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BMP signaling is required for postnatal murine hematopoietic stem cell self-renewal

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Abstract

Life-long production of blood from hematopoietic stem cells (HSCs) is a process of strict modulation. Intrinsic and extrinsic signals govern fate options like self-renewal – a cardinal feature of HSCs. Bone morphogenetic proteins (BMP) have an established role in embryonic hematopoiesis, but less is known about its functions in adulthood. Previously, SMAD-mediated BMP signaling has been proven dispensable for HSCs. However, the BMP Type-II receptor (BMPR-II) is highly expressed in HSCs, leaving the possibility that BMPs function via alternative pathways. Here, we establish that BMP signaling is required for self-renewal of adult HSCs. Through conditional knockout we show that BMPR-II deficient HSCs have impaired self-renewal and regenerative capacity. BMPR-II deficient cells have reduced p38 activation, implying that non-SMAD pathways operate downstream of BMPs in HSCs. Indeed, a majority of primitive hematopoietic cells do not engage in SMAD-mediated responses downstream of BMPs in vivo. Furthermore, deficiency of BMPR-II results in increased expression of TJP1, a known regulator of self-renewal in other stem cells, and knockdown of TJP1 in primitive hematopoietic cells partly rescues the BMPR-II null phenotype. This suggests TJP1 may be a universal stem cell regulator. In conclusion, BMP signaling, in part mediated through TJP1, is required endogenously by adult HSCs to maintain self-renewal capacity and proper resilience of the hematopoietic system during regeneration.
Introduction

Hematopoietic stem cells (HSCs) have dual capacity to self-renew and give rise to differentiating progeny\textsuperscript{1-2}. Self-renewal pertains to the ability of HSCs to duplicate without losing developmental potential. Maintenance of the stem cell pool is dependent on self-renewal and loss thereof leads to erosion of regenerative capacity and hematopoietic failure. To ensure homeostasis, HSCs are tightly regulated by internal factors and external signaling cues from the BM niche\textsuperscript{3}. Although many regulatory mechanisms have been identified, deeper understanding of the self-renewal machinery is required to fully utilize the therapeutic potential of HSCs.

Bone morphogenetic proteins (BMPs) belong to the TGF-\(\beta\) superfamily of secreted cytokines, which during embryogenesis regulate a wide variety of biological processes\textsuperscript{4-7}. Mechanistically, BMPs signal through cell surface receptors, which activate receptor-regulated SMAD transcription factors (R-SMADs) through phosphorylation\textsuperscript{8}. Phosphorylated R-SMADs form complexes with SMAD4 resulting in nuclear accumulation of activated complexes, which together with cofactors regulate target gene transcription\textsuperscript{8}. Two classes of receptors have been identified; type-I and type-II. BMPs bind to and signal via the BMP type-II receptor (BMPR-II), in association with any type-I receptor (ALK2, ALK3, or ALK6)\textsuperscript{8}.

The importance of BMP signaling during development is well established and reflected in early embryonic lethality of mice with targeted deletions of components of the pathway\textsuperscript{9-12}. Similarly, deletion of \textit{SMAD1} and \textit{SMAD5} results in embryonic lethality\textsuperscript{13-16}. Beyond development, the TGF-\(\beta\) superfamily
regulates tissue homeostasis and adult regeneration of a variety of organ
systems. Several lines of evidence suggest that BMPs play a role in adult HSC
regulation, but conclusive evidence for direct BMP-rerequirement by HSCs is still
lacking. For instance, ALK3-mediated signaling is required by the HSC
osteoblastic niche, with loss of ALK3 leading to increased HSC numbers\(^1\). By
contrast, decreased levels of BMP4 in the BM results in reduced HSC numbers, as
shown in a hypomorphic BMP4 mutant mouse model\(^2\). Additionally, BMP4
maintains cord blood-derived human hematopoietic stem and progenitor cells
(HSPCs) during \textit{ex vivo} culture, by acting as a survival factor\(^3\). Recently, Khurana
\textit{et al.} showed that BMP4 exposure \textit{in vitro} maintains the expression of ITGA4 in
murine HSCs, thereby preventing culture-induced loss of homing capacity\(^4\).
However, \textit{SMAD1} and \textit{SMAD5} are dispensable for adult HSCs, leading to the
conclusion that BMP signaling is not endogenously required by adult HSCs\(^5,6\).
Interestingly, \textit{BMPR-II} is reportedly highly expressed in adult HSCs, suggesting
that BMPs may signal via alternative circuitries in HSCs\(^7\). Indeed, several
pathways can be activated by BMPs, including components of the MAPK
pathway, such as p38 and JNK\(^8,9\). A role for p38 has been suggested in
maintenance of ITGA4 expression in HSCs \textit{in vitro}, but a conclusive role for BMPs
in the regulation of HSCs \textit{in vivo} has never been shown\(^10\). Therefore, to
investigate the complete role of BMP signaling in HSCs \textit{in vivo}, we conditionally
deleted \textit{BMPR-II} in hematopoietic cells using the Cre/loxP system. We report
here that BMPR-II is essential for self-renewal of HSCs with mutants displaying
significantly reduced regenerative capacity upon BM transplantation. Steady
state hematopoiesis is normal in mice deficient of BMPR-II and the
differentiation capacity upon transplantation is likewise unaltered, indicating a
specific role for BMPR-II in HSC self-renewal. In addition, we map the transcriptional activity of SMAD-mediated signaling in hematopoietic cells by using a BRE-GFP reporter mouse\textsuperscript{26}, which suggests a failure to engage SMAD-dependent transcriptional response upon BMP exposure. Furthermore, our findings indicate that loss of BMPR-II results in up-regulation of tight junction protein 1 (TJP1) and that knockdown of TJP1 partly rescues the BMPR-II knockout phenotype. TJP1 is a protein previously implicated in self-renewal regulation of several types of stem cells, including both embryonic and adult stem cells. Together, our findings show that BMP signaling, via BMPR-II, is endogenously required by adult HSCs to maintain self-renewal capacity \textit{in vivo}. 
Methods

Mice

Mice on C57Bl/6 background with loxP flanking one allele of exon 4-5 of the BMPR-II gene (MMRRC, University of North Carolina, Chapel Hill)\textsuperscript{27} were bred to homozygosity and mated with Vav-Cre\textsuperscript{28} transgenic mice to generate conditional Vav-Cre;BMPR-II\textsuperscript{fl/fl} mice. Detection of Cre, floxed (fl), wild-type (WT), and excised alleles was done by PCR as previously described\textsuperscript{22,27}. Mice were housed and bred in ventilated cages in the BMC animal facility. All experiments involving animals were approved by the regional Animal Ethical Committee in Lund.

