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Hedgehog signaling is dispensable for adult hematopoietic stem cell function

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Summary

The Hedgehog (Hh) signaling pathway is a developmentally conserved regulator of stem cell function. Several reports suggested that Hh signaling is an important regulator of hematopoietic stem cell (HSC) maintenance and differentiation. Here we test this hypothesis *in vivo* using both *gain*- and *loss-of-function* Hh genetic models. Surprisingly, our studies demonstrate that conditional *Smoothened (Smo)* deletion or over-activation has no significant effects on adult HSC self-renewal and function. Moreover, they indicate a lack of synergism between the Notch and Hh pathways in HSC function, as compound *RBPJ*- and *Smo*-deficiency does not affect hematopoiesis. In agreement with this notion, detailed genome-wide transcriptome analysis reveals that silencing of Hh signaling does not significantly alter the HSC-specific gene expression "signature". Our studies demonstrate that the Hh signaling pathway is dispensable for adult HSC function and suggest that the Hh pathway can be targeted in future clinical trials addressing the effect of Hh inhibition on leukemia-initiating cell maintenance.

Introduction

Hematopoietic stem cells (HSC) are able to self-renew as well as give rise to all blood lineages. HSCs mainly reside in specialized bone marrow microenvironments, called HSC niches. The niche is thought to provide appropriate signals that maintain the balance between self-renewal and differentiation of HSCs (Adams and Scadden, 2006; Lessard et al., 2004; Moore and Lemischka, 2006; Morrison and Spradling, 2008; Wilson and Trumpp, 2006; Yin and Li, 2006). However, the identity of these signals and the molecular

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mechanisms governing HSC fate largely remain elusive. Thus, identification of regulators of HSC function is a central issue in stem cell biology.

The roles of developmentally imprinted signaling pathways, more specifically Notch, Wingless (Wnt) and Hedgehog (Hh), in HSC homeostasis have been studied extensively (Maillard et al., 2008; Stier et al., 2002); (Cobas et al., 2004; Reya et al., 2003). Hh is a secreted protein family with 3 members in higher vertebrates (Shh, Ihh, Dhh). In the absence of Hh, the Patched (Ptch) receptor acts as a negative regulator of signaling as it inhibits the action of Smoothened (Smo) (Hammerschmidt et al., 1997). Hh protein binds and inhibits Ptch action, inducing signaling transduction through Smo. This signaling cascade results in the nuclear localization and activation of the Gli family of transcription factors. Although Hh is a major regulator of cell fate decision and body segment polarity (Nusslein-Volhard and Wieschaus, 1980), its role in HSC homeostasis and differentiation remains controversial. Several reports have suggested that Hh signaling is critical for HSC and hematopoietic progenitor differentiation. A study of zebrafish hematopoiesis revealed that embryo mutants of the Hh pathway display defects in HSC formation (Gering and Patient, 2005), indicating that Hh is required for definitive hematopoiesis. Consistent with these data, *in vitro* studies found that antibodies to Hh inhibited the cytokine-induced proliferation of human primitive hematopoietic stem cells, whereas Shh induced the expansion of human hematopoietic repopulating cells (Bhardwaj et al., 2001). In addition, analysis of *Ptch1*$^{+/-}$ mice showed that Hh activation expanded primitive bone marrow cells, but continued Hh activation led to HSC exhaustion (Trowbridge et al., 2006). Furthermore, a recent study using an *in vivo* model of Hh deficiency suggested that HSCs require Smo-mediated signals for their homeostasis (Zhao et al., 2009a). In contrast to these studies, it was proposed that Hh signaling is involved at the level of lymphocyte lineage commitment as a defect in the common lymphoid progenitor (CLP) population was observed upon deletion of *Ptch1* (Uhmann et al., 2007). Moreover, Hh signaling has been demonstrated to be important for the differentiation and proliferation of hematopoietic progenitors in the thymus (Crompton et al., 2007; El Andaloussi et al., 2006). Finally, a recent report suggested that Hh signaling is essential for the differentiation of leukemia-initiating cells, introducing Hh inhibitors in clinical trials targeting BCR-ABL^{+} leukemia (Dierks et al., 2008; Dierks et al., 2007).

As none of these studies directly targeted Hh function specifically in adult HSCs, we decided to address HSC-specific Hh function *in vivo*. To this end, both *gain-* and *loss-of-function* conditional *Smo* genetic models were used, as the Smo receptor is the only non-redundant element of the Hh pathway. Surprisingly and contrary to the consensus view, Hh signaling appeared to be dispensable for the self-renewal and differentiation of adult bone marrow HSC. Indeed, neither conditional deletion of the Smo signal transducer nor hyper-activation of the Hh pathway had an affect in adult HSC maintenance and function. Interestingly, Hh signaling also appeared to be dispensable for the function of putative leukemia-initiating cells in T-cell leukemia as induction and progression of the disease was unaffected by silencing of the pathway.

Results

Conditional deletion of Smo fails to affect HSC maintenance *in vivo*

To study the role of Hh signaling in adult HSCs, we generated a Cre-regulated conditional model of *Smo* deletion (Smo$^{F/F}$Mx1-Cre^{+}, Figure 1A), in which expression of the Cre recombinase is under the control of myxovirus-resistance 1 (Mx1) gene promoter (Mx1-Cre) (Gu et al., 1994) and is induced by interferon-α (via stimulation with polyI:polyC). In these mice, the first exon of the Smo locus is flanked by loxP sites and is deleted upon Cre-mediated recombination (Long et al., 2001). Smo$^{F/F}$Mx1-Cre^{-}(control) and Smo$^{F/F}$Mx1-Cre^{+} littermate mice were treated with polyI:polyC. This treatment resulted in the efficient

deletion of *Smo* floxed alleles and the generation of a recombined *Smo* deleted (Δ) alleles (Figure 1B, lane 4). At the mRNA level, *Smo* was not detectable in SmoΔ/Δ bone marrow cells, and the expression of *Ptch1*, a key target gene of Hh activation, was significantly reduced compared to the control mice (Figure 1C).

Initial analysis of control and *Smo*-deficient mice at 4 weeks post polyI:polyC injection demonstrated no significant alteration in the overall bone marrow cellularity (p=0.10). Further analysis showed that Smo deletion had no effect on the relative frequency (p=0.35) or the absolute number of $Lin^-Sca1^+cKit^+$ (LSK), a cell population enriched for HSCs (p=0.49) (Figure 1D). HSCs differentiate and give rise to myeloid progenitors (MP, $Lin^-Sca1^-cKit^+$), which can be subdivided into common myeloid progenitors (CMP, $Lin^-Sca1^-cKit^+CD34^+Fc\gamma R^{low}$), granulocyte-monocyte progenitors (GMP, $Lin^-Sca1^-cKit^+CD34^+Fc\gamma R^{hi}$), and megakaryocyte/erythrocyte progenitors (MEP, $Lin^-Sca1^-cKit^+CD34^-Fc\gamma R^{low}$). Our analysis showed that CMP, GMP and MEP compartments were comparable between *Smo*-deficient and control mice (Figure 1E). Moreover, the percentages of terminally differentiated B- and T-lymphocytes appear normal in *Smo*-deficient spleen and bone marrow (Figure S1). In the thymus, the distribution of mature ($CD4^+$, $CD8^+$) and immature ($CD4^+8^+$, $CD4^-8^-$) compartments appeared similar to controls. Further subdivision of the $CD4^-8^-$ compartment using the CD25 and CD44 surface antigen expression also revealed a normal distribution (Figure S1). The lack of a perturbation of the hematopoietic compartment was not due to early time-point analysis as *Smo*-deficient mice at 16 weeks post polyI:polyC injection also displayed normal populations of LSK, progenitors and lymphocytes despite the complete absence of *Smo* mRNA (Figure S2).

