Smad4 is critical for self-renewal of hematopoietic stem cells

Göran Karlsson,[1] Ulrika Blank,[1] Jennifer L. Moody,[1] Mats Ehinger,[2] Sofie Singbrant,[1] Chu-Xia Deng,[3] and Stefan Karlsson[1]

[1]Department of Molecular Medicine and Gene Therapy, Institute of Laboratory Medicine, Lund University Hospital and Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, 22184 Lund, Sweden
[2]Department of Pathology, Helsingborgs Lasarett, 251 87 Helsingborg, Sweden
[3]National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, 20892

Members of the transforming growth factor β (TGF-β) superfamily of growth factors have been shown to regulate the in vitro proliferation and maintenance of hematopoietic stem cells (HSCs). Working at a common level of convergence for all TGF-β superfamily signals, Smad4 is key in orchestrating these effects. The role of Smad4 in HSC function has remained elusive because of the early embryonic lethality of the conventional knockout. We clarify its role by using an inducible model of Smad4 deletion coupled with transplantation experiments. Remarkably, systemic induction of Smad4 deletion through activation of MxCre was incompatible with survival 4 wk after induction because of anemia and histopathological changes in the colonic mucosa. Isolation of Smad4 deletion to the hematopoietic system via several transplantation approaches demonstrated a role for Smad4 in the maintenance of HSC self-renewal and reconstituting capacity, leaving homing potential, viability, and differentiation intact. Furthermore, the observed down-regulation of *notch1* and *c-myc* in *Smad4*$^{-/-}$ primitive cells places Smad4 within a network of genes involved in the regulation HSC renewal.

CORRESPONDENCE
Stefan Karlsson:
Stefan.Karlsson@med.lu.se

On the cell surface, TGF-β superfamily growth factors, including TGF-βs, activins, and bone morphogenetic proteins (BMPs), bind to type I and type II serine/threonine kinase receptors. Upon association with the ligand, type II receptors form a complex with their respective type I receptors. Subsequently, TGF-β and activin signals progress through phosphorylation of receptor-activated Smads (R-Smads) 2 and 3, whereas R-Smads 1, 5, and 8 mediate BMP signaling. R-Smads of both pathways eventually heteroligomerize with the common mediator Smad4, and the resulting complex translocates to the nucleus and recruits transcriptional cofactors to control gene expression. Additionally, in a negative feedback mechanism, Smad6 and 7 inhibit TGF-β superfamily signaling by competing with R-Smads for Smad4 interaction and receptor binding and by targeting receptors for ubiquitination and degradation. In addition to the canonical Smad circuitry, TGF-β superfamily members have been reported to signal through other pathways, predominantly including members of the mitogen-activated protein kinase (MAPK) family (1).

In the hematopoietic system, numerous TGF-β family members have been established as modulators of hematopoietic stem cell (HSC) fate decisions (2–4). BMP-4 is critical for mesoderm formation and, thus, hematopoietic development in various model organisms, as well as from human embryonic stem cells. Furthermore, BMP stimulation affects proliferation and differentiation of human hematopoietic progenitors and can prolong the maintenance of functional human cord blood HSCs in vitro through as of yet unidentified mechanisms. Conversely, the TGF-βs are generally context-dependent, potent inhibitors of primitive hematopoietic cell proliferation in vitro. The mechanisms behind TGF-β–induced growth arrest in hematopoietic progenitors are intricate, involving both down-regulation of cytokine receptors and modulation of genes involved in the cell cycle. Previously, it has been reported that TGF-β1–null mice and inducible TGF-β receptor (TβR) knockout models develop a transplantable lethal inflammatory disorder affecting multiple organs (5–7).

The online version of this article contains supplemental material.

However, transplantation experiments using BM from *TβRI*$^{-/-}$ mice failed to reveal any effect on HSC proliferation and differentiation in vivo, even though TβRI-deficient primitive hematopoietic cells had increased proliferative capacity in vitro under low stimulatory conditions (8). Redundant mechanisms within the Smad signaling network or cross talk with other pathways relevant in the more complex and enduring in vivo setting may account for this discrepancy between in vitro and in vivo findings. Indeed, members of the Wnt-, Notch-, and MAPK-signaling pathways have been suggested to interact with the Smads in other contexts (9–11). Recent work identifies the nuclear protein transcription intermediary factor 1 (TIF1) γ as a competitor to Smad4 for R-Smad binding in human CD34^{+} cells, demonstrating additional complexities in the interaction between the canonical Smad pathway and other regulatory circuits in hematopoietic cells (12).