Transplantation assays

For competitive transplantation assays, 0.2x10\textsuperscript{6} unfractionated BM cells from BMPR-II\textsuperscript{fl/fl}, Vav-Cre, BMPR-II\textsuperscript{fl/+}, Vav-Cre, and WT littermates (BMPR-II\textsuperscript{fl/fl} or BMPR-II\textsuperscript{fl/+}) (CD45.2) were transplanted with 0.2x10\textsuperscript{6} congenic CD45.1 BM cells by tail vein injection to lethally irradiated (900cGy) congenic CD45.1/2 recipients (3 recipients/donor). Donor, competitor, and recipient cells were monitored by peripheral blood (PB) samplings at several time points at 4-16 weeks. Sixteen weeks post-transplantation mice were killed, BM was analyzed and 2x10\textsuperscript{6} cells were transplanted to secondary recipients, monitored as above. After another 16 weeks secondary mice were killed and tertiary transplantations were performed using 20x10\textsuperscript{6} BM cells. Tertiary recipients were monitored as above for 16 weeks, after which final analyses of BM and PB were performed. For transplantations using purified HSCs, 10 LSK/CD48\textsuperscript{-}/CD150\textsuperscript{+} cells from BMPR-II\textsuperscript{fl/fl}, Vav-Cre or WT littermates were transplanted together with 0.2x10\textsuperscript{6} whole
BM support cells (CD45.1/2) to CD45.1 recipients. Reconstitution was monitored as above and BM analyzed at 16 weeks. Homing assays were performed by transplantation of 15x10^6 unfractionated BM cells to congenic CD45.1 recipients; BM analysis was done 20 hours post-transplantation. For competitive homing 10x10^6 BM cells from donors were transplanted with an equal number of WT competitor cells.

**Knockdown of TJP1**

For knockdown of TJP1, lentiviral plasmid pGFP-C-shLenti containing shRNA targeting TJP1 or scrambled shRNA (OriGene) was used to produce lentiviral particles at the Stem Cell Center Vector Core Facility (Lund University). C-kit-enriched BM cells (CD45.2; BMPR-II^{fl/fl};Vav-Cre or WT) were placed into virus-loaded plates at an MOI of 30-50 and incubated overnight (37°C, 5% CO2). Transduced cells were collected and transplanted into lethally irradiated CD45.1 recipient mice (2 recipients/donor). An aliquot of cells was cultured for flow cytometry analysis of transduction efficiency after 48 hours. BM of transplanted animals was analyzed at 16 weeks.

Additional information in supplementary methods.
Results

**BMPR-II is highly expressed in LT-HSCs**

To map the extent of **BMPR-II** expression in distinct populations of primitive adult hematopoietic cells, we performed qPCR analyses on sorted long-term HSCs (LT-HSCs; LSK-CD34-FLT3-), short-term HSCs (ST-HSCs; LSK-CD34+FLT3-), lymphoid-primed multipotent progenitors (LMPPs; LSK-CD34+FLT3+), as well as various progenitor populations\(^{29}\). Robust expression of **BMPR-II** was detected in all subsets, although LT-HSCs exhibited highest expression on average between tested populations (Figure 1A). Similarly, we examined expression of type-I receptors **ALK2**, **ALK3**, and **ALK6** in HSC populations (Figure 1B). In LT- and ST-HSCs, both **ALK2** and **ALK3** were expressed, but expression of both receptors was more abundant in LT-HSCs. In LMPPs, **ALK2** was the dominating receptor. **ALK6** was undetectable in all hematopoietic subsets tested.

**Normal steady state hematopoiesis despite reduced progenitor activity upon deletion of BMPR-II**

Given the robust expression of **BMPR-II** in LT-HSCs, we hypothesized that its deletion would blunt most signaling events initiated by BMPs in HSCs, allowing us to probe the full role of BMPs in adult hematopoiesis. We conditionally deleted **BMPR-II** in hematopoietic cells, employing the Cre/loxP system with the \textit{Vav-Cre} driver strain\(^{27,28}\). Efficient deletion of exon 4-5 of the **BMPR-II** gene in hematopoietic cells was confirmed by PCR analysis of individual colonies from BM, reaching 98.85% efficiency (n=160 alleles; Supplement 1A). Recombination at the **BMPR-II** locus resulted in efficient reduction of **BMPR-II** mRNA in purified
LT-HSCs (Supplement 1B-C). Vav-Cre mediated deletion in mice homozygous for floxed BMPR-II alleles (BMPR-IIfl/fl; Vav-Cre, hereafter referred to as BMPR-II/-) did not result in embryonic lethality although the Vav promoter is active from E10.5, indicating that BMPR-II signaling is not endogenously required in HSCs for normal development after E10.5. All PB parameters were normal in adult BMPR-II/- and BMPR-II+/ mice at steady state compared to WT littermates (Figure 2A-B). Similarly, B/T/myeloid lineage distribution and number of cells in the BM of mutant mice were unaltered compared to WT littermates (Figure 2C and data not shown). Megakaryocytic lineage distribution and progenitor populations were also unaltered (Supplement 2A-B). To further analyze HSPCs lacking BMPR-II, we performed flow cytometry analyses on BM from BMPR-II/-, BMPR-II+/+, and WT littermate mice. Interestingly, BMPR-II+/ mice had significantly fewer LSK cells in the BM as compared to WT mice (Figure 2D-E). Further analyses by SLAM markers did not reveal significant differences in more primitive subsets of LSK cells, such as LT-HSCs (Figure 2D-E). Similarly, when assessing HSC phenotypic aging by CD41 expression we saw no significant differences between WT and BMPR-II/- LT-HSCs (Supplement 2C). However, in agreement with the reduced number of LSK cells, the colony forming capacity of BM cells from BMPR-II/- mice was significantly reduced compared to that of WT littermates (Figure 2F, Supplement 2D), suggesting that primitive hematopoiesis might be altered in BMPR-II/- mice.
BMPR-II deficient HSCs exhibit reduced regenerative potential upon transplantation

