in a limiting dilution fashion, which would also enable independent functional confirmation of phenotypical results shown in Figure 3B. These experiments showed that the number of functional HSCs is unchanged in *Sfrp1*$^{-/-}$ mice (Figure 6A). In addition, the pattern of lymphomyeloid engraftment was unchanged with regard to recipients receiving control 129B6 cells (Figure 6B and Figure S6), indicating there are no intrinsic defects in the ability of *Sfrp1*$^{-/-}$ to engraft myeloid and lymphoid lineages.

In order to assess the extent of the extrinsic effect, we analyzed engraftment of wild-type Ly5.1 HSC in *Sfrp1*$^{-/-}$ and control 129Ly5.1 mice (primary transplants, 1°) (Figure 7A). Sixteen weeks after transplantation in 1° recipients, we observed a significant increase of engrafted wild-type cells in BM and PB of *Sfrp1*$^{-/-}$ mice (Figure 7B). The number of mature myeloid cells was increased in spleen and PB of *Sfrp1*$^{-/-}$ mice compared to control (Figure 7C). The maintenance of donor LSK and MPP cells in 1° *Sfrp1*$^{-/-}$ mice was slightly higher, but not significantly changed (Figure 7D).

As it was recently shown that mild effects in primary recipients may, in fact, reflect major and irreversible changes in HSC behavior and number only observed at later time points (Fleming et al., 2008; Miyamoto et al., 2007), we also performed secondary transplantation (2°). In these experiments, we used wild-type recipients, so that any phenotypical changes would depend only on the microenvironment of the 1° recipient. In 2° recipients reconstituted with donor cells from *Sfrp1*$^{-/-}$ 1° mice, we observed decreased total as well as HSC engraftment (Figure 7B and Table S2). Interestingly, we observed a significant increase in the number of B220-positive B cells in all tissues examined: BM, spleen, and blood (Figure 7E). In addition, contrary to the 1° *Sfrp1*-deficient recipients, the number of donor Gr1+ cells (Figure 7E), as well as LSK and MPP cells, were severely decreased in 2° transplants (Figure 7F), indicating that in the primary *Sfrp1*$^{-/-}$ recipients, the ability of the microenvironment to maintain LT-HSCs was reduced.