In this paper, we have studied the complete role of Smad4-dependent signaling in HSCs and hematopoiesis by means of inducible *MxCre/Smad4*$^{-/-}$ mice. Previously, several groups demonstrated that conventional *Smad4*$^{-/-}$ embryos die at embryonic day 7.5 because of an impaired proliferation of the ectoderm, resulting in a lack of mesoderm formation (13, 14). Adult *Smad4* heterozygote mice develop polyps and tumors of the gastrointestinal tract (15, 16), findings that have also been observed in human patients with juvenile polyposis syndrome caused by *SMAD4* mutations (17). Correspondingly, we observed that MxCre-induced deletion of Smad4 in hematopoietic and extrahematopoietic tissues results in histopathological changes in the colonic mucosa and intestinal bleeding, reinforcing the importance of Smad signaling in colon homeostasis. By using cells from these mice in BM transplantation settings, we have isolated

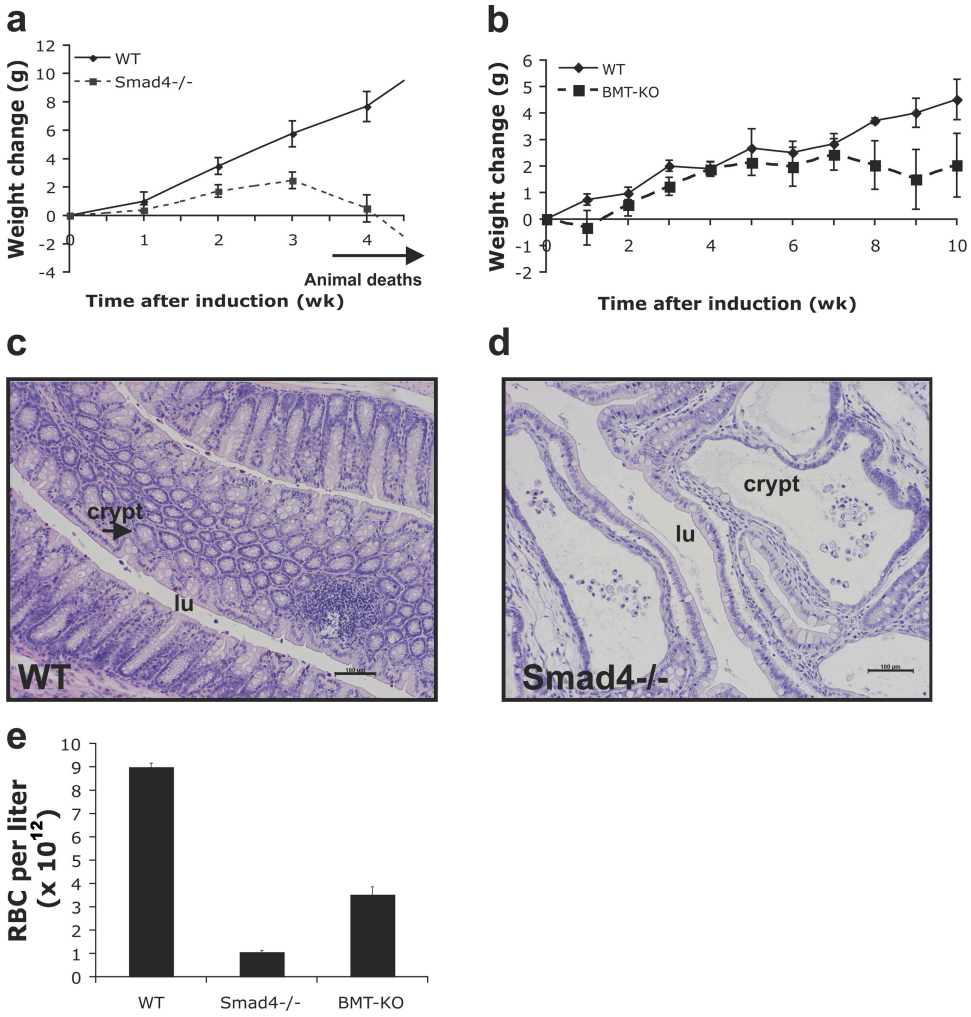

Figure 1. Induction of Smad4 deficiency leads to death caused by a gastrointestinal disorder. (a) Animal weight change after *Smad4* deletion. Results are shown as the mean ± SEM from two independent experiments (WT, $n = 4$; *Smad4*$^{-/-}$, $n = 9$). (b) Animal weight change after gene deletion in *Smad4*$^{-/-}$ mice with WT BM and control mice. Results are shown as the mean ± SEM of one experiment ($n = 3$ for both genotypes). (c and d) Pathological changes in the colon submucosa after *Smad4* deletion. Bar, 100 μm. Lu, lumen; crypt, crypts of Lieberkuhns. (e) RBC counts for *Smad4*$^{-/-}$ and BMT-KO mice. Results are shown as the mean ± SEM (WT, $n = 4$; *Smad4*$^{-/-}$, $n = 4$; BMT-KO, $n = 3$).

hematopoietic-intrinsic and extrahematopoietic defects associated with Smad4 deficiency. Importantly, we reveal a novel role for Smad4-dependent signaling as a cell-autonomous critical regulator of HSC self-renewal in vivo.

