ARTICLE

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 (HSC) is a process of strict modulation. Intrinsic and extrinsic signals govern fate options like self-renewal – a cardinal feature of HSC. 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 HSC. However, the BMP type-II receptor (BMPR-II) is highly expressed in HSC, leaving the possibility that BMP function via alternative pathways. Here, we establish that BMP signaling is required for self-renewal of adult HSC. Through conditional knockout we show that *BMPR-II* deficient HSC have impaired self-renewal and regenerative capacity. *BMPR-II* deficient cells have reduced p38 activation, implying that non-SMAD pathways operate downstream of BMP in HSC. Indeed, a majority of primitive hematopoietic cells do not engage in SMAD-mediated responses downstream of BMP *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 HSC to maintain self-renewal capacity and proper resilience of the hematopoietic system during regeneration.

Introduction

Hematopoietic stem cells (HSC) have dual capacity to self-renew and give rise to differentiating progeny.[1,2] Self-renewal pertains to the ability of HSC 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. In order to ensure homeostasis, HSC are tightly regulated by internal factors and external signaling cues from the bone marrow (BM) niche.[3] Although many regulatory mechanisms have been identified, deeper understanding of the self-renewal machinery is required to fully utilize the therapeutic potential of HSC.

Bone morphogenetic proteins (BMP) belong to the TGF-β superfamily of secreted cytokines, which during embryogenesis regulate a wide variety of biological processes.[4-7] Mechanistically, BMP signal through cell surface receptors, which activate receptor-regulated SMAD transcription factors (R-SMAD) through phosphorylation.[8] Phosphorylated R-SMAD form complexes with SMAD4 resulting in nuclear accumulation of activated complexes, which together with cofactors regulate target gene transcription.[8] Two classes of receptors have been identified; type-I and type-II. BMP bind to and signal via the BMP type-II receptor (BMPR-II), in association with any type-I receptor (ALK2, ALK3, or ALK6).[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.[9-12] Similarly, deletion of *SMAD1* and *SMAD5* results in embryonic lethality.[13-16] Beyond development, the TGF-β superfamily regulates tissue home-

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ostasis and adult regeneration of a variety of organ systems. Several lines of evidence suggest that BMP play a role in adult HSC regulation, but conclusive evidence for direct BMP-requirement by HSC is still lacking. For instance, ALK3-mediated signaling is required by the HSC osteoblastic niche, with loss of *ALK3* leading to increased HSC numbers.[17] By contrast, decreased levels of BMP4 in the BM results in reduced HSC numbers, as shown in a hypomorphic BMP4 mutant mouse model.[18] Additionally, BMP4 maintains cord blood-derived human hematopoietic stem and progenitor cells (HSPC) during *ex vivo* culture, by acting as a survival factor.[19] Recently, Khurana *et al.* showed that BMP4 exposure *in vitro* maintains the expression of ITGA4 in murine HSC, thereby preventing culture-induced loss of homing capacity.[20] However, *SMAD1* and *SMAD5* are dispensable for adult HSC, leading to the conclusion that BMP signaling is not endogenously required by adult HSC.[21,22] Interestingly, *BMPR-II* is reportedly highly expressed in adult HSC, suggesting that BMP may signal via alternative circuitries in HSC.[23] Indeed, several pathways can be activated by BMP, including components of the MAPK pathway, such as p38 and JNK.[24,25] A role for p38 has been suggested in maintenance of ITGA4 expression in HSC *in vitro*, but a conclusive role for BMP in the regulation of HSC *in vivo* has never been shown.[20] Therefore, in order to investigate the complete role of BMP signaling in HSC *in vivo*, we conditionally deleted *BMPR-II* in hematopoietic cells using the Cre/loxP system. We report here that BMPR-II is essential for self-renewal of HSC 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,[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 HSC to maintain self-renewal capacity *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, NC, USA)[27] were bred to homozygosity and mated with *Vav-Cre*[28] transgenic mice to generate conditional *Vav-Cre;BMPR-II$^{fl/fl}$* mice. Detection of *Cre*, floxed (fl), wild-type (WT), and excised alleles was done by polymerase chain reaction (PCR) as previously described.[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, $0.2x10^6$ unfractionated BM cells from *BMPR-II$^{fl/fl}$;Vav-Cre*, *BMPR-II$^{fl/+}$;Vav-Cre*, and WT littermates (*BMPR-II$^{fl/fl}$* or *BMPR-II$^{fl/+}$*) (CD45.2) were transplanted with $0.2x10^6$ congenic CD45.1 BM cells by tail vein injection to lethally irradiated (900 cGy) congenic CD45.1/2 recipients (three recipients per 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 $2x10^6$ cells were transplanted to secondary recipients, monitored as above. After another 16 weeks secondary mice were killed and tertiary transplantations were performed using $20x10^6$ 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 HSC, ten LSK/CD48$^-$/CD150$^+$ cells from *BMPR-II$^{fl/fl}$;Vav-Cre* or WT littermates were transplanted together with $0.2x10^6$ whole BM support cells (CD45.1/2) to CD45.1 recipients. Reconstitution was monitored as above and BM was 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*