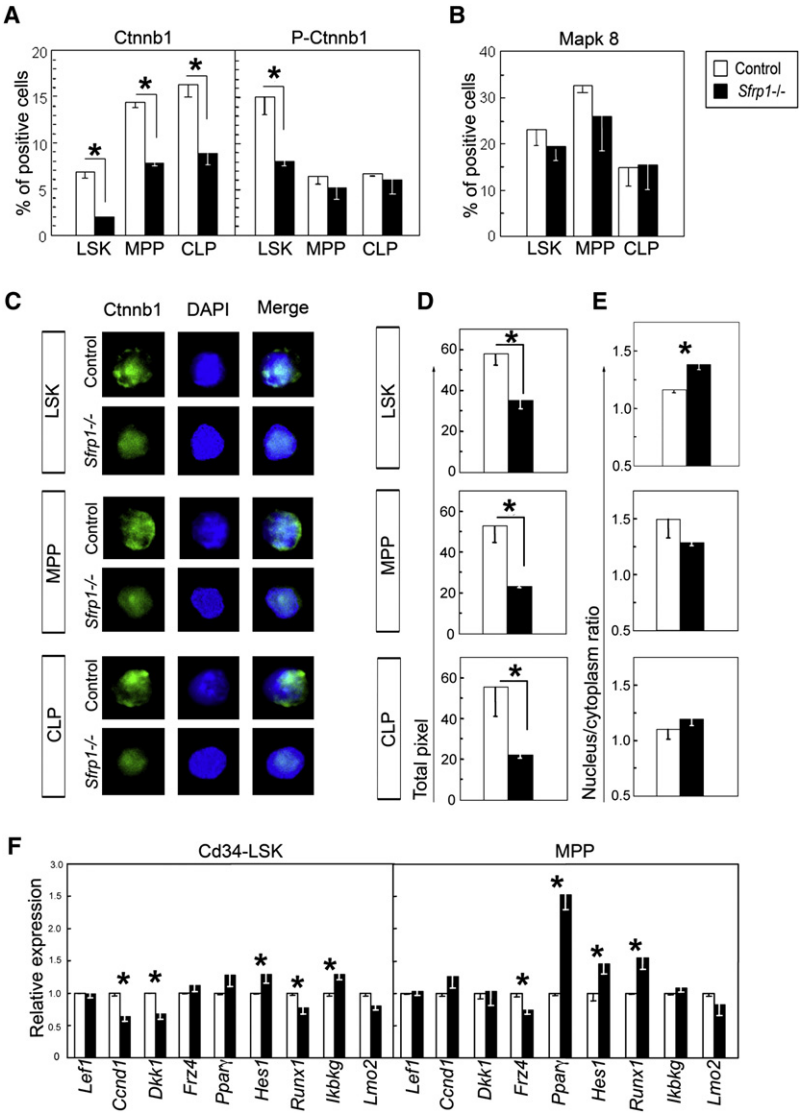


Figure 5. Alterations in β-Catenin-Dependent Signaling in *Sfrp1*$^{-/-}$ Mice

(A) Intracellular stain of Ctnnb1 and P-Ctnnb1 in LSKs, MPPs, and CLPs of control and *Sfrp1*$^{-/-}$ mice (n = 3).

(B) Intracellular stain of Mapk8 in LSKs, MPPs, and CLPs.

(C) Single-cell stains showing the expression of Ctnnb1 in sorted LSK, MPP, and CLP cells (n = 3).

(D) Quantification of Ctnnb1 stain (total pixel number) using ImageJ in LSKs (n = 10), MPPs (n = 8), and CLPs (n = 5).

(E) Ratio of nuclear and cytoplasmic Ctnnb1 expression in sorted LSKs, MPPs, and CLPs.

(F) Normalized expression of *Lef1*, *Ccnd1*, *Dkk1*, *Fzd4*, *Pparg*, *Hes1*, *Runx1*, *Ikbkg*, and *Lmo2* in Cd34-LSKs and MPPs of control and *Sfrp1*$^{-/-}$ mice. Results were normalized relative to the combined expression of the three housekeeping genes *Hprt1*, *Rps21*, and *Ythdf1*. All values are shown as mean ± SEM in 3 to 5 independent experiments. *$p < 0.05$.

DISCUSSION

In previous studies, we generated embryo-derived stromal cell lines capable of supporting HSCs in culture (Oostendorp et al., 2002). By comparing the expression profiles of these cell lines with nonsupportive ones, we previously identified upregulation of Sfrp2 (Oostendorp et al., 2005). In the current study, we identified an additional member of Sfrp family, Sfrp1, to be overrepresented in HSC-supporting stromal cells. Both Sfrp1 and Sfrp2 are known modulators of Wnt signaling. The members of the Wnt-signaling pathway have emerged as regulators of HSC self-renewal and proliferation (Murdoch et al., 2003; Reya et al., 2003; Nemeth et al., 2007; Liang et al., 2003; Luis et al., 2009). In particular, the Wnt factors play an important role in the complex interplay of intrinsic signals from HSCs and extrinsic stimuli from the surrounding stromal microenvironment, also called the stem cell niche (reviewed in Yin and Li, 2006).

Here, we analyzed the effect of Sfrp1 on hematopoiesis and found that the downregulation of *Sfrp1* in the embryo-derived stromal cell lines UG26-1B6 and EL08-1D2 led to an increased production of hematopoietic progenitors in culture. Since previous studies showed that Sfrp1 is expressed in bone-lining osteoblast-like cells (Yokota et al., 2008) and the endosteal surfaces of trabecular bones function as a niche in hematopoiesis (reviewed in Yin and Li, 2006), we hypothesized that Sfrp1 might play an important role in the extrinsic regulation of hematopoiesis. Hence, we decided to analyze early hematopoietic cells and the intrinsic and extrinsic contribution of Sfrp1 deficiency to hematopoietic regulation.