RESULTS AND DISCUSSION

Induced Smad4 deletion is lethal in a manner distinct from the inflammatory phenotype of upstream TGF-β signaling knockouts

In addition to the *MxCre*-induced gene deletion being essentially 100% efficient in the BM, the *Mx1* promoter is active in numerous other tissues (18). Hence, we created two experimental models: one in which steady-state WT or transgenic *MxCre/Smad4*$^{fl/fl}$ were induced (hereafter referred to as *Smad4*$^{-/-}$ or Smad4 deficient), and another in which lethally irradiated *Smad4*$^{fl/fl}$ mice were transplanted with WT BM before induction (BMT-KOs), effectively isolating *Smad4* deletion to tissues outside the BM. Subsequent to gene deletion, the two models were monitored for weight gain as an indicator of health. Approximately 3 wk after induction, Smad4-deficient mice began to lose weight and most were moribund 4 wk after induction (Fig. 1 a). The BMT-KOs remained healthy for up to 7 wk after induction, as measured by weight gain, after which they slowly started to lose weight compared with WT controls (Fig. 1 b). Histopathological examination of the liver, kidney, heart, lungs, esophagus, pancreas, and small intestines of *Smad4*$^{-/-}$ mice did not reveal evidence of tissue damage or inflammatory infiltration, as has been previously reported in models deficient in TGF-β signaling (unpublished data) (5–7). However, all *Smad4*$^{-/-}$ animals examined ($n = 6$) demonstrated apparent pathological changes in the colonic mucosa (Fig. 1 d), characterized by dilated glands and an enlarged lamina propria surrounding the crypts of Lieberkuhns. Furthermore, the crypts were increased in size but reduced in number and were infiltrated with macrophages. This finding had similarities with the phenotype seen in the small intestines of inducible *MxCre/Bmpr1a*-deficient mice (19). Surprisingly, colon histology of BMT-KO mice was normal 10 wk after induction (unpublished data), suggesting a mechanistic link between Smad4 deletion in the BM with the colon phenotype of Smad4$^{-/-}$ mice. This result is especially intriguing in light of recent findings by Kim et al., demonstrating a requirement for Smad4 in T cells in the maintenance of colon homeostasis (20).

Peripheral blood (PB) cell counts revealed a substantial reduction in RBCs (Fig. 1 e) and hemoglobin levels (not depicted) in *Smad4*$^{-/-}$ mice, whereas myeloid and lymphoid lineage distributions were normal (not depicted). Similarly, PB analysis revealed that BMT-KOs were also anemic, suggesting that *Smad4*$^{-/-}$ BM-derived cells are not required to generate this particular phenotype (Fig. 1 e). Feces from *Smad4*$^{-/-}$ and BMT-KO mice stained positive for hemoglobin 3 wk after gene deletion (unpublished data), suggesting that Smad4 deletion in the colon resulted in intestinal hemorrhage. Interestingly, these symptoms are paralleled in patients suffering from juvenile polyposis syndrome (17).

Smad4$^{-/-}$ HSCs possess normal differentiation capacity but impaired BM reconstitution

To determine the effect of Smad4 deficiency on hematopoietic potential, we performed in vitro assays and BM transplantations. Colony and single-cell proliferation assays demonstrated that clonogenicity and proliferation of hematopoietic progenitors in vitro were unaffected by Smad4 deficiency despite the insensitivity of *Smad4*$^{-/-}$ cells to TGF-β1 (Fig. S1, a–d, available at http://www.jem.org/cgi/content/full/jem.20060465/DC1). Furthermore, *Smad4*$^{-/-}$ hematopoietic progenitors had normal homing potential (Fig. S2, a and b), and recipients transplanted with *Smad4*$^{-/-}$ BM were healthy and displayed normal PB cell counts and lineage distribution over the 16 wk they were monitored, suggesting that Smad4 is dispensable for the differentiation capacity of HSCs (Fig. 2, a and b). Intriguingly, we did not observe any anemia nor changes in the colon of recipients engrafted with *Smad4*$^{-/-}$ BM, suggesting that Smad4 deletion in both hematopoietic and colon cells is required for the observed disruption of homeostasis of the colon submucosa in *Smad4*$^{-/-}$ steady-state mice. Earlier studies of *TGF-β–null* and inducible *TβR*-deficient mice resulted in a lethal multifocal inflammatory disorder in primary mice and recipients of BM transplantations (5–7). Although this phenotype was mediated by T cells (6, 21), the signaling mechanisms of the disease remain unknown. Importantly, recipients of *Smad4*$^{-/-}$ donor BM showed no signs of this inflammatory disease, suggesting that TGF-β–regulated T cell homeostasis is mediated through Smad-independent mechanisms. This conclusion is supported by a previous study in which Smad signaling was repressed through overexpression of the inhibitory Smad7 in HSCs without any observed inflammatory phenotype (22). Collectively, we conclude that canonical Smad signaling is dispensable for the inflammatory phenotype earlier observed in transgenic mice with upstream disruptions of the TGF-β pathway. Instead, loss of Smad4 in both the hematopoietic system and in the colon leads to death of the mice because of a gastrointestinal disorder and intestinal hemorrhage with subsequent anemia.