To test the regenerative capacity of BMPR-II deficient HSCs, we transplanted unfractionated BM cells from *BMPR-II*−/−, *BMPR-II*+/−, and WT mice at a 1:1 ratio with congenic WT competitor cells to lethally irradiated recipients (Figure 3A). *BMPR-II*−/− BM cells exhibited significantly reduced reconstitution capacity in PB short term at 4 weeks (data not shown) and a similar, though non-significant, reduction in PB long term at 16 weeks post-transplantation (Figure 3A-C). Deficiency of BMPR-II did not affect lineage distribution, though a slight decrease in donor contribution to myeloid cells could be observed (Figure 3D). To further investigate the ability of *BMPR-II*−/− cells to contribute to primitive hematopoietic cells, we quantified the number of donor-derived LSK-SLAM cells in BM. Interestingly, *BMPR-II*−/− cells exhibited a significantly reduced contribution to the entire LSK compartment including all LSK-SLAM populations, including the LT-HSCs (LSK-CD150⁺CD48−) (Figure 3E-F).

Deletion of BMPR-II results in compromised self-renewal capacity and altered LT-HSC quality

To assay the self-renewal ability of BMPR-II deficient HSCs, secondary and tertiary BM transplantations were performed. We transplanted a fixed number of cells from primary recipients to lethally irradiated secondary recipients. Similarly, BM from secondary recipients was transplanted to lethally irradiated tertiary recipients. The overall donor contribution of *BMPR-II*−/− HSCs dropped dramatically upon secondary transplantation, as compared to WT cells, which exhibited stable reconstitution across consecutive transplantations (Figure 3G).
Upon tertiary transplantation, \textit{BMPR-II/-} cells dropped further, indicating a severely compromised ability to self-renew under stressed conditions (Figure 3G). \textit{BMPR-II+/} BM cells displayed sustained donor contribution in secondary recipients, but appeared to drop upon tertiary transplantation, although not significantly so (Figure 3G). Furthermore, quantification of LT-HSCs revealed decreasing numbers of \textit{BMPR-II/-} derived cells across consecutive transplantations and in tertiary recipients the contribution to LT-HSCs was essentially nonexistent (Figure 3H). These data show that BMPR-II-mediated signaling is essential for self-renewal of LT-HSCs \textit{in vivo}.

In agreement with above \textit{in vivo} transplantation data is the \textit{in vitro} serial replating assay which shows a significant decrease in \textit{BMPR-II/-} colony number after three platings (Supplement 2E).

To verify that the observed defect in regenerative capacity was caused by a qualitative defect of HSCs, we transplanted ten sorted \textit{BMPR-II/-} or WT LT-HSCs in conjunction with congenic WT support BM cells (Figure 3I). In agreement with previous transplantations, overall donor contribution of \textit{BMPR-II/-} LT-HSCs was significantly reduced at 16 weeks post-transplantation in PB (Figure 3J) and the lineage distribution was unaffected (Figure 3K). Furthermore, the LSK compartment in BM was significantly reduced, as was the CD150\textsuperscript{-}CD48\textsuperscript{-} and CD150\textsuperscript{-}CD48\textsuperscript{+} subsets of LSK cells (Figure 3L). The LT-HSCs showed a similar reduction, though it did not reach significance (\(p=0.09\)) (Figure 3L).
Loss of BMPR-II causes transcriptional cell cycle perturbation but has little or no effect on cell cycle and apoptosis in LT-HSCs

To investigate the biological properties of BMPR-II/- primitive hematopoietic cells, we analyzed apoptosis and cell cycle parameters of BM cells from BMPR-II/- and WT mice by flow cytometry. The fraction of apoptotic (Annexin V+) cells within LSK/LSK-SLAM populations did not differ between BMPR-II/- and WT BM (Figure 4A-B). Cell cycle distribution, analyzed using Ki67 and DAPI, was mostly unaltered in all hematopoietic populations tested between BMPR-II/- and controls (Figure 4C). We observed a slight decrease in quiescent G0-phase LT-HSCs and a slight increase in LT-HSCs in G1-phase, though these differences did not reach significance (Figure 4D). Similar results were seen in other primitive hematopoietic populations, with a significant decrease of cells in G0 in the CD150-CD48- and CD150-CD48+ subsets of LSK cells (Supplement 3A-C). In contrast to the lack of significant cell cycle perturbation in LT-HSCs was the observed enrichment in gene sets pertaining to cell cycling (Supplement 4A). When the hematopoietic system was put under stress following in vivo treatment with 5-fluorouracil, the blood, BM, and spleen were mostly unaffected. Though white blood cells and splenic LT-HSCs were reduced, this was not significant (Supplement 5A-D). Furthermore, the proliferative capacity of BMPR-II/- c-kit+ BM cells in vitro was normal when assayed under serum-free conditions in the presence of SCF, IL-3, and IL-6 (Figure 4E).

Homing is unaffected by deletion of BMPR-II

As BMP signaling has been linked to HSC homing via maintenance of ITGA4 expression during ex vivo culture, we investigated if loss of BMPR-II resulted in a
homing defect20. We transplanted unfractionated $BMPR-II^{-/-}$ and WT BM cells, with or without competitor cells, to lethally irradiated recipients. Following 20 hours, BM was analyzed by flow cytometry. The donor contribution to Lin$^{-}$ Sca1$^{+}$CD150$^{+}$ cells as well as to the overall Lin$^{-}$ population was not significantly altered between $BMPR-II^{-/-}$ and WT cells (Figure 4F, Supplement 6A). Donor contribution following competitive transplantation was also not significantly altered (Supplement 6B-C). Likewise, the expression of ITGA4 (CD49d) was unaltered between $BMPR-II^{-/-}$ and control LT-HSCs, indicating that BMP signaling does not regulate ITGA4 expression in vivo (Supplement 6D).

Reduced phosphorylation of SMAD1 upon BMPR-II deletion

To investigate the SMAD signaling status of $BMPR-II^{-/-}$ hematopoietic cells, we performed western blots of purified c-kit$^{+}$ cells incubated with/without BMP4 in vitro. As expected, $BMPR-II^{-/-}$ cells exhibited significantly reduced phosphorylated SMAD1/5, both in the presence and absence of BMP4 stimulation (Figure 5A). WT cells exhibited robust levels of phosphorylated SMAD1/5, but the level was not further increased upon BMP4 exposure, suggesting already saturated levels. These data confirm that deletion of $BMPR-II$ translates into a functional reduction of SMAD signaling.