We further examined the LSK population, which can be subdivided into long term (LT)-HSC ($LSKCD34^-Flt3/Flk2^-$), short term (ST)-HSC ($LSKCD34^+Flt3/Flk2^-$) and multipotent progenitors (MPP, $LSKCD34^+Flt3/Flk2^+$). We observed comparable numbers of LT-, ST-HSC and MPPs between control and *Smo*-deficient mice (Figure 1E). Also similar analysis using CD150 and CD48 as markers of LT-HSC ($LSKCD48^-CD150^+$) did not reveal any abnormalities in *Smo*-deficient mice (Figure 1E). Additionally, we investigated the pro-survival and pro-proliferative functions of the Hh pathway. Staining for the pro-apoptotic marker AnnexinV did not reveal abnormal induction of cell death in *Smo*-deficient LSKs. Furthermore cell cycle analysis of the marker for proliferation Ki67 in conjunction with DAPI to measure DNA content did not reveal any differences in the cell cycle profiles between control and *Smo*-deficient LSKs (Figure 1E). These findings strongly suggested that *Smo*-mediated Hh signaling is dispensable for adult HSC and progenitor homeostasis and differentiation. To further test this hypothesis, we analyzed $Gli1^{lacZ/lacZ}$ mice (Bai et al., 2002), in which *Gli1*, a key transcription activator of the Hh pathway, is deleted and replaced by a *lacZ* allele. We did not detect any defects in the HSC compartment or in T and B lymphopoiesis in the bone marrow and the thymus of $Gli1^{lacZ/lacZ}$ mice (Figure S3), suggesting that *Gli1* function is dispensable for hematopoiesis.

Smo-deletion does not alter differentiation ability of progenitor cells

To test functionality of *Smo*-deficient stem cells and progenitors, LSKs were flow-purified from either control or *Smo*-deficient bone marrows and methylcellulose assays were performed in presence of the appropriate cytokines. Both types of LSK cells generated similar numbers of colony-forming units (CFU) in both primary and secondary platings (Figures 2A and 2B). The deletion of *Smo* was confirmed by colony-specific PCR. The results showed that 14 out of the 15 studied colonies derived from *Smo*-deficient LSKs deleted the *Smo* allele. The expression of *Smo* mRNA was not detectable by quantitative RT-PCR; moreover, the expression of *Ptch1* mRNA was significantly reduced in *Smo*-

deficient LSK-derived colonies (Figures 2C and 2D). These results suggest that *Smo* is dispensable for short-term differentiation ability of hematopoietic progenitor cells.

ST-HSC cells are able to give rise rapidly to colonies in the spleen when transplanted into lethally irradiated hosts. To study the effect of *Smo* deletion in this process, CFU-spleen units (CFU-S) were scored after transplanting either control or *Smo*-deficient bone marrow cells. We obtained identical CFU-S scores for the two groups (Figure S4), again indicating that *Smo* function is dispensable for rapid progenitor differentiation. To test the capability of *Smo*-deficient progenitors to expand and replenish the immune system, control and *Smo*-deficient mice were challenged weekly with a dose of 5-fluorouracil (5-FU) to eradicate cycling cells (Berardi et al., 1995) and the survival of these mice was observed. Similar survival percentage in the two groups (Figure 2E) suggested that the *Smo*-deficient progenitor cells were able to enter into cell cycle at a comparable level as wild-type cells. Collectively these results demonstrate that *Smo* is dispensable for short-term differentiation of adult progenitor cells both *in vitro* and *in vivo*.

Smo deletion does not affect HSC self-renewal and reconstitution ability

One explanation for the lack of an overt effect of *Smo* loss on HSC maintenance or function is that the potential Hh function is masked due to the nature of the analysis utilized, and that it can be revealed only in a competitive setting. To test the reconstitution capacity of *Smo*-deficient HSCs, competitive bone marrow transplantations (BMT) were performed. Bone marrow cells from either control or *Smo*-deficient mice ($CD45.2^+/Ly5.2^+$) were competed with an equal number of bone marrow cells from isogenic $CD45.1^+/Ly5.1^+$ mice and transplanted into lethally irradiated $Ly5.1^+$ recipients (Figure 3A). Peripheral blood analysis of chimerism of the recipients showed that *Smo*-deficient HSCs were able to compete with wild-type HSCs, in a manner similar to control HSCs (Figure 3B). Similar assays were performed by mixing flow-purified LSKs from either control or *Smo*-deficient mice with competing Ly5.1 bone marrow cells. Once more, no significant differences were observed between control and *Smo*-deficient LSKs 14 weeks after BMT (Figure 3C). This lack of phenotype was not due to partial or inefficient deletion of the *Smo*, as quantitative RT-PCR in flow-purified $Ly5.2^+Lin^-$ bone marrow cells of recipient mice 16 weeks after BMT showed a complete loss of *Smo* mRNA expression (Figure 3D). Indeed, at week 16 after BMT, $Ly5.2^+$ *Smo*-deficient donor cell-derived LSK cells were present in the bone marrow, $B220^+$ B and $CD3^+$ T cells in the spleen of recipients (Figure 3E), demonstrating the repopulation ability of *Smo*-deficient HSCs.

To more rigorously test the repopulation ability *Smo*-deficient HSCs, a secondary competitive BMT was performed using donor-derived $Ly5.2^+$ bone marrow cells isolated from the recipients of the primary transplant. We observed that the reconstitution ability of *Smo*-deficient HSCs was identical to that of control HSCs even in this sensitive serial transplantation setting. As shown in Figure 3F, at 12 weeks post secondary BMT, the chimerism in peripheral blood was comparable between recipients that had received control or *Smo*-deficient cells, and donor-derived $B220^+$, $CD3^+$ and $Mac1^+$ cells were present at similar percentages. Taken together these data indicate that deletion of *Smo* has no significant effect on HSC repopulation ability.

Smo deletion does not alter HSC-specific gene expression signature

The absence of a phenotypic defect in HSCs that lack *Smo* led us to search for a putative role for Hh signaling in stem cell and progenitor gene expression patterns. To examine whether the loss of *Smo* results in changes at the molecular level in HSCs, microarray analysis was performed using flow-purified LSK and MP populations from either control or *Smo*-deficient mice. The array analysis (Figure S5C) and qRT-PCR studies (data not shown)

showed a complete loss of *Smo* expression in both LSK and MP compartments. When control LSKs were compared with control MPs in duplicate experiments, 739 genes changed expression levels by 2-fold or greater (Figure S5A). For the purpose of this analysis, these 739 genes were regarded as an HSC-enriched gene expression "signature". As a proof of principal, it was shown that this specific gene "signature" was lost upon deletion of *Fbw7*, a ubiquitin ligase that is essential for the maintenance of HSC quiescence (Thompson et al., 2008). Indeed, 43% (315 out of 739) of these selected genes were significantly down-regulated in *Fbw7*-deficient LSKs. In contrast, less than 10% (70 out 739) of these genes changed (up- or down-regulated) in response to *Smo* deletion (Figure S5B), suggesting that the HSC gene signature is largely preserved in *Smo*-deficient LSKs.