In the present investigation, we found that Sfrp1 loss leads to increased peripheral blood cell numbers, particularly B220+ and granulopoietic (Gr1+) cells. This observation suggested a dysregulation of hematopoiesis, which is in line with the proposed role of Sfrp1 as an inhibitor of B lymphopoiesis (Yokota et al., 2008), but which was previously not detected in mice overexpressing another Wnt-antagonist, Dkk1, in osteoblasts (Fleming et al., 2008), or in mice with conditional deletion of Ctnnb1 (Cobas et al., 2004). However, severe decreases in blood cell numbers were noted in studies in which Ctnnb1 was stabilized (Kirstetter et al., 2006, Scheller et al., 2006). In the latter, it was noted that in the LSK cells, the number of G0/G1 cells was decreased, suggesting the earliest hematopoietic cells were recruited to enter the cell cycle, but that the number of HSCs required to maintain hematopoiesis was, in fact, depleted, perhaps because the cells did not return to G0 (Scheller et al., 2006). We clearly show that the general level of Ctnnb1 is decreased in *Sfrp1*$^{-/-}$ LSKs, MPPs, and CLPs and that P-Ctnnb1 is decreased in LSK cells. Others have shown that Sfrp1 treatment increases β-catenin levels in Lin−Kit+ cells (Yokota et al., 2008). Although these observations suggest that Sfrp1 may directly inhibit degradation of Ctnnb1, which is normally associated with stimulation of canonical Wnt signaling, these findings contrast to the general designation of Sfrp1 as a canonical Wnt inhibitor.

A

Limiting dilution	Frequency of positive animals	
	Control	Sfrp1-/-
150000	3/4	4/6
50000	1/3	2/5
20000	1/5	1/6
Frequency	1 in 107,000	1 in 121,000
Confidence interval	67,000 ➔172,000	81,000 ➔180,000

B

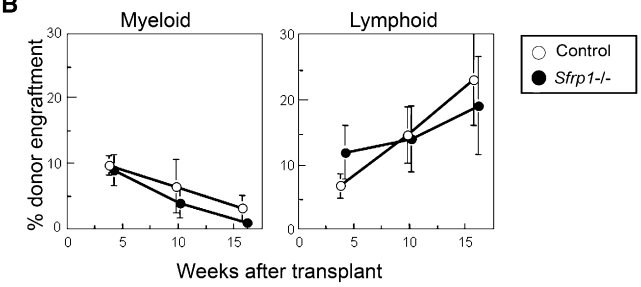


Figure 6. No Intrinsic Defect in the Engraftment of *Sfrp1*$^{-/-}$ HSC

(A) Limiting dilution analysis of engrafted cells in the PB of mice transplanted with 150,000, 50,000, or 20,000 control or *Sfrp1*$^{-/-}$ donor cells. Shown are the number of positive animals per total transplanted as well as the frequency and 95% confidence interval of this frequency determined using the L-Calc software.

(B) The pattern of myeloid and lymphoid engraftment in mice transplanted with 50,000 donor cells. Values are shown as mean ± SEM.

Most likely, the balance between canonical and noncanonical signaling dictates the intracellular level of Ctnnb1. An alternative explanation for decreased Ctnnb1 levels with Sfrp1 might be our recent observation that Sfrp1 binds and inhibits Wnt5a (Matsuyama et al., 2009), an interaction which potentially increases the level of Ctnnb1 (Topol et al., 2003). Loss of Sfrp1 could, therefore, increase Wnt5a-dependent signaling, thereby indirectly affecting Ctnnb1 stability. A finding in favor of this hypothesis is our observation that a central target of the calcium-dependent noncanonical Wnt pathway, *Pparg*, is increasingly expressed in the absence of Sfrp1 in MPPs. Pparg was shown to suppress Ctnnb1 levels in colon cancer (Girnun et al., 2002) and during adipogenesis (Moldes et al., 2003), most probably through a proteasome-dependent mechanism (Sharma et al., 2004). More importantly, Pparg is transcriptionally repressed by noncanonical Wnt signaling through the calcium-dependent Camk2-Tak1-Tab2-Nlk in bone marrow mesenchymal progenitors (Takada et al., 2007). These findings suggest that, at least in MPP, in the absence of Sfrp1, the noncanonical calcium-dependent Wnt pathway is upregulated. Considering the complex interplay of Wnt agonists and antagonists within the microenviroenment, the identification of the precise mechanism by which Sfrp1 may be stimulating Ctnnb1 signaling will be difficult and lies beyond the scope of the present paper.