Intriguingly, FACS analysis of long-term reconstituted recipients revealed a slight but significant decrease in *Smad4*$^{-/-}$ donor engraftment in the BM as compared with recipients of WT donor cells (89.32 ± 1.6% vs. 94.6 ± 1.5%, respectively; $P < 0.03$), with a corresponding increase of the endogenous Ly5.1$^+$ fraction (Fig. 2 c).

To confirm that the reconstituting HSCs and progenitors were truly *Smad4*$^{-/-}$, we performed PCR analysis of CFU-GM colonies from recipient BM 16 wk after receiving BM transplants from six different *Smad4*$^{-/-}$ donors (Fig. 2 d). Out of the 60 colonies analyzed, all were positive either for the *null* or the endogenous WT allele, whereas none were positive for floxed alleles, demonstrating highly efficient *MxCre*-induced deletion of the Smad4 gene. Moreover, FACS analysis revealed that even though *Smad4*$^{-/-}$ donor and recipient mice had normal fractions and absolute numbers of total HSC-enriched Lin$^-$ Sca1$^+$ cKit$^+$ (LSK) cells as compared with controls (unpublished data), the proportion of

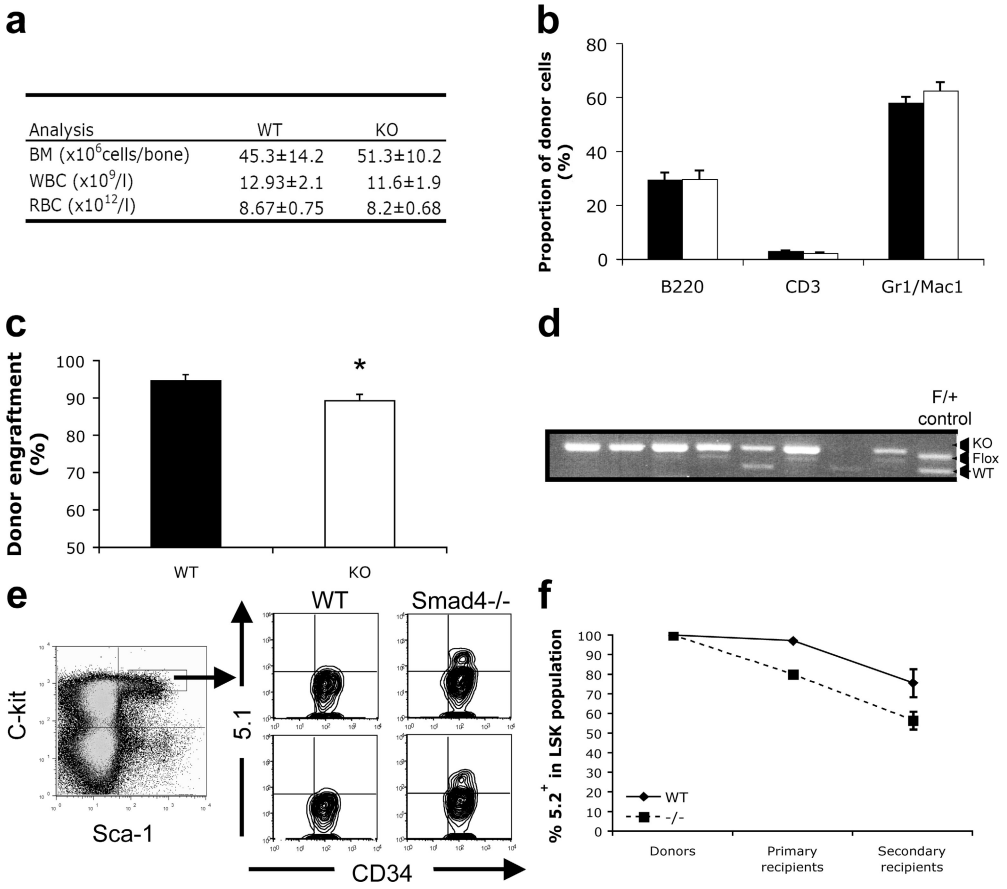

Figure 2. Smad4-deficient HSCs exhibit impaired repopulation capacity. (a) Hematopoietic cell counts and (b) lineage distribution. (c) Reconstitution of the hematopoietic system as measured by FACS analysis of engrafted Ly5.2$^+$ donor cells in recipient BM after transplantation. Results shown are the mean ± SEM of three independent experiments ($n = 8$ donors per genotype and 2–6 recipients per donor). *, $P < 0.03$ as measured by the Student's *t* test. WBC, white blood cell. (d) A representative figure of PCR analysis on CFU-GM colonies from BM 16 wk after transplantation. (e) FACS analysis showing the fraction of endogenous cells (Ly5.1$^+$) in the LSK population in irradiated recipients after two noncompetitive transplantations. (f) *Smad4*$^{-/-}$ donor LSK cells (Ly 5.2$^+$) are more quickly outcompeted by remaining endogenous cells during noncompetitive serial BM transplantations. Results shown are the means ± SEM using two donors per genotype and six recipients per donor. Shaded bars, WT; open bars, *Smad4*$^{-/-}$.