Previous reports (Forsberg et al., 2005; Jankovic et al., 2007; Mansson et al., 2007; Terskikh et al., 2003; Thompson et al., 2008) have defined a list of genes closely associated with LT-HSC activity. These genes are highly expressed in LT-HSCs but are down-regulated as HSCs lose their self-renewal abilities. These genes include transcription factors/cofactors important for HSC self-renewal and differentiation (Meis1, Egr1, Eya1/2), surface receptors (Mpl, Thy1, Agpt) as well as regulators of HSC survival (Mcl1). Our array analyses showed that the expression of these genes was not altered by the inducible deletion of *Smo* (Figure S5C). These data demonstrate that *Smo* is not required for the maintenance of adult HSC properties at the molecular level and support our findings that HSCs are phenotypically normal in the absence of *Smo*.

Absence of functional redundancy between the Notch and Hh pathways in hematopoiesis

The possibility remains that redundancy between signaling pathways masked a potential function for Hh in the early stages of hematopoiesis. We have shown previously that the Hh and Notch pathways share similar expression patterns and putative functions. Also, *Smo* mRNA expression appears to be significantly induced in response to Notch activation in Lin^{neg} bone marrow progenitors (El Andaloussi et al., 2006; Vilimas et al., 2007). These observations suggest a functional redundancy between the two pathways in adult HSC function. To test this hypothesis, we generated mice deficient for both *Smo* and *RBPJ*, a DNA binding factor required for canonical Notch signaling and performed competitive BMT. We injected polyI:polyC into $RBPJ^{F/F}Smo^{F/F}Mx1\text{-}Cre^{+}$ mice, and confirmed the excision of Smo- and RBPJ-floxed alleles as well as the recombination of both alleles in the bone marrow (Figure 4A). Next we performed competitive BMT and found that *RBPJ/Smo*-deficient (DKO) cells were able to efficiently reconstitute irradiated hosts. Analysis of chimerism in the peripheral blood 6 to 10 weeks after BMT did not reveal any defects for DKO cells (Figure 4B). In fact, donor cells derived from DKO bone marrow were able to give rise to LSKs in bone marrow (Figure 4C) and $B220^{+}$ cells in the spleen (Figure 4F). There were no significant differences in the number of donor-derived LSKs between the two groups 12 weeks post BMT (Figure 4D). As expected, donor-derived T cells ($CD4^{+}CD8^{+}$ T cells in thymus, and $TCR\beta^{+}$ cells in spleen) (Figures 4E and 4F) and marginal zone B cells ($B220^{+}CD21^{high}CD23^{low/-}$ cells in spleen) (Figure 4F) were reduced in recipients transplanted with DKO bone marrow, since Notch signaling is required for the development of these two lineages. Collectively these results show that neither Notch nor Hh signaling are necessary for adult HSC maintenance and differentiation. Furthermore these data suggest that these two pathways are not redundant in governing HSC fate.

Mapping of Hh signaling component expression in HSC and their niche

The absence of a phenotype in *Smo*-deficient HSCs suggested that the Hh signaling might not be active or of low activity in these cells. To determine whether elements of the Hh signaling network can be detected in either HSCs or the HSC "niche", the expression of the components of this pathway was examined in flow-purified LSKs and differentiated MPs.

We found that both the *Smo* transducer and *Ptch1* receptor mRNAs (which is also a target gene of Hh signaling) were expressed in LSKs and MPs, (Figure S6A). In contrast, the members of Hh ligand family, *Ihh* and *Dhh* but not *Shh* mRNA was detected in primary preparations of calvarial osteoblasts, cells that comprise the osteoblastic HSC niche (Figure S6B), demonstrating that Hh ligands are available to HSCs. Several mouse and human osteoblastic lines showed similar Hh expression profiles (not shown). However, the expression of the downstream transcription factors *Gli1*, *Gli2* and *Gli3* was not detectable in either LSKs or MPs by quantitative PCR (Figure S6C) and microarray analysis (data not shown), a result that suggested low levels of Hh activity in both LSK and MP populations. Therefore, these data indicate that HSCs and progenitors have the ability to receive Hh signaling since they express both the *Smo* and *Ptch1* receptors and Hh ligands are present in the niche. Nevertheless, there is little ongoing Hh activity as the transcription factors are not expressed, which is consistent with the described lack of HSC phenotype in *Smo*-deficient mice.

Hh pathway activation does not expand HSC or enhance their engrafting ability

It has been proposed previously that Hh morphogens could be used for *in vitro* expansion of primitive stem cell and progenitor populations and thus could be beneficial in transplantation protocols (Bhardwaj et al., 2001). Our results have shown an incomplete Hh activation in HSCs suggesting that Hh pathway activation could either expand HSC or provide them with competitive advantage in transplantation settings. To directly test this hypothesis, we used a Hh *gain-of-function* model ($R26^{SmoM2}$), in which enhanced yellow fluorescent protein (EYFP) was fused with the constitutively active W539L point mutation of the mouse smoothened homolog gene (SmoW539L), and "knocked" into the ubiquitously-expressed ROSA26 locus (Jeong et al., 2004). The expression of SmoM2/EYFP fusion gene is blocked by a loxP-flanked STOP fragment inserted between the ROSA26 promoter and the SmoW539L/EYFP sequence (Figure 5A). We crossed these mice to the Mx1-Cre stain and generated $R26^{SmoM2/SmoM2}$Mx1-Cre^{+} (referred to hereafter as Cre^{+}) or $R26^{SmoM2/SmoM2}$ Mx1-Cre^{-} (referred to as Cre^{-}), and induced SmoW539L/EYFP expression by injecting polyI:polyC. As shown in Figure 5B, YFP expression was detected by flow cytometry in LSKs of Cre^{+} mice after polyI:polyC administration. At the mRNA level, both *Smo* and *Ptch1* expression were significantly increased in the LSKs of Cre^{+} compared to Cre^{-} mice (Figure 5C), demonstrating the over-expression of *Smo* and activation of the Hh pathway. Also, it was found that elements of the Hh pathway (*Ptch*, *Gli1*) were aberrantly expressed in differentiated hematopoietic cells (thymic $CD4^{+}8^{+}$ cells) in which the pathway is normally silent (not shown). An additional indication of non physiological Hh activation was that the majority of Cre^{+} mice died later in life due to the development of tumors (primarily medulloblastomas and skin tumors, data not shown). However, Cre^{+} mice did not show any increase in absolute numbers of LSKs (Figure 5D), illustrating that the hyper-activation of Hh signaling was unable to result in expansion of the LSK compartment. Moreover, LSKs of Cre^{+} mice did not show any signs of enhanced (or suppressed) apoptosis or aberrant cell cycle profiles as shown by AnnexinV or Ki67 staining. Finally, no major defects in lymphopoiesis were detected (Figure 5E).

To further examine whether hyper-activation of Hh influences the ability of LSKs to differentiate, CFU methylcellulose-based assays were performed. We observed that Smo-mutant LSKs gave rise to similar number of colonies as controls. Moreover, replating of the colonies that originated from the Hh hyper-active LSKs also generated identical number of colonies as controls (Figure 5F).

To directly test the reconstitution ability of Hh hyper-active HSCs, we transplanted bone marrow cells from polyI:polyC-treated Cre^{+} mice ($Ly5.2^{+}$) mixed with $Ly5.1^{+}$ competing bone marrow into lethally irradiated $Ly5.1^{+}$ hosts. Bone marrow cells used for competitive

BMT were confirmed to express YFP as shown in Figure 5G. The chimerism in the peripheral blood 4-12 weeks after transplant was similar between Cre^- and Cre^+ groups (Figure 5H), indicating that over-expression of an activated Smo does not provide a competitive advantage to HSCs. These observations argued against the suggestion that Hh hyper-activation affects HSC expansion and *in vivo* fitness.