We found that Ccnd1, a "classical" transcriptional target of canonical Wnt signaling, was decreased in Cd34-LSK cells. Hence, our finding that Sfrp1 deficiency affected cell cycling was not surprising. However, it was unanticipated that the effects of Sfrp1 on early hematopoietic cells (LSKs and MPPs) appeared to favor retention in G0/G1, whereas S phase entry was increased in more mature B220+ and Gr1+ cells. This suggests that early and mature cells are, in fact, regulated through different Sfrp1-modulated signaling pathways. Whereas the decreased cycling activity of LSKs and MPPs can be attributed by decreased canonical signaling as well as with increased Pparg (Altiok et al., 1997), the increase on cycling in mature cells is not explained by this mechanism. Previously, the haploinsufficiency of the noncanonical intermediate Wnt5a in adult mice was shown to lead to increased peripheral blood cell numbers, especially B220+ and Gr1+ cells (Liang et al., 2003). This observation shows similarities with our observations in *Sfrp1*$^{-/-}$ mice. Hence, the alterations of cell cycling in more mature populations could be explained by deficient noncanonical signaling with little contribution of catenin-dependent canonical signaling.

Our results also imply a possible involvement of Sfrp1-dependent signaling events in the regulation of non-Wnt pathways. It was reported that Wnt and Notch signaling may collaborate to regulate self-renewal of HSCs (Duncan et al., 2005). Indeed, our results show that Sfrp1 deficiency causes an upregulation of the Notch target *Hes1* in both Cd34-LSKs and MPPs. Since *Hes1* overexpression induces quiescence (Yu et al., 2006), this observation suggests collaborating Notch signals could be involved in the observed cell-cycle changes seen in early hematopoietic cells. Unexpectedly, we also saw changes in the expression of *Ikbkg*, an NF-κB modulator involved in B cell development, and *Runx1*, which is important in hematopoietic specification and lineage maturation, but does not appear to affect HSC behavior (Ichikawa et al., 2004). The latter was surprising: though *Runx1* and *Lef1* are both regulated by Groucho family members (Levanon et al., 1998), it was previously unknown that *Runx1* transcription was regulated through the Wnt pathway. We clearly observe a slightly decreased expression of *Runx1* in Cd34-LSKs and an increase of *Runx1* in MPPs, indicating a tightly developmentally regulated program for *Runx1* expression from Cd34-LSK to MPP cells, which, in part, depends on Sfrp1.

Our transplantation experiments revealed that there are no intrinsic defects in HSCs caused by Sfrp1 deficiency. The kinetics of lymphomyeloid lineage engraftment were, in fact, the same in control and *Sfrp1*$^{-/-}$ HSC donors. However, we found a severe loss of maintenance of wild-type HSCs in *Sfrp1*$^{-/-}$ recipients, suggesting extrinsic Sfrp1-dependence of lymphomyeloid engraftment. This finding is puzzling with regard to its similarity with the results of transgenic mice overexpressing Dkk1 in osteoblasts (Fleming et al., 2008). In both models, initial engraftment in primary recipients is increased, with a significant drop in engraftment in 2° recipients, suggesting that engrafted HSCs are in cycle in both models. Our coculture experiments suggest that progenitors are indeed recruited into cell cycle more on Sfrp1 knockdown cell lines or Sfrp1-deficient primary marrow stromal. Most likely, differences between steady-state hematopoiesis and hematopoietic repopulation are, in fact, not comparable. During the regenerative response after myeloablation, the microenvironment undergoes significant remodeling (Slayton et al., 2007). Also, there is a rapid upregulation of Wnt10b in both stromal and hematopoietic cells, with a concomitant stabilization of Ctnnb1 and upregulation of Myc and Axin2 (Congdon et al., 2008). Since downregulation of canonical