Limited SMAD-dependent transcriptional activity in hematopoietic populations

Although SMAD1/5-mediated BMP signaling is dispensable for HSC function, transcriptional activity of SMADs downstream of BMP has not been characterized in detail in hematopoietic cells. Using a BRE-GFP reporter mouse, a well-established model for gauging in vivo transcriptional activity of
SMAD1/5/8, cells responding transcriptionally to BMPs through SMADs were measured by GFP, allowing in vivo analysis. BRE-GFP BM cells displayed limited activation of the SMAD pathway, with the highest proportion of GFP* cells reaching only 2.79% on average (LSK CD150+CD48- population) (Figure 5B). To investigate whether hematopoietic cells could respond to BMP signaling via the SMAD pathway, BRE-GFP cells were stimulated in vitro for 16 hours with/without BMP4. No significant difference in GFP* cells was found in any BM population (Figure 5C).

Loss of BMPR-II results in a reduction of p38

As p38 has been implicated downstream of BMPs in hematopoietic cells, we evaluated the level of phosphorylated p38 in c-kit+ progenitor cells by western blot. Phospho-p38 was reduced in un-stimulated BMPR-II-/- cells, though it did not reach significance, and its level did not change following stimulation with BMP4 (Figure 5D). We could not detect robust increase of phospho-p38 in BMP4-stimulated WT cells. Instead, phospho-p38 was reduced following BMP4 stimulation in WT c-kit+ cells (Figure 5D). Additionally, the reduction of phospho-p38 in BMPR-II-/- cells may be a reflection of significantly reduced total p38 (Figure 5E). We found no significant differences in protein levels of other known signaling mediators such as phospho-Limk, phospho-Cofilin, or RhoA/B (Supplement 7A-B). To further investigate whether the reduction in LSK cell numbers in BMPR-II null mice is related to known downstream BMP signaling mediators such as the MAPK pathway, gene expression was evaluated in sorted LSK cells. No significant differences were found among the investigated genes (Supplement 7C). Finally, we assessed expression levels of BMP type-I and other
type-II receptors in sorted WT and \textit{BMPR-II}^{-/-} primitive hematopoietic cells (LSK CD48⁻) to determine whether \textit{BMPR-II} deletion leads to up- or down-regulation of other BMP receptors. We found no significant differences, despite a trend of increased Alk3 in the absence of BMPR-II (Supplement 7D-E).

\textit{Deficiency of BMPR-II results in up-regulation of TJP1 in LT-HSCs}

To further explore underlying mechanisms behind the \textit{BMPR-II}^{-/-} phenotype, we performed microarray analysis on highly purified LT-HSCs (LSK-CD150⁺CD48⁻CD9₃⁵) from adult mice. The analysis generated a number of differentially expressed genes (Supplement 8 and 4B) and enriched gene sets (Supplement 4A). Select genes, based on relevant known connections to stem cell function, hematopoiesis or BMP, were further validated. qPCR analyses confirmed a significant 2.4-fold up-regulation of \textit{TJP1} in \textit{BMPR-II}^{-/-} LT-HSCs (Figure 5F).

To further investigate whether the reduction in LSK cell numbers in \textit{BMPR-II} null mice is related to factors known to associate with TJP1 such as SRC and STAT3, gene expression was evaluated in sorted LSK cells. No significant differences were found among the investigated genes (Supplement 7C). We also found no significant differences in expression of Alpk or microRNAs 15a/23b/27a, which were other hits in the array (Supplement 7F-G).

\textit{TJP1 knockdown partly rescues the BMPR-II knockout phenotype}

To evaluate the contribution of \textit{TJP1} up-regulation to the observed \textit{BMPR-II}^{-/-} phenotype, \textit{TJP1} knockdown was performed using shRNA lentiviral vectors in ckit-enriched BM cells from \textit{BMPR-II}^{-/-} and WT mice. Transduced cells were transplanted to WT recipients. Using shRNA-C knockdown of \textit{TJP1} was achieved
to at least 0.51-fold level (compared to un-transduced cells) (Supplement 9A).

Average transduction efficiency at transplantation was 36 % and 33 % for
scrambled-shRNA transduced WT and BMPR-II−/− groups respectively; 21 % and
26 % for TJP1-shRNA transduced WT and BMPR-II−/− groups (Supplement 9B).

In transplanted mouse BM the donor LSK compartment showed a partial rescue,
as TJP1-shRNA transduced BMPR-II−/− cells no longer showed reduced
engraftment in comparison to Scrambled-shRNA transduced WT cells (Figure
6A). A trend of increased engraftment was seen among HSCs, although this did
not reach significance (Figure 6B). In hierarchically lower populations no similar
effect on engraftment was seen (Figure 6C-D), nor in PB (data not shown).
Discussion

A large body of work from a variety of model systems has established a critical role for BMP signaling during early development\textsuperscript{5-7}. Studies performed \textit{in vitro} indicate that BMP signaling continues to function in the regulation of HSCs beyond development\textsuperscript{19,20,36}. However, \textit{SMAD1} and \textit{SMAD5} are dispensable for adult HSC function in mice, leading to the conclusion that BMPs play a limited role, if any, in adult HSC regulation \textit{in vivo}\textsuperscript{21,22}. The SMAD circuitry is undoubtedly the best characterized pathway downstream of BMPs, but the lack of HSC phenotype in mice deficient of \textit{SMAD1} and \textit{SMAD5} does not automatically rule out a role for BMP signaling in adult HSCs, as non-SMAD pathways can also be activated by BMPs\textsuperscript{24,25}. The fact that \textit{BMPR-II} is highly expressed in LT-HSCs has left a gap in knowledge between the BMP circuitry and its function in adult HSCs \textit{in vivo}\textsuperscript{23}.