Hedgehog signaling is dispensable for the induction or maintenance of lymphoblastic leukemia

Our studies so far do not support a role for Hh signaling in physiological adult HSC function. Recent reports (Dierks et al., 2008; Zhao et al., 2009b) suggested that Hh could affect $BCR\text{-}ABL^+$ leukemia stem cell function and disease progression. These conclusions led us to study the potential role of Hh in the induction and maintenance of a different leukemia type, acute lymphoblastic leukemia (ALL). It has been shown that the majority of primary cases of T cell ALL (T-ALL) carry activating *NOTCH1* mutated alleles (Weng et al., 2004). The study of these tumors have revealed that the Hh pathway was active in T-ALL, since both *GLI1* and *PTCH1* were highly expressed in many Notch1 mutant T-ALL cell lines (unpublished data) and *PTCH1* were expressed in majority (38 out of 48) of primary T-ALL cases (Figure 6A). Thus, to test whether Hh signaling is required for the transformation of hematopoietic progenitors in T-ALL, a well characterized transplantation model (Vilimas et al., 2007) was used. Lineage-depleted bone marrow from either polyI:polyC injected $Smo^{F/F}Mx1\text{-}Cre^-$ or $Smo^{F/F}Mx1\text{-}Cre^+$ mice was isolated, and infected with a bicistronic retroviral vector expressing the intracellular domain of Notch1 (Notch-IC), and green fluorescent protein (GFP). As expected, peripheral blood analysis of recipients of Notch-IC infected control cells revealed that the majority of cells were GFP^+, a marker of Notch-IC expression, and most of which were $CD4^+8^+$, a manifestation of T-ALL (Figure 6B). Examination of recipients that had received Notch-IC infected *Smo*-deficient cells showed similar percentages of $GFP^+CD4^+CD8^+$ cells in the peripheral blood as well as kinetics of leukemogenesis (Figures 6B and 6C). We further confirmed that T-ALLs developed from *Smo*-deficient cells deleted *Smo*-floxed and harbored *Smo*Δ allele (Figure 6D), which demonstrated that Hh signaling is dispensable for T-ALL generation.

To address the ability of *Smo*-deficient tumors to regenerate, we performed secondary BMTs using Notch1-transformed (GFP^+) leukemic cells. No significant differences in the induction of secondary leukemia were noted, in opposition to a role for Hh signaling in the regulation of putative “leukemia-initiating” cells. (Figure 6E). To further demonstrate that Hh signaling is not essential for the maintenance of the transformed cells, several T-ALL lines (*PTCH1* and *GLI1* positive) were incubated with the potent and specific Smo inhibitor, cyclopamine. In agreement with our *in vivo* data, the presence of cyclopamine did not affect the leukemic cell line survival or the rate of proliferation (not shown). Taken together, these results indicate that Hh activation is dispensable for the transformation of hematopoietic progenitors and the progression of Notch-induced T-ALL.

Discussion

In this study, we demonstrate that inducible genetic deletion of the only non-redundant element of the Hh cascade, *Smo*, was unable to affect adult hematopoiesis, specifically at the level of the HSC. *Smo*-deficient HSCs display normal abilities to differentiate, self-renew and regenerate the immune system. In agreement with these phenotypic and functional studies, gene expression profiling analysis demonstrated that HSC-specific gene expression “signature” was preserved in *Smo*-deficient HSCs. Interestingly, the simultaneous ablation of both the Hh and Notch pathways was also unable to affect HSC differentiation and function. Moreover, using a gain-of-function model, we found that Hh hyper-activation did not lead to expansion of the HSC compartment. Finally, *Smo* deletion had no effect on the

ability of the Notch1 oncogene to transform early hematopoietic stem cells and progenitors and to induce T-ALL. All of these findings are of unique importance as they directly question the current consensus on the role of Hh signaling in adult hematopoiesis.

Our studies are in contrast to a recent report by Zhao et al. that also used a conditional *Smo* allele deletion (Zhao et al., 2009). One possible explanation for this discrepancy is the utilization of a distinct mode of deletion. Zhao et al. use the Vav-cre deleter strain that appears to be hematopoietic specific; however it is able to delete the *Smo* alleles in both adult and fetal hematopoiesis. Indeed it was previously shown that the Vav promoter can efficiently drive Cre-recombinase expression in e.d. 13.5 fetal liver HSC (Stadtfeld and Graf, 2005). It is possible that the reported HSC defects in the Vav-creSmo$^{F/F}$ model reflect Hh signaling functioning not in adult but in fetal HSC function and hematopoiesis. Although future work is required to identify putative Hh roles in fetal hematopoiesis, our data clearly demonstrate that Hh signaling is dispensable for adult HSC function.

Our observations suggest that Hh hypeactivation is unable to expand bone marrow stem cells and progenitors, a conclusion that is inconsistent with a report by Trowbridge and colleagues (Trowbridge et al., 2006). A potential reason for this discrepancy could be the utilization of different animal models. In the germline *Ptch*$^{+/-}$ model both HSC and/or the HSC “niche” could contribute to phenotype, whereas in the inducible SmoM2 model expression of the activated allele is largely restricted to the hematopoietic compartment. Moreover, putative differences on the effect of Hh hyper-activation on HSC/LSK cell cycle progression could be explained by the differential analysis performed. Indeed, Trownbridge et al. study the cell cycle status of *Ptch*$^{+/-}$ LSKs after transplantation while we study steady-state LSKs shortly after SmoM2 activation. Finally, it is important to note that the *gain-of-function* of a pathway effector (SmoM2) may well engender a different hematopoietic phenotype than the *loss-of-function* of a negative regulator (*Ptch*) that may have effects on other signaling pathways that could influence hematopoiesis.

Our analyses also failed to demonstrate a significant effect of *Smo* deletion on T cell differentiation, as proposed previously by several studies including one from our own laboratory, in which *Smo* was deleted in early T cell progenitors using the Lck-cre strain (Crompton et al., 2007; El Andaloussi et al., 2006). This discrepancy could be due to the differential mode of Cre-recombinase activation and pathway deletion. Indeed, Lck-cre is only active in early thymocytes and it ensures deletion in both fetal and adult thymus, suggesting again that fetal and adult hematopoiesis has unique and distinct Hh signaling requirements. Another reason for the phenotypic discrepancy could be that the Lck-cre-driven thymic effect was only partial. It is thus possible that our current studies, aimed mainly at HSC function, were not quantitative enough to reveal slight alterations of early T cell differentiation. It is more difficult to explain the differential effects on thymic size and progression of T cell maturation of the Mx1-cre-mediated Smo deletion reported by El Andaloussi et al. The timing of the analysis could provide a potential explanation. In this study thymi were analyzed at week 4 and 16 post-deletion, while El Andaloussi et al analyzed mice only one week after the last polyI:polyC injection. It is thus possible that the outcome of these studies was dictated by the timing of the analysis, especially as the thymus is a tissue with enormous regenerative capacity. Additional explanations could also include background differences as the mice studied here (and by Hoffman et al.) are C57Bl/6 Smo$^{F/F}$ while El Andaloussi et al. utilized 129X1/SvJ Smo$^{F/null}$ animals. It is thus possible that the effects on T cell development were influenced by the genetic background of the analyzed mice. Future studies that directly compare T cell development in the different Hh deficient strains are necessary to address the extent of Hh function in T cell development.

Is there any role for Hh in hematopoiesis? The strongest evidence supporting a pivotal role of Hh signaling in hematopoiesis came from the study of zebrafish embryo Hh mutants. (Gering and Patient, 2005). As zebrafish hematopoiesis shares striking similarities to the mammalian fetal blood development, it is possible that the Hh pathway, as previously suggested, plays a more prominent role during fetal blood development. Moreover, it is possible that Hh function is masked by the synergistic function of other signaling pathways. Indeed, pathways such as Notch and Wnt, which have been previously shown to be capable of interacting with Hh (Hallahan et al., 2004; Mak et al., 2006; Yang and Niswander, 1995; Yokota et al., 2004), could collaborate with each other to ensure self-renewal and specify differentiation (Duncan et al., 2005). In this report, we showed that deletion of both RBPJ and Smo did not affect HSC function, suggesting Notch and Hh signaling do not play synergistic roles. However, we cannot exclude potential redundancy with other signaling cues.