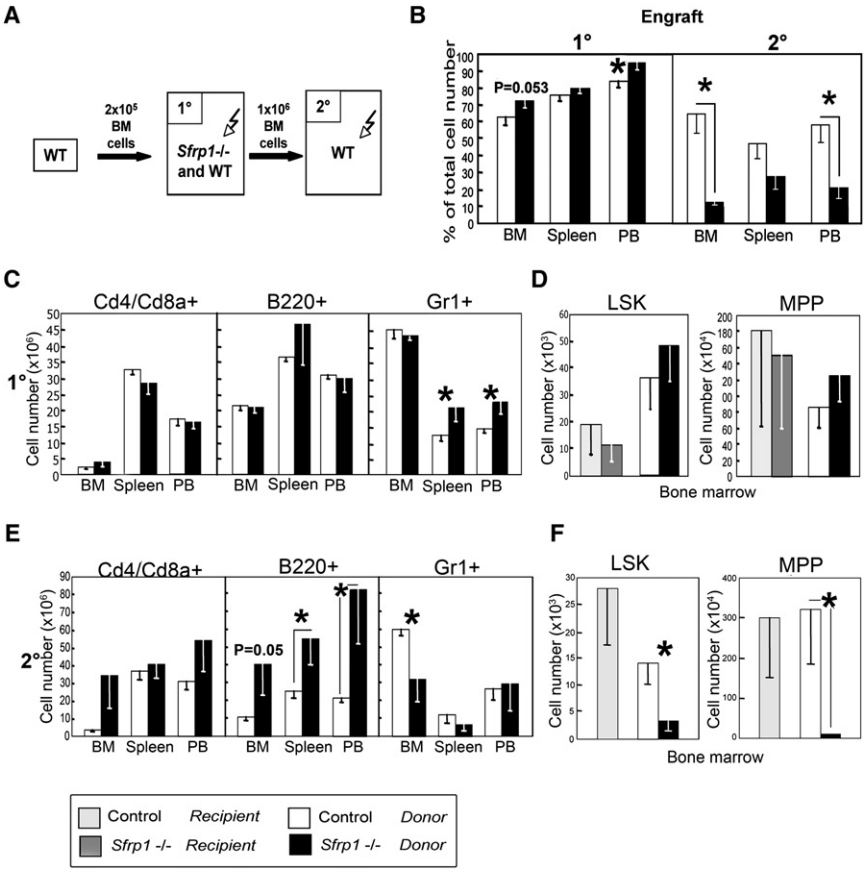


Figure 7. Extrinsic Regulation of Wild-Type HSC Engraftment in the *Sfrp1*$^{-/-}$ Microenvironment

(A) Serial transplantation flow chart. The wild-type BM cells were injected into *Sfrp1*$^{-/-}$ or wild-type (Sfrp1$^{+/+}$) recipient mice (1° transplants). Sixteen weeks post-transplantation, BM was isolated and 1 × 10^6 BM cells were transplanted into wild-type recipients (2° transplants). Transplantations were carried out as three independent experiments each.

(B) The engraftment levels in the BM, spleen, and PB of 1° (control [n = 9] and *Sfrp1*$^{-/-}$ mice [n = 5]) and 2° (cells from 1° BM into n = 9 [1° recipient, control] or n = 13 [1° recipient: Sfrp1$^{-/-}$]).

(C) Absolute numbers of engrafted T (Cd4$^+$ and/or Cd8a$^+$ cells), B (B220$^+$ cells), and myeloid (Cd11b$^+$ and/or Gr1$^+$) cells in the BM (cells per four long bones), spleen, and peripheral blood (PB, cells per μl) of primary recipients.

(D) Absolute numbers of engrafted LSKs and MPPs in the BM (four long bones) of primary recipients.

(E) Absolute numbers of engrafted T, B, and myeloid cells in the BM (per four long bones), spleen, and PB (per μl) of secondary recipients.

(F) Absolute numbers of engrafted LSKs and MPPs in the BM of secondary recipients (per four long bones). All values are shown as mean ± SEM. *p < 0.05.

signaling is what the Dkk1-transgenic and Sfrp1-deficient models have in common, it appears that canonical Wnt signaling is the main driving force in hematopoietic regeneration after myeloablative insult.

In conclusion, our study shows the value of studying stromal cell lines to uncover factors involved in extrinsic regulation of HSCs. The present study demonstrates that microenvironmental Sfrp1 affects both catenin-dependent canonical and Pparg-dependent noncanonical Wnt signaling to regulate cell cycling and gene expression in HSCs and lineage-committed MPPs. Our observations show that Sfrp1 does not have major intrinsic roles in lymphomyeloid engraftment and that loss of Sfrp1 severely impairs HSC maintenance in an extrinsically regulated manner.