FFor knockdown of *TJP1*, lentiviral plasmid pGFP-C-shLenti containing short hairpin RNA (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 a multiplicity of infection (MOI) of 30-50 and incubated over night (37°C, 5% CO2). Transduced cells were collected and transplanted into lethally irradiated CD45.1 recipient mice (two 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 can be found in the *Online Supplementary Appendix*.

Results

BMPR-II is highly expressed in long-term hematopoietic stem cells

In order to map the extent of *BMPR-II* expression in distinct populations of primitive adult hematopoietic cells, we performed quantitative PCR (qPCR) analyses on sorted long-term HSC (LT-HSC) (LSK-CD34$^-$FLT3$^-$), short-term HSC (ST-HSC) (LSK-CD34$^+$FLT3$^-$), lymphoid-primed multipotent progenitors (LMPP) (LSK-CD34$^+$FLT3$^+$), as well as various progenitor populations.[29] Robust expression of *BMPR-II* was detected in all subsets, although LT-HSC exhibited the 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-HSC, both *ALK2* and *ALK3* were expressed, but expression of both receptors was more abundant in LT-HSC. In LMPP, *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-HSC, we hypothesized that its deletion would blunt most signaling events initiated by BMP in HSC, allowing us to probe the

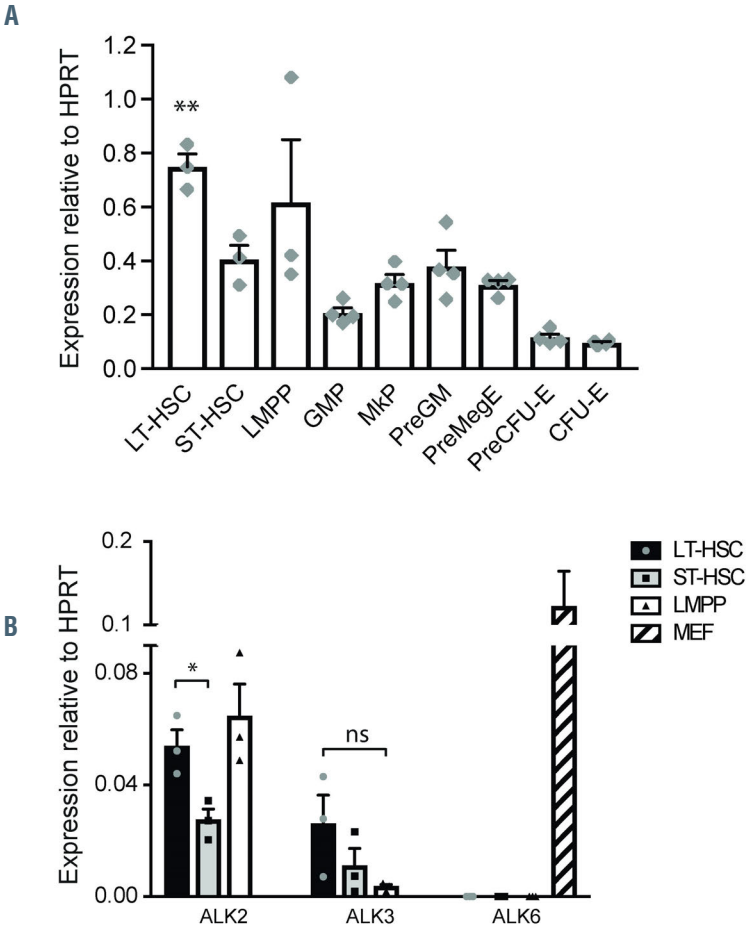


Figure 1. Expression of bone morphogenetic protein receptors in primitive hematopoietic subsets. (A) Expression of bone morphogenetic protein (BMP) receptor *BMPR-II* relative to *HPRT* in indicated subsets of bone marrow (BM) hematopoietic cell populations (n=3-4). LT-HSC: long term-hematopoietic stem cells; 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, *ALK2*, *ALK3*, and *ALK6* in indicated hematopoietic cell populations (n=3). $^{**}P<0.01$ in comparison to all other populations except for LMPP (A); $^{*}P<0.05$; ns:not significant; ALK6 data was not statistically tested (B). MEF: mouse embryonic fibroblasts.