Two recent reports (Dierks et al., 2008; Zhao et al., 2009) have identified Smo as a drug target for the targeting of BCR-ABL^{+} human leukemic stem cells, introducing the notion that the Hedgehog pathway could be important for malignant hematopoiesis and the maintenance of leukemia. In the light of these seminal findings, our results are of further importance as they prove that pharmacological targeting of Hedgehog in leukemia is feasible as physiological HSC function and progression of hematopoiesis remains unaffected. They also suggest that not all blood malignancies can be treated using similar therapeutic protocols as the progression of T-ALL is not affected by the silencing of Hedgehog function.

Materials and Methods

Animals

Smo$^{F/F}$ mice (Long et al., 2001) were a gift of Dr. A. McMahon (Harvard University, Boston). Genotyping of Smo$^{F/F}$ (Long et al., 2001; Zhang et al., 2001) and RBPJ$^{F/F}$ mice (Han et al., 2002; Tanigaki et al., 2004) was performed as previously reported. Smo$^{F/F}$Mx1-Cre animals were injected with 20μg polyI:polyC per gram of body weight for a total of 3 injections. The injections were initiated 14 days after birth and done every two days. Animals were analyzed 4-6 weeks after the last injection unless indicated otherwise. All animal experiments were done in accordance to the guidelines of the NYU School of Medicine. Gli1^{lacZ} mice were a gift of Dr. A. Joyner (Memorial Sloan Kettering Cancer Center, New York). ROSA26^{SmoM2} mice (Jeong et al., 2004) were purchased from Jackson laboratory. For 5-FU experiments, 150 μg of 5-FU per gram of body weight were intra-peritoneally injected every week.

Antibodies and FACS analysis

Antibody staining and FACS analysis was performed as previously described (Aifantis et al., 1999). All antibodies were purchased from BD-Pharmingen or e-Bioscience. We used the following antibodies: c-kit (2B8), Sca-1 (D7), Mac-1 (M1/70), Gr-1 (RB6-8C5), NK1.1 (PK136), TER-119, CD3 (145-2C11), CD19 (1D3), IL7Rα(A7R34), CD34 (RAM34), FcγII/III (2.4G2), Flk-2/Flt-3 (A2F10.1), CD4 (RM4-5), CD4 (H129.19), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD150 (9D1), CD48 (HM481), Ki67, AnnexinV, 7-AAD. Bone marrow lineage antibody cocktail includes: Mac-1, Gr-1, NK1.1, TER-119, CD3, CD19. For Ki67 and DAPI staining, briefly, the cells were first treated with Fix and Perm reagents according to manufacturer's instruction (Invitrogen), stained with Ki67 for 20 minutes at room temperature, then washed and resuspended in PBS with 5μg/ml RNaseA and 2μg/ml DAPI.

RT-PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and cDNA was synthesized using the SuperScript First-Strand Kit (Invitrogen). Quantitative PCR was performed using iQ SYBR Green Supermix and an iCycler (Bio-Rad) using the primer sequences (Tm=60°C used for all primers) provided in supplemental table 1. T-ALL patient samples were provided by collaborating institutions in the United States (St. Jude Children's Research Hospital, Memphis, TN) and Canada (Hospital for Sick Children, Toronto, Canada) (Thompson et al., 2007).

Methylcellulose assay

LSK cells were flow-purified from polyI:polyC injected mice. LSK cells were plated in duplicate (500 LSK/35mm dish) into cytokine-supplemented methylcellulose medium (MethoCult 3434, Stem Cell Technologies), and the number and morphology of colonies were scored 7 days later. For secondary plating, cell colonies were pooled from the first plating, and 4,000 cells were plated in duplicate.

Bone marrow transplantation

5×10^5 bone marrow cells ($Ly5.2^+$) or 500 LSKs ($Ly5.2^+$) were transplanted by retro-orbital i.v. injections into lethally irradiated (960 cGy) BL6SJL ($Ly5.1^+$) recipient mice in competition with 5×10^5 B6SJL ($Ly5.1^+$) bone marrow cells. Peripheral blood of recipient mice was collected at 4, 8, and 12 weeks after transplant. For secondary transplanst, recipient mice were sacrificed 16 weeks after primary transplant. $Ly5.2^+$ bone marrow cells were flow-purified and 5×10^5 cells were transplanted by retro-orbital i.v. injections into lethally irradiated (960 cGy) BL6SJL ($Ly5.1^+$) recipient mice in competition with 5×10^5 B6SJL ($Ly5.1^+$) bone marrow cells.

Microarray analysis

A group of four mice was pooled for each condition. Microarray analysis was performed as previously described (Thompson et al., 2008). Briefly, freshly isolated cells were sorted by surface marker expression, and total RNA was extracted using the RNeasy kit (QIAGEN, CA). In order to generate sufficient sample quantities for oligonucleotide gene chip hybridization experiments, we used the GeneChip Two-Cycle cDNA Synthesis Kit (Affymetrix, San Jose, CA) for cRNA amplification and labeling. The amplified cRNA was labeled and hybridized to the MOE430 Plus 2 oligonucleotide arrays (Affymetrix). The Affymetrix gene expression profiling data was normalized using the previously published Robust Multi-array Average (RMA) algorithm using the GeneSpring 7 software (Agilent, Palo Alto, CA). The gene expression intensity presentation was generated with MeV software (http://www.tm4.org). Microarray data were deposited under GEO database with the accession number GSE15194.

Retroviral infection of lineage-negative bone marrow cells

Bone marrow cells were enriched for lineage-negative cells using EasySep kit (StemCell Technology), and cultured in OPTI-MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml of SCF and Flt3l, 10 ng/ml of IL6 and IL7. For retroviral production, phoenix cells were transfected with pMigNotch-IC by calcium phosphate method. Virus supernatant was collected 48 hr post transfection and used directly for spin infection of lineage-depleted bone marrow cells at 2500 rpm for 90 minutes. Forty-eight hours after infection, 1×10^5 lineage-negative GFP-positive cells were i.v. injected into one lethally irradiated (960 cGy) C57BL/6J host mouse.