EXPERIMENTAL PROCEDURES

Mice

Sfrp1$^{-/-}$ mice were bred on a 129 × C57BL/6.J (129B6) background (Satoh et al., 2006). Age- and sex-matched 129B6, C57BL/6.Pep3b.Ptprc (Ly5.1), and (129 × Ly5.1) F1 (129Ly5.1) mice were used as controls in all experiments. Mice were kept in microisolators under SPF conditions according to FELASA recommendations.

Cell Lines

The embryo-derived stromal cell lines EL08-1D2, UG26-1B6, UG15-1B7, AM20-1B4, EL28-1B3, and AM30-3F4 were cultured as described previously (Oostendorp et al., 2002). Stable knockdown cells for Sfrp1 were made using lentiviral shRNAmir (Open Biosystems, Huntsville, AL, USA), followed by puromycin-selection. Puromycin was removed from the medium 3 days prior to the experiments.

Tissue Samples

Blood samples were taken retro-orbitally with 0.8 mm capillaries (Neolab, Heidelberg, Germany) and collected in 1.2 ml heparinised tubes (Sarstedt AG, Nümbrecht, Germany). Blood cells were counted on a Scil Vet ABC (Scil Animal Care, Viernheim, Germany). Bone marrow cells were flushed from femurs and tibias with HF2+ buffer (Hank's balanced salt solution, supplemented with 2% FCS, 10 mM HEPES buffer, and antibiotics). Spleen and thymus were passed through 70 μm nylon Cell Strainer (BD Biosciences, Erembodegem, Belgium). WBCs were counted after erylysis with ammonium chloride solution (Stemcell Technologies, Vancouver, Canada). Viable cell were counted using Trypan Blue (Invitrogen) in a Neubauer hemocytometer.

FACS Analysis

Cell suspensions were stained with antibodies in HF2+ buffer for 15 min on ice in the dark. Hematopoietic populations were separated with the following antibodies: biotinylated Gr-1; phycoerythrin (PE)-labeled Cd4, Cd8, and Cd117 (Kit); PE-Cy5-labeled B220; fluorescein isothiocyanate (FITC)-labeled Cd34 and Cd48; Pacific-Blue-labeled Cd16/32 (FcγR), Alyophycocyanin (APC)-labeled Cd117 and IL7R; and PE-Cy7-labeled anti Ly-6A/E (Sca1) were all obtained from eBiosciences (San Diego, CA, USA) and PE-labeled Cd150 from BioLegend (San Diego, CA, USA). Lineage stains were performed with biotinylated lineage cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with biotin-labeled CD3 and IL-7R antibodies (eBiosciences). Blood samples were analyzed with following antibodies: biotinylated Gr-1 (eBioscience) labeled with PECy5 streptavidin (Caltag), Cd4 – PECy5 (BD PharMingen), Cd8a – PECy5 (eBioscience), or B220 – PECy5 (BD PharMingen). In

transplantation experiments, Cd45.2 – FITC (BD) and Cd45.1 – PE (BD PharMingen) antibodies were used additionally.

FACS analyses were performed on a Coulter EPICS XL (Beckman Coulter GMBH, Krefeld, Germany) or CyAn ADP Lx P8 (Beckman Coulter) flow cytometers. FACS data were analyzed using FlowJo 8.8.3 software (Tree Star, Inc. Ashland, OR, USA).

Cell populations were sorted on MoFlo (Cytomation-Beckman Coulter) supplied with Summit 4.3 software (Beckman Coulter).

Hematopoietic Colony Assays

For colony forming assay, BM cells (2.5×10^4) or cocultures initiated with 1×10^4 of Lin− cells were cultivated in methylcellulose (MethoCult GF M3434, Stemcell Technologies, Vancouver, Canada) on 3.5 mm dishes for ten days at 37°C, 5% CO_2. Colonies formed were counted under a microscope.

The stromal cells used for cocultures were irradiated with 30 Gy (UG26-1B6) and 15 Gy (primary stroma) by a Mevatron KD2 (Siemens, Munich, Germany).

In the rescue experiments, half of the medium was replaced on each third day with conditioned medium (CM) and harvested, and 0.22 μm was filtered from confluent-grown Sfrp1$^{+/+}$ primary BM stroma.

Human recombinant SFRP1 (R&D Systems, Minneapolis, USA) was added at a concentration of 1 μg/ml when used to evaluate its effect.