Here we aimed to elucidate the endogenous role of BMP signaling in adult murine HSCs, by conditional deletion of \textit{BMPR-II} specifically in hematopoietic cells. Unlike deletion of \textit{SMAD1} and \textit{SMAD5}, we report here that BMPR-II is essential for self-renewal of adult HSCs. It is likely that this non-SMAD signal in HSCs is mediated by BMPR-II associated with the BMP type-I receptor ALK2 or possibly ALK3, based on our transcriptional profiling of receptor expression in WT LT-HSCs and that we find no significant change in expression levels of other BMP receptors in primitive hematopoietic cells from \textit{BMPR-II}\textsuperscript{-/-} mice.

Additionally, there is limited knowledge about BMPR-II being able to activate downstream signaling pathways independently of type-I receptors. Despite the absence of significant differences in our measurements of above mentioned transcript levels, a trend of increased Alk3 seemed to be observed following
BMPR-II deletion. This will require further studies to fully decipher the relation between BMP receptors and their cross-regulation, and to understand their relative function in the context of HSC regulation. Though it is possible that cross-talk and feedback regulation occurs within the BMP signaling pathway, BMPR-II deletion does not seem to have a regulatory effect at transcript level on other BMP family receptors in HSCs.

In this study we show that BMPR-II deficient HSCs fail to efficiently generate additional HSCs upon transplantation, thus causing a significant reduction in hematopoietic regeneration following serial BM transplantation. Loss of BMPR-II did not affect homing capacity of HSCs to the BM, suggesting that the reduced regenerative capacity observed upon transplantation derives from compromised self-renewal ability of LT-HSCs. During steady state hematopoiesis, BMPR-II/- mice display essentially normal hematopoietic parameters, lending further evidence to a specific role for BMPR-II in self-renewal of LT-HSCs. Furthermore, as cell cycle distribution among LT-HSCs is more or less unaffected by loss of BMPR-II and the hematopoietic system recovers almost normally following stress, our data suggest that a possible effect on cell cycle progression plays only a small part in HSC regulation by BMPs. Instead, LT-HSCs deficient of BMPR-II fail to maintain stemness during conditions when self-renewal divisions are required. This is in agreement with previous data, which shows that BMP stimulation does not affect proliferation of HSCs in vitro\textsuperscript{23}.

By investigating the transcriptional activity of the SMAD pathway, our data reveals that a majority of hematopoietic cells fail to respond transcriptionally to BMPs and thus do not employ SMAD-dependent transcriptional response, despite phosphorylation of SMADs. We hypothesize that other regulatory
mechanisms limit the ability of the SMAD pathway to engage transcriptionally in response to BMP stimulation and that BMPs preferentially signal through non-SMAD circuitries in hematopoietic cells. The BRE-reporter study is in agreement with the lack of hematopoietic phenotype seen upon deletion of SMAD1/SMAD5. The p38 signaling pathway is an alternative signaling circuitry implicated downstream BMP receptors. Khurana et al. showed that p38 is phosphorylated in both human and mouse HSPCs cultured in the presence of BMP4 in vitro. In agreement with this, we observed a reduction in phosphorylation of p38 in hematopoietic progenitor cells lacking BMPR-II. However, we could not detect a robust induction of phosphorylation in response to BMP4 in WT progenitor cells. This may be due to the length of BMP stimulation, as Khurana et al. measured p38 signaling following five days of continuous BMP4 exposure. We assayed p38 after 30 minutes of BMP4 stimulation, a time point measuring direct activation. Reduced phosphorylation of p38 is therefore in agreement with a more long-term loss of BMPR-II, and may thus be due to secondary effects.

Interestingly, we observed a significant increase in expression of TJP1 in purified BMPR-II−/− LT-HSCs. TJP1 has previously been linked to regulation of self-renewal in embryonic stem cells where loss of TJP1 results in increased self-renewal. Expression of TJP1 is shared between HSCs, ES cells, and neural stem cells, indicative of a universal role for TJP1 in self-renewal of stem cells. Additionally, TJP1 is downregulated in a multipotent hematopoietic cell line upon differentiation. Taken together, these data substantiate the link between TJP1 and HSC self-renewal. Contrary to what is seen in hematopoietic cells in vitro, our data suggests that loss of BMPR-II leads to disruption of HSC self-renewal via excessive expression of TJP1, which is in line with previous findings in ES cells.
It is possible that fine-tuned HSC regulation in vivo requires very specific levels of TJP1. Our findings further show that knockdown of TJP1 partly rescues the BMPR-II null phenotype. Following transplantation of BMPR-II-/- cells with TJP1 knockdown, we observed an increase in cell contribution to the donor LSK compartment. A similar trend was seen in the HSC compartment, but not in more differentiated populations. Our data suggests that the up-regulation of TJP1 is, at least in part, one of the key mechanisms behind the observed BMPR-II-/- hematopoietic phenotype. Complete reversal of the phenotype may not have been achieved due to incomplete knockdown or that in addition to TJP1 there could be other mechanisms playing a part in generating the phenotype.

To increase the therapeutic applicability of HSCs, more detailed information is required regarding mechanisms controlling fate options such as self-renewal. In human hematopoiesis BMPs have been shown to have an important role in adhesion to stroma, differentiation potential and ex vivo maintenance19,20,36.

Here, we identify BMPR-II and TJP1 as important players regulating murine LT-HSC self-renewal in vivo. In light of our findings, further work should focus on investigating the role for BMPR-II and in particular TJP1 in human HSC self-renewal.
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Authorship contributions

SW, UB, and SK designed experiments. SW, UB, MD, THMG, LS, and SA performed experiments. SW and UB analysed data. SW, UB, and SK wrote the paper. SK supervised the study.

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Disclosure of conflicts of interest

The authors declare no competing financial interests.
References


Figure legends

Figure 1. Expression of BMP receptors in primitive hematopoietic subsets.
(A) Expression of \textit{BMPR-II} relative to \textit{HPRT} in indicated subsets of BM hematopoietic cell populations (n=3-4). LT-HSC (long term-HSC), ST-HSC (short term-HSC), LMPP (lymphoid-primed multipotent progenitor), GMP (granulocyte-macrophage progenitor), MkP (megakaryocyte progenitor), PreGM (pre-granulocyte-macrophage progenitor), PreMegE (pre-megakaryocyte-erythroid progenitor), PreCFU-E (pre-colony-forming unit-erythroid progenitor), CFU-E (colony-forming unit-erythroid progenitor). (B) Expression of Type-I receptors, \textit{ALK2}, \textit{ALK3}, and \textit{ALK6} in indicated hematopoietic cell populations (n=3).
MEF indicates mouse embryonic fibroblasts. ** indicates P-value < .01 in comparison to all other populations except for LMPP (fig 1A). * indicates P-value < .05, ns = not significant, ALK6 data was not statistically tested (fig 1B).