Statistical analysis

The means of each data set were analyzed using the Student's t test, with a two-tailed distribution and assuming equal sample variance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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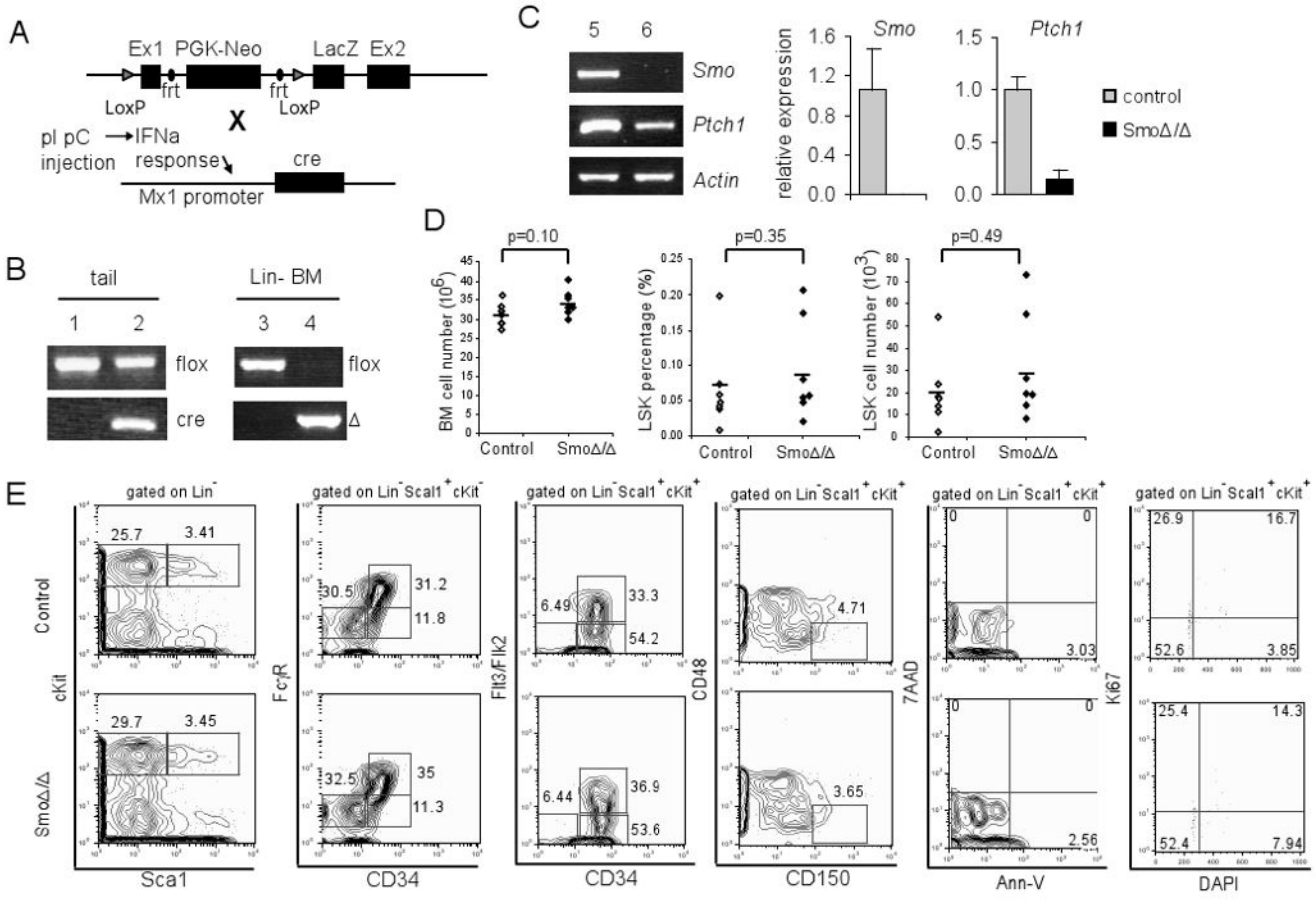

Figure 1. Phenotypically normal HSCs and progenitors in *Smo*-deficient mice

(A) Schematic representation of *Smo*-floxed allele (upper lane) and Mx1-Cre allele (lower lane). (B) PCR of genomic DNA extracted from mouse tails or lineage negative bone marrow cells to detect the Smo floxed, deleted (Δ), or Cre allele. Lane 1, 3: $Smo^{F/F}MxCre^{-}$; Lane 2, 4: $Smo^{F/F}MxCre^{+}$. (C) RT-PCR and quantification of *Smo* and *Ptch1* mRNA in lineage negative bone marrow cells from polyI:polyC injected $Smo^{F/F}MxCre^{-}$ (lane 5) and $Smo^{F/F}MxCre^{+}$ (lane 6) mice. The expression levels were normalized against *β-actin*. (D) Total number of bone marrow cells and LSK cells in control and SmoΔ/Δ mice. Each diamond represents a single mouse, and the bar indicates the average numbers. (E) FACS plots of bone marrow from control and SmoΔ/Δ mice. Representative plots (from at least 20 individual experiments) are shown.

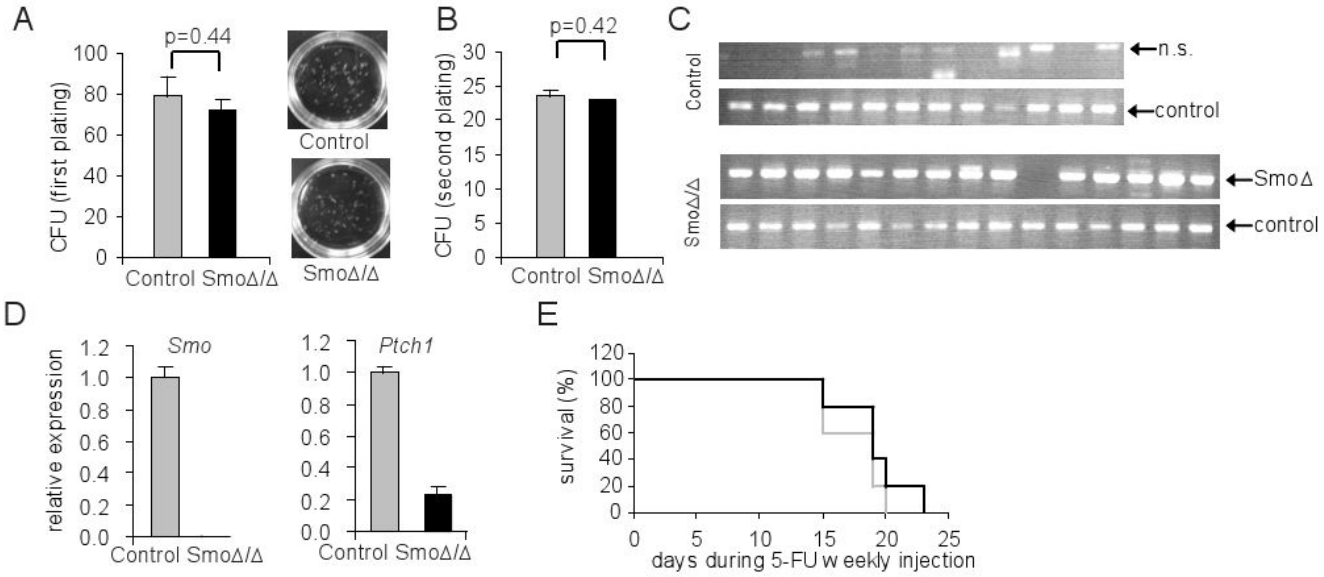

Figure 2. Physiological differentiation of *Smo*-deficient progenitors

(A) Number of colonies was scored on day 7 of methylcellulose assay, and images of plates are shown on right panel. (B) Number of colonies was scored on day 7 after re-plating cells from A. (C) PCR on genomic DNA of colonies formed from A. The Smo Δ allele and a loading control genomic allele are shown. (D) Quantitative RT-PCR of *Smo* and *Ptch1* on colonies formed from A. The expression levels were normalized against *β-actin*. (E) Survival curve of control (grey) or SmoΔ/Δ (black) mice after weekly 5-FU injection (n=5).