donor-derived LSK cells was decreased in recipients receiving Smad4-deficient BM compared with those receiving WT BM (82.7 ± 0.2% vs. 96.7 ± 0.05%, respectively; Fig. 2, e and f). We followed this cell population through serial transplantations, passaging 2×10^6 BM cells to new lethally irradiated recipients. Intriguingly, FACS analysis of secondary recipients demonstrated that *Smad4*$^{-/-}$ donor LSK cells were more quickly outcompeted by endogenous HSCs than were WT LSK cells (Fig. 2 f), thus indicating HSC self-renewal defects. Importantly, these analyses confirmed that *Smad4*$^{-/-}$ HSCs were capable of reconstituting the hematopoietic system but suggested that they were compromised in their ability to do so.

Smad4$^{-/-}$ HSCs have impaired self-renewal

We further challenged the repopulative capacity of *Smad4*$^{-/-}$ cells using competitive assays. 2×10^5 *Smad4*$^{-/-}$ or WT BM cells were transplanted into lethally irradiated recipients together with an equal number of normal competitor cells. In agreement with the impaired reconstitution capacity in noncompetitive assays, PB from recipients of *Smad4*$^{-/-}$ BM analyzed 4 wk after transplantation had a 2.5-fold lower hematopoietic reconstitution as compared with recipients receiving WT BM (15.1 ± 3.72% vs. 34.8 ± 4.8%, respectively; $P < 0.02$). Moreover, the contribution of *Smad4*$^{-/-}$ PB cells slightly decreased over time, reaching a level of 11.4 ± 3.5% at 16 wk after BM transplantation, as opposed to WT donor cells, which displayed a stable PB distribution over time (40.2 ± 8%; $P < 0.01$; Fig. 3, a and b). Lineage distribution of the remaining *Smad4*$^{-/-}$ BM was normal at all time points after transplantation up to and including 16 wk (Fig. 3 c). Strikingly, *Smad4*$^{-/-}$ cells demonstrated a 40-fold reduction in engraftment compared with WT controls upon secondary transplantation (Fig. 3 d).

Furthermore, competitive repopulation units (CRUs) were quantified by transplanting limiting doses of Smad4-deficient or WT donor cells in competition with a set number (2×10^5) of normal cells to lethally irradiated recipients. The CRU