Figure 2. Normal steady state hematopoiesis in BMPR-II deficient mice. (A) PB cell counts of adult mice. Y-axis indicates number of cells per L (liter) PB. RBC indicates red blood cell, and WBC denotes white blood cell. (n=3-9). (B) Lineage distribution in PB of adult mice at steady state by flow cytometry analysis. CD3 indicates T-cells, B220 denotes B-cells, and Gr1/Mac1 shows myeloid cells (n=3-10). (C) Lineage distribution in BM of adult mice at steady state by flow cytometry analysis (n=3-10). (D) Representative FACS plot of an LSK-SLAM stain of BM cells from adult mice. (E) Quantification of LSK-SLAM populations in BM of adult mice at steady state. Number of LSK cells: 54341 ± 4939 for WT (n=8) vs. 41052 ±3398 for BMPR-II⁻/⁻ (n=10). (F) BM colony forming assay in vitro. Total number of colonies: 111.5 ±5.07 for WT (n=8) vs. 86.2 ±3.37 for BMPR-II⁻/⁻ (n=10).
* indicates $P$-value < .05  *** $P$-value < .001

**Figure 3. Reduced self-renewal capacity of HSCs upon loss of BMPR-II.** (A) Competitive transplantation. (B) Representative FACS plots of PB showing CD45.1 competitor vs. CD45.2 donor contribution, and lineage distribution within the CD45.2 subset of cells. T indicates T-cell, B represents B-cells, and M shows myeloid cells. (C) CD45.2 donor contribution in PB of primary recipients at 16 weeks post transplant. Mean engraftment 50.6% ± 6.2 for WT vs. 36.5% ± 7.6 for BMPR-II−/−. Heterozygotes (BMPR-II+/−) did not differ from WT in PB engraftment (50.4% ± 7.1). Due to statistically detectable variability between experiments, paired t-test was used to compare WT and BMPR-II−/−. (n=4-7) (D) Lineage distribution within CD45.2 donor subset of PB in primary recipients at 16 weeks post transplant. (n=4-7) (E) Representative FACS plots of LSK-SLAM CD45.1/2 stain of BM of primary recipients at 16 weeks post transplant. (F) Quantification of CD45.2+ LSK-SLAM populations in BM of primary recipients at 16 weeks post transplantation. (n=4-7) (G) CD45.2 donor contribution in BM at 16 weeks post transplant in primary, secondary, and tertiary recipients. (n=4-7) (H) Quantification of CD45.2+ LT-HSCs in BM at 16 weeks post transplantation in primary, secondary, and tertiary recipients. (n=4-7) (I) Transplantation of sorted LT-HSCs to lethally irradiated recipients. (J) CD45.2 donor contribution in PB of recipients at 16 weeks post sorted LT-HSC transplant (n=6). (K) Lineage distribution within CD45.2 donor subset of PB in recipients at 16 weeks post sorted LT-HSC transplant. (n=6) (L) Quantification of CD45.2+ LSK-SLAM populations in BM of recipients at 16 weeks post sorted LT-HSC transplantation. (n=6) * $P<.05$  ** $P<.01$ † indicates $P$-value = .078
Figure 4. BMPR-II deficient mice exhibit normal apoptosis and cell cycle parameters of primitive hematopoietic cells. (A) Representative FACS plots of LSK-SLAM/Annexin V stain of BM. (B) Percentage of Annexin V+ cells within indicated LSK-SLAM subsets of BM of adult mice at steady state (n=5). (C) Representative FACS plots of LSK-SLAM/Ki67/DAPI stain of BM. (D) Cell cycle distribution in % within LT-HSCs (n=9-10). (E) In vitro growth of c-kit+ cells (n=3). (F) Homing assay. Percentage of Lin-/Sca1+/CD150+/CD45.2+ cells in BM of transplanted recipients (n=5).

Figure 5. BMPR-II deficient cells have reduced p38 levels and up-regulation of TJP1. (A) Western blot analysis of SMAD1/5 phosphorylation in WT and BMPR-II deficient c-kit+ cells, with and without BMP4 stimulation in vitro (n=3). ψ indicates P < .05 compared to WT stimulated with BMP4 and P < .01 compared to WT (without BMP4). (B) GFP+ cells in BM from BRE-GFP reporter mice by flow cytometry analysis (n=3). (C) GFP+ cells following over night in vitro culture with or without BMP4 (n=3). (D) Western blot analysis of phospho-p38 in WT and BMPR-II-/- cells cultured over night with or without BMP4 (n=3). (E) Western blot analysis of total p38 in WT and BMPR-II-/- cells cultured over night with or without BMP4 (n=3). ψ indicates P < .05 compared to WT stimulated with BMP4 and P < .01 compared to WT (without BMP4). (F) Two separate qPCR analyses of TJP1 expression in WT and BMPR-II deficient LT-HSCs (n=3-4). Due to statistically detectable variability between experiments and consistent ratio based changes in expression levels, ratio paired t-test was used to compare groups. TJP1 protein levels could not be investigated as all tested commercially available antibodies yielded no results (data not shown). ** P < .01 *** P < .001
**Figure 6. TJP1 knockdown in BMPR-II deficient cells increases primitive cell engraftment.** Engraftment of GFP+ cells (%) in bone marrow following transplantation of transduced cells in (A) CD45.2+ (donor) GFP+ LSK population, (B) CD45.2+ GFP+ HSC (LSKCD48-CD150+) population, (C) CD45.2+ GFP+ Lin-population, and (D) CD45.2+ GFP+ population, analyzed at 16 weeks post transplant (n=8-9). In data set A and B outliers (one and two respectively) were detected using Grubb’s test (alpha=0.01) and removed; this did not alter outcomes of statistical analyses.* $P < .05$
Supplementary methods

Cell preparation
Peripheral blood was collected from the tail vein and analyzed on a Boule Medonice CA 530-16 blood analyzer to determine blood counts. Single-cell suspension of bone marrow (BM) and spleen were obtained by crushing and passage through a 40-µm nylon mesh cell strainer (Fisher Scientific). Cells were kept in phosphate buffered saline (PBS; Thermo Scientific) containing 2% fetal calf serum (FCS; HyClone Thermo Scientific). When necessary, red blood cells were lysed with ammonium chloride (NH₄Cl; Stem Cell Technologies). For c-kit enrichment, cells were incubated with anti-CD117 micro beads (Miltenyi Biotec). C-kit+ cells were enriched using magnetic separation columns (MACS 25 LS; Miltenyi Biotec) according to the manufacturer’s instructions.