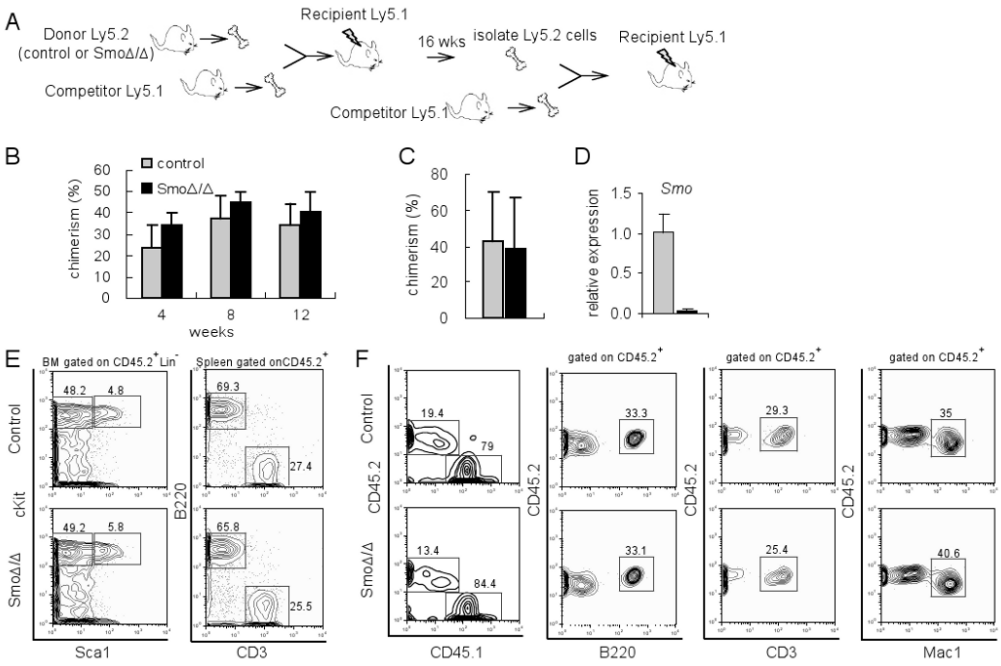

Figure 3. Physiological competitive ability of *Smo*-deficient hematopoietic progenitors

(A) Scheme of primary and secondary bone marrow transplantation (BMT). (B) Percentage of chimerism in peripheral blood of recipient mice at different time points after primary competition BMT. Donor cells were total bone marrow cells. Mean ± S.D. are shown (n=8). (C) Chimerism of peripheral blood of recipient mice 14 weeks after primary competition BMT. Donor cells were flow-purified LSK cells. Mean ± S.D. are shown (n=4). (D) Quantitative RT-PCR of *Smo* in flow-purified $Ly5.2^{+}Lin^{-}$ bone marrow of recipients 16 weeks after BMT. Grey: control; Black: SmoΔ/Δ. The expression levels were normalized against *β-actin*. (E) Representative FACS plot of bone marrow and spleen of primary recipient mice 16 weeks after BMT. (F) Representative FACS plot of peripheral blood in the recipient (n=4 for each genotype) 12 weeks after secondary competition BMT.

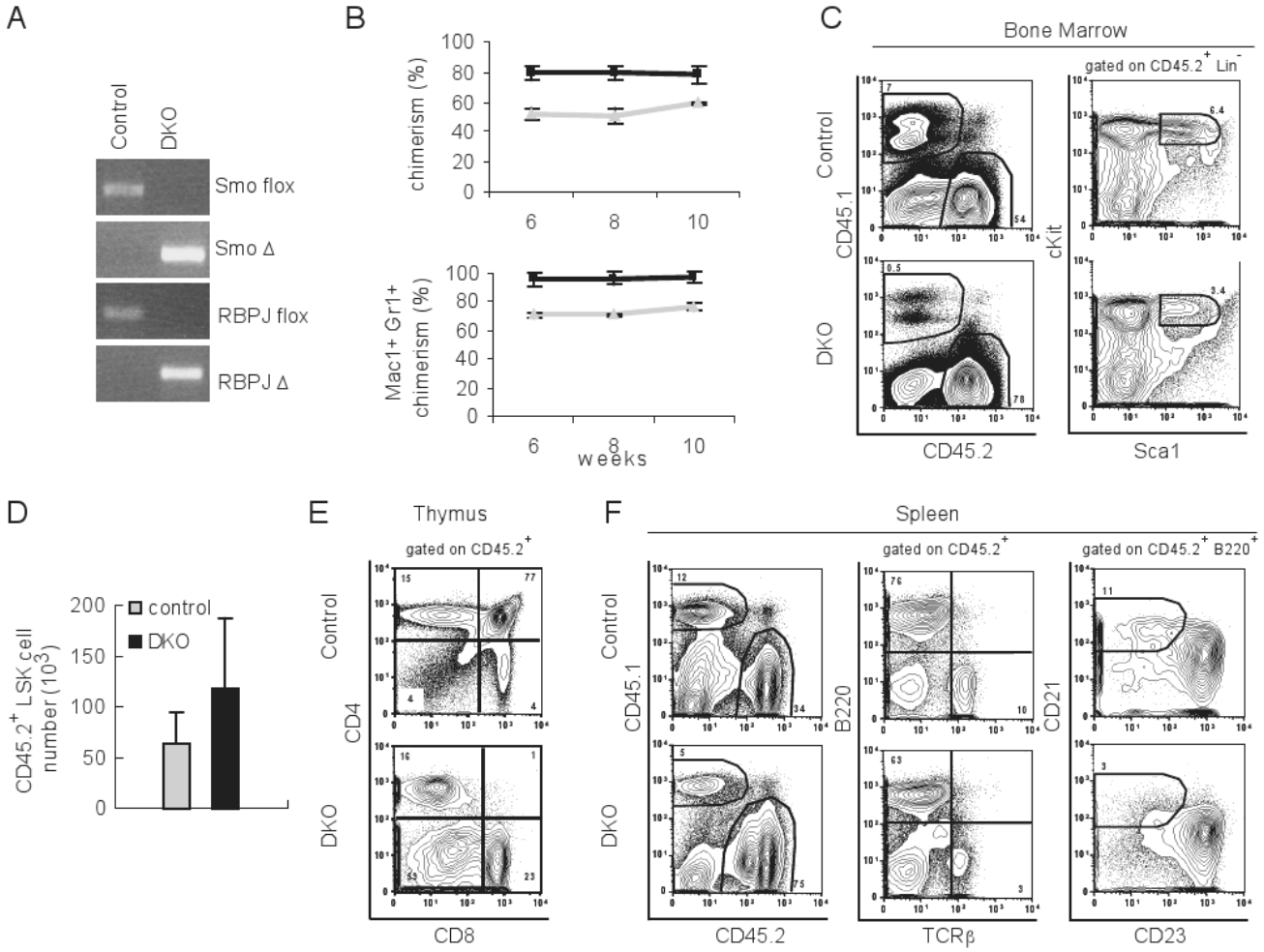

Figure 4. Physiological competitive ability of *RBPJ*- and *Smo*- double deficient progenitors

(A) PCR of genomic DNA extracted from bone marrow cells of polyI:polyC injected $RBPJ^{F/F}Smo^{F/F}Mx1\text{-}Cre^{-}$ mice (control) and $RBPJ^{F/F}Smo^{F/F}Mx1\text{-}Cre^{+}$ mice (DKO). Smo and RBPJ floxed and deleted (Δ) alleles were detected. (B) Chimerism of total peripheral blood (upper panel) or $Mac1^{+}Gr1^{+}$ cells in peripheral blood (lower panel) of recipient mice at different time points after primary competition BMT. Donor cells were mixed of 1:2 ratios of $Ly5.1^{+}$ cells and $Ly5.2^{+}$ cells. $Ly5.2^{+}$ cells were either from control (grey) or DKO mice (black). Mean ± S.D. are shown (n=2 for control, n=6 for DKO). (C) Representative FACS plot of bone marrow in the recipient mice 12 weeks after competitive BMT. (D) Number of donor derived LSK cells in bone marrow 12 weeks after BMT. Mean ± S.D. are shown (n=10 for control, n=4 for DKO). (E) Representative FACS plot of thymus in the recipient at 12 weeks after competition BMT. (F) Representative FACS plot of spleen in the recipient 12 weeks after competitive BMT.