Cell-Cycle Analysis

For analysis of cell cycle, animals were intraperitoneally injected with 1 mg of BrdU. After 3 hr, BM cells were flushed and stained with surface markers prior to BrdU detection with a BrdU labeling kit as described by the manufacturer (BD Biosciences).

Transplantation Assay

The Cd45 congenic system (Cd45.1 and Cd45.2) was used to distinguish donor from recipient cells. Ten- to twelve-week-old lethally irradiated recipient mice received donor cells via the tail vein on the same day. In primary transplantations (1°), 2×10^5 BM cells were used and in secondary transplants (2°), 1×10^6.

For the limiting dilution assay, 1.5×10^5, 5×10^4 or 2×10^4 BM cells from WT or *Sfrp1*$^{-/-}$ mice were injected with 2×10^5 (129Ly5.1) competitor cells into WT recipients. Mice with engraftment over 1% of myeloid and 1% lymphoid lineages are defined as positive mice.

Transplanted mice received 1 mg/ml neomycin sulfate (Sigma) and 500 units/ml Polymyxin B (Sigma) in the drinking water. At week 16 weeks post-transplantation, mice were sacrificed, and BM, spleen, and blood cells were analyzed.

Intracellular Stains

For intracellular staining, three million BM cells were stained for surface markers prior to fixation and permeabilization with BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). In brief, cells were fixed and permeabilized in Cytofix/Cytoperm buffer for 20–25 min at room temperature in the dark. After fixation and permeabilization, cells were washed with Perm/Wash buffer and incubated with primary antibody diluted in the staining buffer overnight at 4°C. Antibodies used are as follows: rabbit anti-Ctnnb1, rabbit anti-Ser33/Thr41 P-Ctnnb1 (both from Cell Signaling Technology, Boston, USA), rabbit anti-Jnk/Mapk8 (Santa Cruz Biotechnology, Heidelberg, Germany), and control preimmune rabbit immunoglobulin (Jackson ImmunoResearch, Newmarket, Suffolk, UK), followed by FITC-labeled anti-rabbit antibodies (Jackson ImmunoResearch). Stained cells were analyzed on CyAn ADP Lx P8 (Beckman Coulter) flow cytometer.

Immunocytofluorescence Staining

For the single-cell staining assay, 200 sorted LSK, MPP, and CLP were spotted on poly-L-lysine-coated glass slides. After a short incubation on ice, cells were fixed with 4% PFA. Fixed cells were incubated in 10% FCS, 0.1% Triton-X in PBS at room temperature for 1 hr and stained overnight with anti-Ctnnb1, L54E2 Alexa Fluor 488-conjugated Mouse antibody (Cell Signaling). The cell nuclei were counterstained with DAPI (4,6-diamino-2-phenylindole, dihydrochloride) (Invitrogen) and mounted in Prolong Gold Antifade Reagent. Staining was assessed using a 100× magnification on a Leica DM RBE fluorescent microscope (Leica, Wetzlar, Germany). The fluorescence intensity of each individual cell was quantified with ImageJ (NIH, Bethesda, USA) software. The nuclear expression was quantified in the area corresponding to the DAPI-positive area. The cytoplasmic expression was quantified in the cell compartment after subtraction of the "nuclear" area.

Gene Expression Analysis

mRNA was isolated from sorted cells by using the Dynabeads mRNA DIRECT Micro Kit (Invitrogen) as described by the manufacturer. cDNA was amplified using Omniscript RT kit (QIAGEN GmbH, Hilden, Germany) as described by the manufacturer. The following Quantitative PCRs were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions, and analyzed on a Applied Biosystems 7900HT (Applied Biosystems). Gene expression is presented as a relative value ($2^{\Delta Ct} \times 100\%$) compared to the expression levels of housekeeping genes Rpl13a, Rps21, Hprt1, and Ytdhf1. Primer sequences are shown in Table S1.

Statistics

Unpaired Student's t test was used for the statistical analyses using the InStat statistical package (GraphPad Software, Inc., La Jolla, CA, USA). The limiting dilution experiment was evaluated using the L-Calc software package (Stemcell Technologies).

SUPPLEMENTAL DATA

Supplemental Data include two tables and six figures and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00229-X.

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