Flow cytometry
Cells were stained with fluorescently labeled rat antibodies (conjugated with either PE, FITC, APC, PE-Cy5, PE-Cy7, Brilliant Violet 421, or eFlour 780) against mouse B220, CD3, Gr1, Mac1, Ter119, Sca1, CD9, CD48, CD150, CD49, CD45.1, CD45.2, CD41, CD105, CD16/32, CD42 (all from BioLegend), c-kit (eBioscience), CD31, CD41, CD61, CD34 and Flt3 (BD Pharmingen, BD Biosciences). Dead cells were excluded by 7-aminoactinomycin D (7-AAD; Sigma-Aldrich). For apoptosis assays, PE Annexin V was used according to the manufacturer’s protocol (BD Pharmingen/BD Biosciences). Cells were sorted by fluorescence-activated cell sorting (FACS) on a FACS Aria or analyzed on a FACS Canto (BD). Data was analyzed using FlowJo software (Treestar).

Cell cycle analysis
Freshly isolated BM was enriched for c-kit+ cells and stained with antibodies against lineage markers, Sca-1, c-kit, CD48, CD150, prior to fixation in 0.4 % formaldehyde (VWR). Following permeabilization with 0.2% Triton-X (Sigma-Aldrich), cells were incubated with fluorescently conjugated anti-Ki67 antibody (BD Biosciences). DNA was stained using DAPI (Molecular Probes, Invitrogen). Cells were analyzed as described for flow cytometry above.
**CFU assay and in vitro cell culture**

For analysis of CFU-GM/E/mix, BM was seeded in methylcellulose (M3434 or M3234; Stem Cell Technologies) at 30 000 cells/ml in 35 mm Petri dishes. To verify recombination at the BMPR-II locus, DNA from individual colonies was analyzed by PCR. For megakaryocytic CFU-Mk assay the MegaCult™-C kit (StemCell Technologies) was used according to manufacturer’s protocol. For liquid cultures, c-kit+ cells were seeded at a concentration of 0.25 x 10^6 cells/ml in serum-free media (Stemspan SFEM; Stemcell Technologies) containing 50 ng/ml mSCF, 10 ng/ml mIL-3 (Peprotech), 10 ng/ml hIL-6 (Peprotech), 100 U/ml penicillin, and 100 µg/ml streptomycin (Hyclone, Thermo Scientific). Cells were counted and re-plated every third day until day 15.

**Serial replating assay**

BM of BMPR-IIfl/fl;Vav-Cre and WT littermate mice was harvested and seeded at a density of 300 000 cells per replicate into cytokine-supplemented methylcellulose medium (Methocult M3434; STEMCELL Technologies). Colonies were scored and replated at day 7-10, except for the fourth replating, which was scored at day 12 and could not be replated due to lack of BMPRII-/- colonies. For replating, cells were resuspended and counted and 40 000 cells per replicate were taken for replating.

**Hematopoietic stress assay using 5-fluorouracil**

Mice (BMPR-IIfl/fl;Vav-Cre and WT littermates) were given one intravenous bolus injection of 0.15 mg/g 5-fluorouracil. On day 12 after 5-fluorouracil injection blood, BM, and spleen was harvested and analyzed for blood counts and using flow cytometry as described above.

**BRE-GFP reporter assay**

Canonical BMP reporter mice (BRE) are previously described1-3. To detect BMP-responsive hematopoietic cells, BM was stained with antibodies as above. For BMP stimulation, c-kit+ cells were incubated for 16 hours in serum-free SFEM containing 50 ng/ml BMP4 (RnD Systems) supplemented with 50 ng/ml mSCF.
and 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were analyzed on a FACS Canto II.

**Cell stimulation**

BM cells (c-kit enriched or FACS sorted) were isolated and subsequently cultured for 16 hours in serum-free SFEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 ng/ml mouse stem cell factor. Subsequently, cells were stimulated with recombinant mouse BMP4 (RnD Systems) at 50-100 ng/ml for 5 to 45 minutes. Cells were then used for western blot analysis, flow cytometry, FACS, or qPCR.

**Western blot analyses**

Lysates were prepared using Laemli buffer (Bio-Rad Laboratories). Samples were loaded on pre-casted gels (Novex NuPage 4-12% Bis Tris gel; Life Technologies) and run according to the manufacturer’s instruction. Transfer was performed using iBlot gel transfer device (Invitrogen/Life Technologies). The following antibodies were used: anti-phosphoSMAD1/5 (Cell Signaling Technology), anti-Actin (BD Transduction Laboratories), anti-β-tubulin (CST), anti-phospho-p38 (CST), anti-total p38 (CST), anti-phospho-Limk (Biorbyt), anti-phospho-Cofilin (CST), Horseradish peroxidase (HPR)-conjugated secondary antibodies against mouse and rabbit IgG (GE Healthcare). Detection was performed using ECL select WB detection reagent (GE Healthcare/Amersham). Data was collected using Chemidoc XRS+ Molecular Imager (Bio-Rad Laboratories) and data was analyzed and quantified using Image Lab software (Bio-Rad Laboratories).

**Microarray**

For microarray analysis, 5000–10000 LSK CD150+CD48-CD9hi BM cells were sorted into 350 µl RLT buffer (Qiagen) and immediately frozen on dry ice. Samples were then shipped on dry ice to the authorized Affymetrix service provider Kompetenzzentrum Fluoreszent Bioanalytik (KFB) Center of Excellence for Fluorescent Bioanalytics (Regensburg, Germany) and subsequent processing was performed at KFB. RNA isolation was done using the RNeasy
Micro Kit (Qiagen) according to manufacturer's protocol. Purity and integrity of the RNA was assessed on the Agilent 2100 Bioanalyzer with the RNA 6000 Pico LabChip reagent set (Agilent). Sample preparation for microarray (Affymetrix Gene Chip; Mouse Gene 1.0 ST array) was done as described in the NuGEN Ovation PicoSL WTA System V2 and NUGEN Encore Biotin Module manuals (NuGEN Technologies). Summarized probe set signals in log2 scale were calculated by using the RMA algorithm with the Affymetrix GeneChip Expression Console v1.4 Software. After exporting into Microsoft Excel, average signal values, comparison fold changes and significance P-values were calculated. Probe sets with a fold change above 2.0 fold and a t-test P-value lower than 0.05 were considered significantly regulated.