full role of BMP in adult hematopoiesis. We conditionally deleted *BMPR-II* in hematopoietic cells, employing the Cre/loxP system with the *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; *Online Supplementary Figure S1A*). Recombination at the *BMPR-II* locus resulted in efficient reduction of *BMPR-II* mRNA in purified LT-HSCs (*Online Supplementary Figure S1B* and *C*). *Vav-Cre* mediated deletion in mice homozygous for floxed *BMPR-II* alleles (*BMPR-II*$^{fl/fl}$;*Vav-Cre*, hereafter referred to as *BMPR-II*$^{-/-}$) did not result in embryonic lethality although the *Vav* promoter is active from embryonic day (E) 10.5,[30] indicating that BMPR-II signaling is not endogenously required in HSC 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 and 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 (*Online Supplementary Figure S2A* and *B*). In order to further analyze HSPC 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 to E). Further analyses by SLAM markers did not reveal significant differences in more primitive subsets of LSK cells, such as LT-HSC (Figure 2D to E). Similarly, when assessing HSC phenotypic aging by CD41 expression[31] we saw no significant differences between WT and *BMPR-II*$^{-/-}$ LT-HSC (*Online Supplementary Figure S2C*). 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; *Online Supplementary Figure 2D*), suggesting that primitive hematopoiesis might be altered in *BMPR-II*$^{-/-}$ mice.

BMPR-II deficient hematopoietic stem cells exhibit reduced regenerative potential upon transplantation

In order to test the regenerative capacity of BMPR-II deficient HSC, 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 to 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). In order 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-