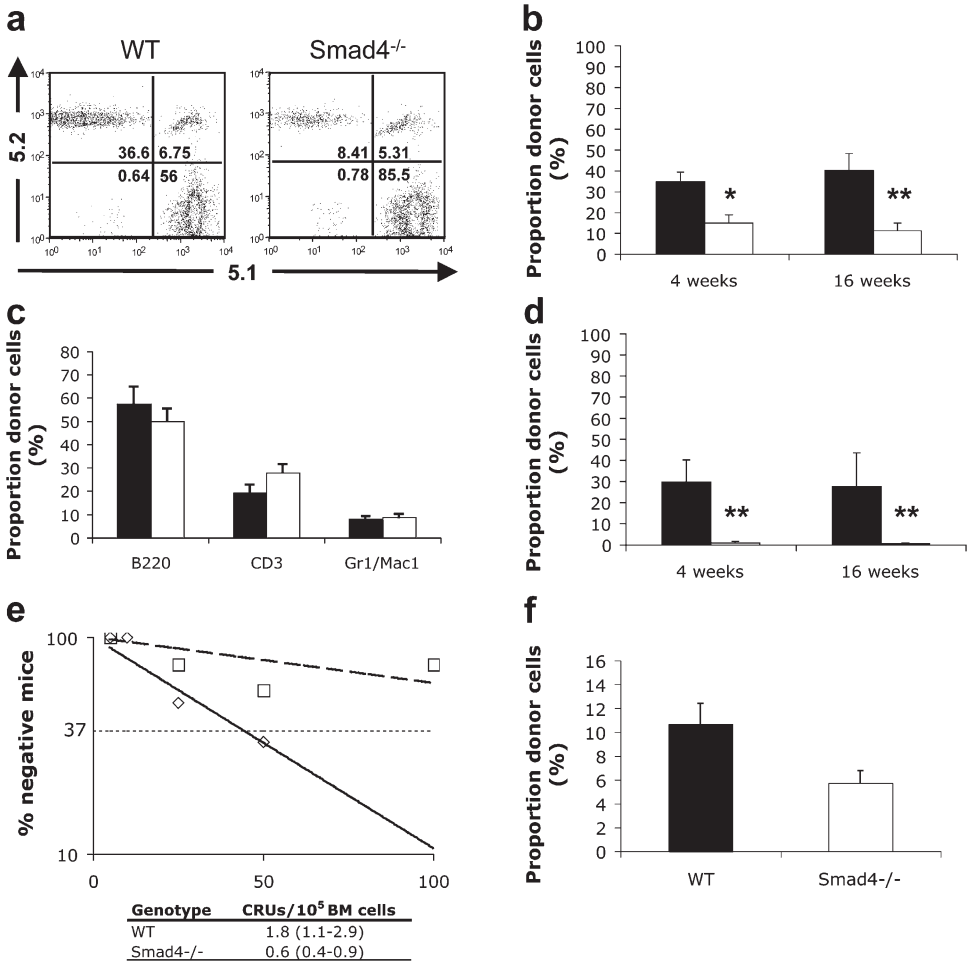

Figure 3. *Smad4*$^{-/-}$ HSCs have a pronounced disadvantage in competitive transplantation settings. (a) Representative Ly5.1/Ly5.2 FACS analysis of PB. Numbers indicate the percentage of cells in each quadrant. (b) Pooled data from two independent competitive transplantation experiments at 16 wk after BM transplantation. Results shown are the mean percentage of PB ± SEM ($n = 6$ donors and 2–3 recipients per donor for each genotype). (c) BM lineage distribution of *Smad4*$^{-/-}$ Ly5.2-reconstituting donor cells after 16 wk of competitive transplantation. (d) Donor contribution to PB 16 wk after secondary transplantations ($n = 5$ donors and 3 recipients per donor). (e) Limiting dilution assay and CRU numbers in *Smad4*$^{-/-}$ BM. The figure is based on four doses per donor, one WT and two *Smad4*$^{-/-}$, and three to four recipients per donor. CRU numbers are presented as the mean ± SE. Squares and dashed line, *Smad4*$^{-/-}$; diamonds and continuous line, WT. (f) Donor contribution 7 wk after competitive transplantation using WT chimeras with *Smad4*$^{-/-}$ BM as donors. Results shown are the mean percentage of PB ± SEM ($n = 4$ donors and 3 recipients per donor for each genotype; $P < 0.02$). Shaded bars, WT; open bars, *Smad4*$^{-/-}$. *, $P < 0.02$; and **, $P < 0.01$ as measured by the Mann-Whitney test.

frequency in the *Smad4*$^{-/-}$ mice was estimated to be threefold lower as compared with control BM (Fig. 3 e), further supporting the findings of impaired reconstitution capacity and reduced numbers of phenotypically defined HSCs/progenitors.

Notably, FACS analyses of HSC-enriched populations demonstrated that cell-cycle status and apoptosis were unaffected by Smad4 deficiency (Fig. S3, a–d, available at http://www.jem.org/cgi/content/full/jem.20060465/DC1). However, in response to myeolablative stress in vivo, *Smad4*$^{-/-}$ cells had a slight but significant decrease in proliferative capacity compared with controls (Fig. S3, e and f), suggesting that their capacity to proliferate may not be as robust as that of WT cells at times of acute demand for a replenished blood system.

To ask whether the *Smad4*$^{-/-}$ phenotype was cell autonomous or dependent on the lack of Smad4 in the HSC niche, 2×10^6 uninduced *Smad4*$^{fl/fl}$ or WT BM cells were transplanted to lethally irradiated mice. 10 wk after transplantation, Smad4 deletion was induced, producing chimeras with *Smad4*$^{-/-}$ or WT HSCs in a normal HSC niche. Competitive transplantation experiments using BM cells harvested from these mice yielded an analogous phenotype as when performing competitive transplantation assays using *Smad4*$^{-/-}$ donors averaging ~50% of the reconstitution levels of WT cells 7 wk after transplantation (Fig. 3 f). Collectively, these experiments reveal a profound cell-autonomous defect in competitive repopulating capacity and self-renewal of *Smad4*$^{-/-}$ HSCs.

Molecular profiling of Smad4$^{-/-}$ LSK cells reveals down-regulation of Notch1 and c-myc

To gain insight into the effect of *Smad4* deletion on HSC function, we performed gene expression profiling using quantitative real-time PCR. LSK cells deficient in Smad4 display reduced expression of the transcription factor and cell-cycle regulator *c-myc*, which was down-regulated 1.6-fold ($P = 0.02$; Table I). A recent loss-of-function study manifested a dual role for c-myc in mouse HSC function. *MxCre*-induced *c-myc* deficiency resulted in an expected decrease in proliferation of hematopoietic progenitors but also in an increase in HSC self-renewal, presumably because of defective migration out of the HSC niche (23). However, earlier work by Satoh et al. demonstrated that ectopic expression of c-myc–induced self-renewal of HSCs in vitro (24). In the same study, *c-myc* was shown to be transcriptionally induced by Notch signaling. Correspondingly, we also observed a significant transcriptional down-regulation of the Notch1 receptor in Smad4-deficient hematopoietic populations enriched for HSCs (Table I). The Notch ligand Jagged1 is expressed on osteoblasts in the HSC niche in the BM and has been demonstrated to be an important regulator of HSC self-renewal in vivo (25, 26). The down-regulation of Notch1 would therefore be consistent with our observation of reduced self-renewal in vivo but unchanged in vitro proliferation of HSC-enriched populations.