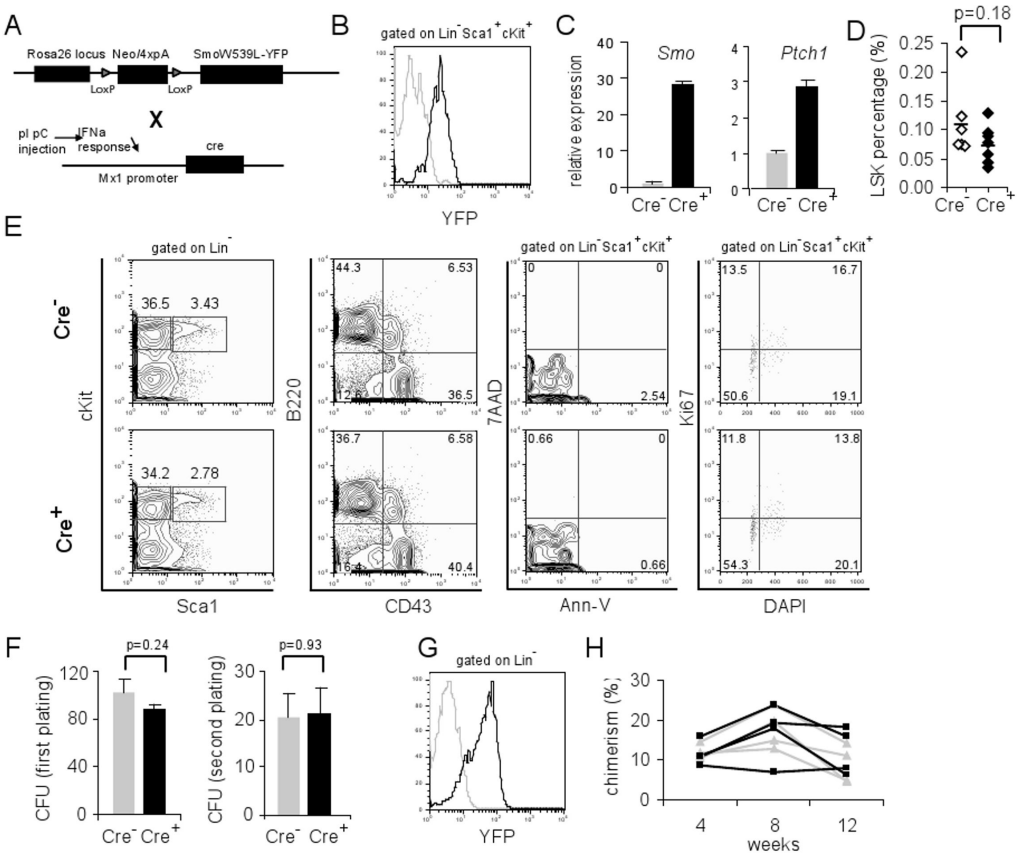

Figure 5. Hyper-activation of the Hh pathway does not expand HSC compartment

(A) Schematic representation of $R26^{SmoM2}$ locus (upper panel) and Mx1-Cre locus (lower panel). (B) Histogram of YFP gated on LSKs. Grey line: $Rosa26^{SmoM2/SmoM2}$Mx1-Cre^{-}. Black line: $R26^{SmoM2/SmoM2}$Mx1-Cre^{+}. (C) Quantative RT-PCR of *Smo* and *Ptch1* in LSKs. The expression levels were normalized against *β-actin*. (D) Frequency of LSK cells. Each diamond represents a single mouse, and the bar indicates the average number. (E) Representative FACS plots of bone marrow from $R26^{SmoM2/SmoM2}$Mx1-Cre^{-} and $R26^{SmoM2/SmoM2}$Mx1-Cre^{+} mice. (F) Number of colonies was scored on first and secondary plating of methylcellulose assay. (G) Histogram of YFP gated on lineage negative cells of bone marrow, which were used for BMT in H. Grey line: $Rosa26^{SmoM2/SmoM2}$Mx1-Cre^{-}. Black line: $R26^{SmoM2/SmoM2}$Mx1-Cre^{+}. (H) Percentage of peripheral blood chimerism in recipient mice after competitive BMT of $R26^{SmoM2/SmoM2}$Mx1-Cre^{-} (grey) and $R26^{SmoM2/SmoM2}$Mx1-Cre^{+} (black) at different time points. One line represents one mouse (n=4).

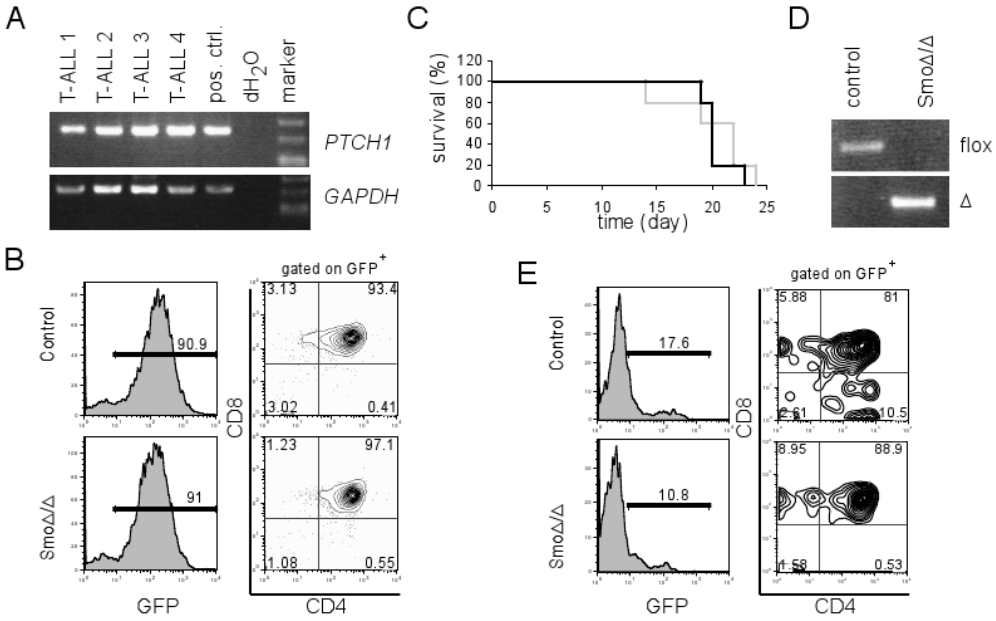

Figure 6. Leukemia (T-ALL) induction and maintenance is not altered by *Smo* deficiency

(A) RT-PCR of *PTCH1* in primary T-ALL samples. GAPDH served as a loading control. (B) Representative FACS plots for CD4 and CD8 staining of peripheral blood from the recipients 2 weeks following transplantation with Notch-IC infected control or SmoΔ/Δ lineage-negative bone marrow cells. Notch-IC infected cells were identified by gating on GFP^+ cells. (C) Survival curve of host mice transplanted with Notch-IC infected control (black) or SmoΔ/Δ lineage-negative bone marrow cells (grey) (n=5). (D) PCR of Smo-floxed and Δ alleles on genomic DNA purified from $GFP^+CD4^+CD8^+$ peripheral blood of host mice in B. (E) Representative FACS plots for CD4 and CD8 staining of peripheral blood from the recipients (n=5) 3 weeks after secondary BMT. 5×10^6 GFP^+ bone marrow cells from primary recipients were used for secondary BMT.