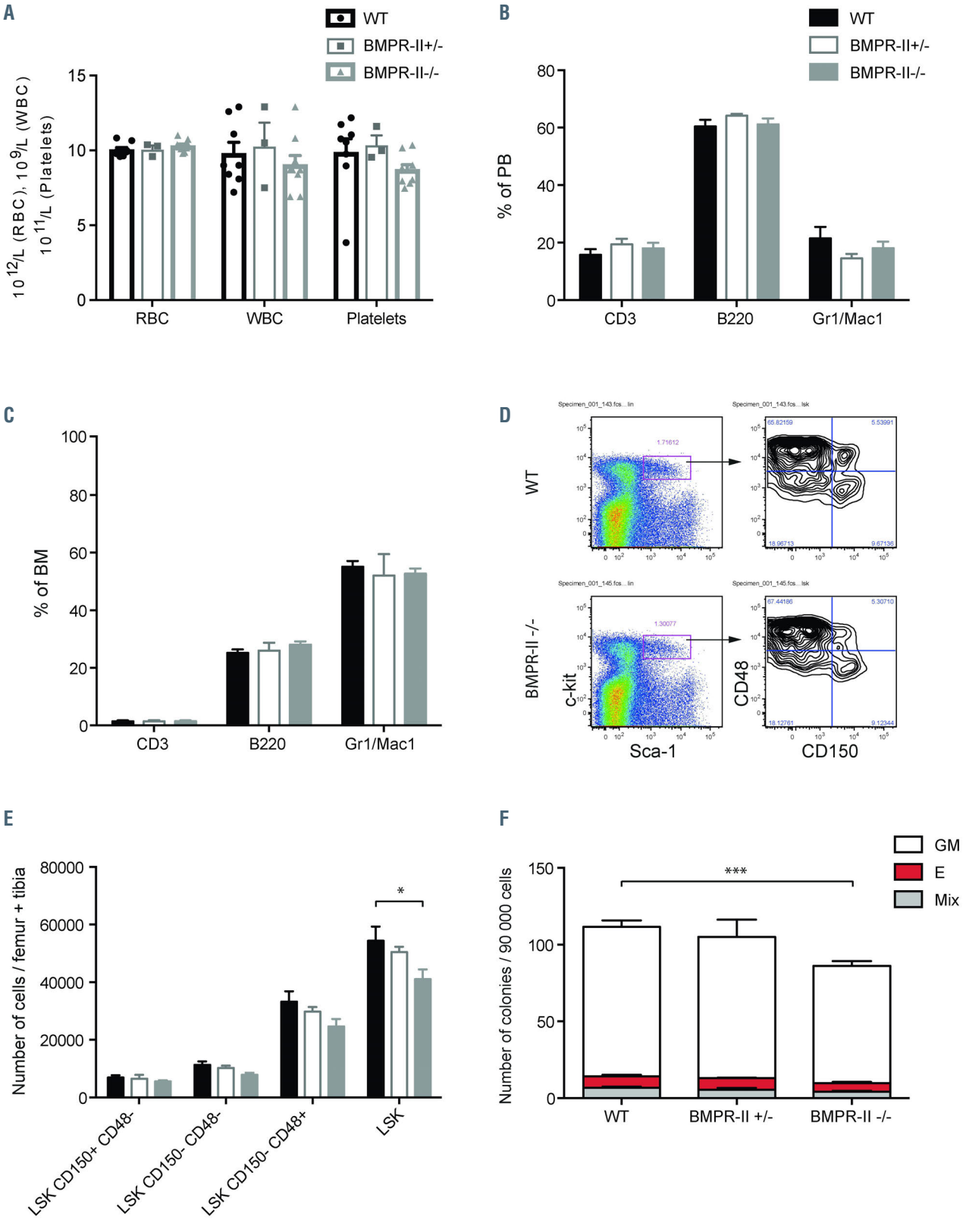


Figure 2. Normal steady state hematopoiesis in BMPR-II deficient mice. (A) Peripheral blood (PB) cell counts of adult mice (n=3-9). Y-axis indicates number of cells per L (liter) PB. RBC: red blood cell; WBC: white blood cell. (B) Lineage distribution in PB of adult mice at steady state by flow cytometry analysis. CD3: T cells; B220: B cells; Gr1/Mac1: 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 fluorescence activated cell sorting (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: 54,341±4,939 for wild-type (WT) (n=8) *vs.* 41,052±3,398 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). **P*<0.05; *** *P*<0.001.

SLAM populations, including the LT-HSC (LSK-CD150^{+}CD48^{-}) (Figure 3E and F).

Deletion of *BMPR-II* results in compromised self-renewal capacity and altered long-term hematopoietic stem cell-quality

In order to assay the self-renewal ability of BMPR-II deficient HSC, 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*$^{-/-}$ HSC dropped dramatically upon secondary transplantation, as compared to WT cells, which exhibited stable reconstitution across consecutive transplantations (Figure 3G). Upon tertiary transplantation, *BMPR-II*$^{-/-}$ cells dropped further, indicating a severely compromised ability to self-renew under stressed conditions (Figure 3G). *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-HSC revealed decreasing numbers of *BMPR-II*$^{-/-}$ derived cells across consecutive transplantations and in tertiary recipients the contribution to LT-HSC was essentially nonexistent (Figure 3H). These data show that BMPR-II-mediated signaling is essential for self-renewal of LT-HSC *in vivo*.

In agreement with the *in vivo* transplantation data stated above is the *in vitro* serial replating assay which shows a significant decrease in *BMPR-II*$^{-/-}$ colony number after three platings (*Online Supplementary Figure S2E*).

In order to verify that the observed defect in regenerative capacity was caused by a qualitative defect of HSC, we transplanted ten sorted *BMPR-II*$^{-/-}$ or WT LT-HSC in conjunction with congenic WT support BM cells (Figure 3I). In agreement with previous transplantations, overall donor contribution of *BMPR-II*$^{-/-}$ LT-HSC 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^{-}CD48^{-} and CD150^{-}CD48^{+} subset of LSK cells (Figure 3L). The LT-HSC 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 long-term hematopoietic stem cells

In order 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 (AnnexinV+) cells within LSK/LSK-SLAM populations did not differ between *BMPR-II*$^{-/-}$ and WT BM (Figure 4A and 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-HSC and a slight increase in LT-HSC 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 (*Online Supplementary Figure S3A to C*). In contrast to the lack of significant cell cycle perturbation in LT-HSC was the observed enrichment in gene sets pertaining to cell cycling (*Online Supplementary Figure S4A*). When the hematopoietic system was put under stress following *in vivo* treatment with 5-fluorouracil, the blood, BM, and spleen were mostly unaffected. Even though white blood cells and splenic LT-HSC were reduced, this was not significant (*Online Supplementary Figure S5A to 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*