Table I. Gene expression analysis of LSK cells

	Gene (number of mice per genotype)	Fold change (KO/WT)
Cell-cycle regulators	*p15* ($n = 2$)	nd
	p18 ($n = 2$)	-1.17 ± 0.04
	p21 ($n = 4$)	1.11 ± 0.4
	p27 ($n = 4$)	1.02 ± 0.23
	p57 ($n = 1$)	nd
	Id1 ($n = 4$)	nd
	Id2 ($n = 2$)	1.25 ± 0.33
	Id3 ($n = 2$)	nd
	c-myc ($n = 5$)	-1.6 ± 0.14**
Transcription regulators	*Gata2* ($n = 4$)	-1.31 ± 0.18
	Hoxb4 ($n = 4$)	1.19 ± 0.93
	Bmi1 ($n = 3$)	1.22 ± 0.1
	Gfi1 ($n = 4$)	-1.19 ± 0.42
	C/EBPα ($n = 4$)	1.3 ± 1.1
Receptors	*Fzd1* ($n = 2$)	nd
	c-kit ($n = 2$)	1.22 ± 0.13
	c-mpl ($n = 2$)	-1.23 ± 0.04
	Notch1 ($n = 5$)	-1.78 ± 0.18*
Signaling molecules	*Lnk* ($n = 2$)	-1.12 ± 0.11
	Spry2 ($n = 2$)	-1.26 ± 0.18

Results show the average fold difference in expression ± SEM. **, $P = 0.02$; and *, $P = 0.03$ as measured by paired *t* test. nd, not detectable.

Smad4 critically regulates adult HSC fate through the canonical Smad pathway and non-Smad regulatory circuits

In this paper, we demonstrate that Smad4 is critical for the regulation of HSC self-renewal in vivo without affecting the ratio of the different hematopoietic lineages. Furthermore, it appears that the *Smad4*$^{-/-}$ HSC phenotype is cell autonomous but dependent on extrinsic signaling from the in vivo microenvironment, because in vitro proliferation was normal in *Smad4*$^{-/-}$ HSCs. Thus, our data suggest a positive role for Smad4 in HSCs in contrast to the documented growth-arresting effect generated by the upstream TGF-β ligand. It is unlikely that this stimulatory effect is caused by the weak HSC growth-promoting signals documented for BMP4 on human cells in vitro, as BMP4 does not seem to have analogous effects in mouse BM (27). In fact, recent work supports a negative role for Smad signaling on the whole in HSC self-renewal, as interference of the pathway through oncoretroviral overexpression of the inhibitory Smad7 resulted in an in vivo expansion of HSCs (22). This, together with the observations presented here, suggest a model for Smad signaling in the regulation of HSC self-renewal, where the positive role of Smad4 is caused by mechanisms outside the canonical Smad signaling pathway (Fig. 4). This idea is supported by the observation that in vivo expansion of Smad7-overexpressing HSCs is dependent on Smad4 (22).

An intriguing recent report demonstrates a previously unknown alternative pathway for TGF-β signaling in which TIF1γ competes with Smad4 to form complexes with Smad2 and 3.

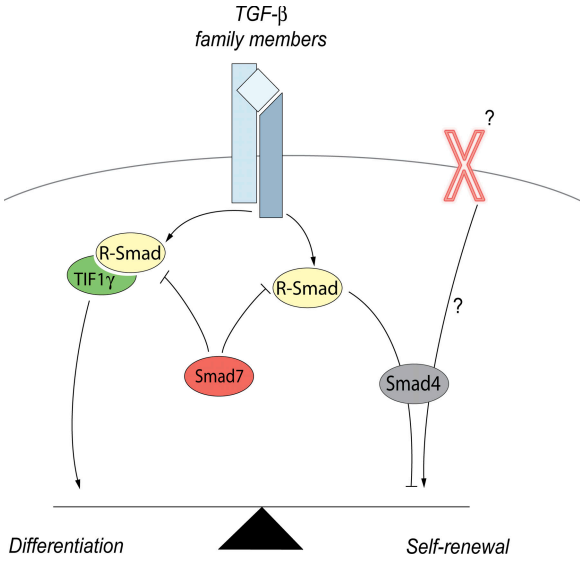

Figure 4. Model for the regulation of HSC self-renewal by the Smad signaling pathway. The canonical Smad pathway inhibits HSC expansion. This inhibition can be relieved upon enforced expression of the inhibitory Smad7. Recently, TIF1γ has been shown to compete with Smad4 for binding of R-Smads, leading to differentiation of primitive cells into erythrocytes. In addition to its inhibitory role in HSCs as a critical partner in the canonical Smad pathway, Smad4 has a positive effect on self-renewal, possibly as a transducer of unknown regulators outside this pathway.