Validation of microarray results was done for selected genes through qRT-PCR as above. The gene list generated by KFB was also further analyzed by gene set enrichment analysis (GSEA) using an online GSEA resource (Broad Institute) and the Molecular Signatures Database (MSigDB) hallmark gene sets collection.

**Quantitative RT-PCR**

Cells were sorted into RLT buffer as described above. RNA isolation, cDNA synthesis, and qRT-PCR were performed as previously described. Gene-specific primers (Taqman probes) were from Applied Biosystems.

**Statistical analyses**

Statistical analyses were performed in Prism (v 6-8, GraphPad) or Excel (Microsoft). Unless otherwise stated unpaired t-tests (parametric) or Wilcoxon-Mann-Whitney tests (non-parametric) were used, or for grouped analyses ANOVA or Kruskal-Wallis tests with post hoc multiple comparisons tests. Assumption of normality was tested on representative data using the D’Agostino & Pearson test where applicable, or the Shapiro-Wilk test as a secondary alternative. Similarly, assumption of homoscedasticity was evaluated using the F-test and appropriate statistical corrections applied where necessary. P-values less than 0.05 were considered statistically significant. Data are shown as mean ± standard error of the mean (SEM).
References (supplementary methods)


Validation of knockout.

A. Representative image from PCR analysis of individual colonies from bone marrow, confirming deletion of exon 4-5 of the BMPR-II gene.

B-C. qPCR analysis using two different probe sets, targeting exon 6 (B) and exon 1 (C), showing reduction of BMPR-II mRNA in purified LT-HSCs (n=3). A small amount of truncated mRNA was detected using the latter probe set (C).

** P < .01 and * P < .05
Supplement 2

Megakaryocytic lineage distribution, Mk progenitors, and CFU-Mk. Assessment of phenotypic aging of LT-HSCs. Serial replating assay.

A. Percentage of various megakaryocytic lineages in bone marrow (n=3).
B. Percentage of megakaryocyte progenitors in bone marrow (n=3).
C. CD41+ LT-HSCs as assessed by flow cytometry (n=6-7).
D. Number of megakaryocytic colonies in CFU-Mk assay.
D. Serial replating of BMPRII deficient and WT littermate bone marrow cells. X-axis indicates the replating at which colony number was counted. Y-axis indicates average number of colonies counted per plate.

* P < .05; *** P < .001
Cell cycle assay results in LSK cells (A), LSK CD48-CD150- cells (B), and LSK CD48-CD150+ cells (C). (n=9-10) * P < 0.05
Analysis of microarray data.
A. GSEA on microarray data to identify gene sets enriched or depleted in BMPRII−/− HSCs compared to WT controls. NES, normalized enrichment score; FDR, false discovery rate.
B. Heat map of top differentially expressed genes in BMPRII−/− HSCs (samples KO1-4) compared to WT controls (samples WT1-4).
Supplement 5

Analysis of hematopoietic stress response following 5-fluorouracil (5-FU) injections in vivo. Analyses done on day 12 after intravenous injection of 5-FU (n=3-4).

A. Sysmex analysis of red blood cells (RBC) and white blood cells (WBC) in peripheral blood of mice.
B. Cellularity, i.e. total number of cells, in bone marrow and spleen.
C-D. Number of primitive hematopoietic cells in bone marrow (C) and spleen (D).
Homing assays and analysis of integrin-a4 (CD49d) expression.

A. Percentage of Lin-/CD45.2+ cells in BM of transplanted recipients in the homing assay (n=5).

B-C. Percentage of Lin-/Sca1+/CD150+/CD45.2+ cells and Lin-/CD45.2+ cells in BM of transplanted recipients in the competitive homing assay (n=3).

D. Representative plot of CD49d expression on LT-HSCs as measured by flow cytometry, showing no differences between BMPR-II knockout and control cells.
Supplement 7

Several known signaling factors associated with TJP1 or canonical BMP signaling are not affected by BMPR-II deficiency. Array hits Alpk and microRNA levels are also not affected.

A. Western blot of Limk & Cofilin phosphorylation in WT and BMPR-II deficient c-kit+ cells (n=3).
B. qPCR of RhoA and RhoB expression in WT and BMPR-II deficient LT-HSCs (n=3).
C. Gene expression relative HPRT in LSK cells (no significant differences). Evaluation of factors known to associate with TJP1 (Stat3 and Src) as well as known downstream BMP mediators.
D-E. Gene expression of BMP type I (D) and type II (E) receptors in primitive hematopoietic (LSK CD48-) cells (no significant differences).
F-G. qPCR of Alpk (D) and microRNA miR-15a, miR-23b, and miR-27a (E) expression in WT and BMPR-II deficient LT-HSCs (n=3). ns = not significant.
Supplement 8

Microarray results from KFB Center of Excellence for Fluorescent Bioanalytics. Genes up- or down-regulated >1.75-fold in BMPR2 knock-out LT-HSCs in comparison to wild type control LT-HSCs.

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TJP1 knockdown using shRNA vectors and transduction efficiency.

A. TJP1 gene knockdown seen as fold change compared to untransduced cells (UT). Four different TJP1-shRNA lentiviral vectors (sh A-D) and one scrambled control shRNA (Scr) were used to transduce ckit+ wild type bone marrow cells, which were grown in vitro for 48 hours before sorting into RLT buffer using a FACS Aria III. Sorting purity of experiments were 50-75% on average. Sorted and lysed cells were subsequently prepared for and analysed by qPCR. (n=6) * P < .05 (in comparison to UT)

shRNA-C was chosen for the following transductions in transplantation experiments.

B. Transduction efficiency (using shRNA-C) for TJP1 knockdown transplantation experiments, analyzed by flow cytometry 48 hours post-transduction. (n=6) * P < .05 (compared to both Scrambled groups). ns = not significant.