In an apparent balancing act, TGF-β–induced R-Smad–Smad4 complexes keep hematopoietic progenitors quiescent, whereas R-Smad–TIF1γ complexes direct human primitive hematopoietic cells toward erythroid differentiation (12). Our data would support a model in which the absence of Smad4 tips the balance in favor of differentiation at the expense of self-renewing divisions. Additionally, emerging data suggest that other intracellular pathways directly affect Smad signal transduction. Such cross talk is well established between MAPK and Smad pathways, where it has been shown that the linker region of Smads can serve as a phosphorylation site for MAPKs, ultimately inhibiting Smad translocation to the nucleus (9). Furthermore, it has been demonstrated that β-catenin and Lef1/TCF, which are downstream mediators of the Wnt signaling cascade, can form a functional complex with Smad4 (11). Moreover, Notch and TGF-β signaling have been demonstrated to converge at the regulation of *Hes1* expression (10). These findings are important in this context, because Wnt and Notch signaling stimulate proliferation of HSCs (25, 26, 28, 29). In summary, we demonstrate that Smad4 is a critical regulator of HSC self-renewal in vivo without affecting lineage choice. Therefore, signaling pathways and other HSC regulators that require Smad4 to mediate their effects will be important topics for future studies of HSC function.

MATERIALS AND METHODS

Mice. Mice with a mixed 129/FVB/Black Swiss background carrying loxP sites flanking one allele of exon 8 of the Smad4 locus (*Smad4*$^{fl/+}$) (30) were mated to homozygosity and subsequently crossed with *MxCre*-transgenic C57BL/6 mice to generate conditional *MxCre/Smad4*$^{fl/fl}$ knockout mice. Poly I:C–induced deletion of the floxed exon was induced in 7–12-wk-old mice, unless other stated, as previously described (8). All animal experiments were approved by the Lund University Animal Ethical Committee.

Histology. 5-wk-old *MxCre/Smad4*$^{fl/fl}$ and WT control mice (*MxCre/Smad4*$^{+/+}$ or Cre^{-} *Smad4*$^{fl/fl}$) were killed 28–32 d after the last poly I:C injection, and organs were embedded after fixation in PBS containing 4% paraformaldehyde. Bones were incubated in Parengy decalcification solution (0.15% chromethrioxid, 4.3% nitric acid, and 30% ethanol; Bie & Berntsen AS) for 48 h. Organs were subsequently sectioned and stained with Erlish eosin for microscopic examination.

BM transplantation assays. BM was harvested from 7–12-wk-old *MxCre/Smad4*$^{fl/fl}$ and littermate control mice (Ly5.2) killed 7–10 d after the last poly I:C injection, and 2×10^6 BM cells were transplanted into the tail vein of lethally irradiated (9 cGy) C57BL/6 × B6SJL recipient mice (Ly5.1/Ly5.2). In the case of serial BM transplantations, 2×10^6 BM cells were passaged into new lethally irradiated recipients 8 wk after transplant.

In the competitive repopulation assay, 2×10^5 *Smad4*$^{-/-}$ or littermate control (WT) donor cells were transplanted into lethally irradiated Ly5.1/Ly5.2 recipients together with 2×10^5 competitor cells from B6SJL (Ly5.1) mice.

In the case of competitive transplantation from the *Smad4*$^{fl/fl}$/WT chimeras, 2×10^6 BM cells from uninduced *MxCre/Smad4*$^{fl/fl}$ were transplanted into WT C57BL/6 × B6SJL mice. 10 wk after transplantation, the mice were induced, and competitive transplantation was performed as described earlier in this section.

CRU assay was performed using four cell doses ranging between 5×10^3 and 10^5 BM cells per donor genotype, together with 2×10^5 normal competitor cells. PB was analyzed 12 wk after transplantation, and a cell dose was considered to contain at least one CRU if donor chimerism was >1% for both the myeloid and lymphoid lineages. The CRU frequency was calculated on the basis of negative recipients using L-calc software (StemCell Technologies Inc.).

FACS analyses. The following fluorochrome-conjugated antibodies were purchased from BD Biosciences: anti-B220, -CD3, -Gr1, -Mac1, -CD45.1, -CD45.2, -CD4, -CD8, -Ter119, -CD71, -Sca1, -Kit, and -CD34. Lineage-positive cells were labeled with unconjugated antibodies and Tri-color–conjugated goat F(ab′)$_2$ anti–rat immunoglobulin G as a secondary antibody.

Quantitative RT-PCR. Gene-specific primers (Applied Biosystems) were used to analyze the expression of 20 genes of interest (Table I), together with the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (*Hprt*).

Online supplemental material. Fig. S1 shows that *Smad4*$^{-/-}$ hematopoietic progenitors have normal in vitro proliferation and colony-forming capacity while being resistant to TGF-β–induced growth suppression. Fig. S2 demonstrates that Smad4 is dispensable for homing of hematopoietic progenitors to the BM. Fig. S3 shows that *Smad4*$^{-/-}$ hematopoietic progenitors have normal apoptosis and cell-cycle status while having a reduced stress-induced proliferative capacity. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060465/DC1.

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