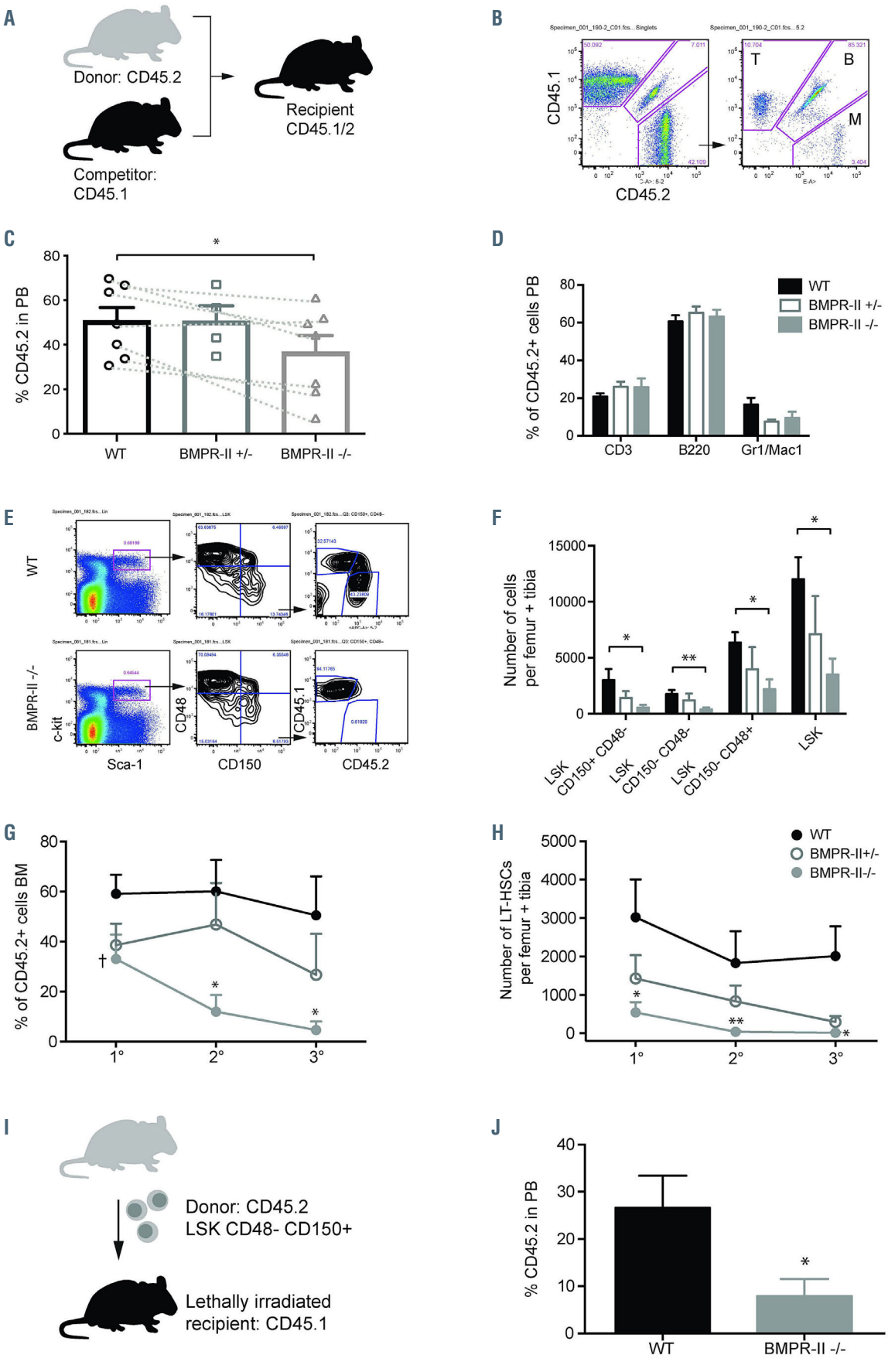
As BMP signaling has been linked to HSC homing via maintenance of ITGA4 expression during *ex vivo* culture, we investigated if loss of BMPR-II resulted in a homing defect.[20] We transplanted unfractionated *BMPR-II*$^{-/-}$ and WT BM cells, with or without competitor cells, to lethally irradiated recipients. 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; *Online Supplementary Figure S6A*). Donor contribution following competitive transplantation was also not significantly altered (*Online Supplementary Figure S6B to C*). Likewise, the expression of ITGA4 (CD49d) was unaltered between *BMPR-II*$^{-/-}$ and control LT-HSC, indicating that BMP signaling does not regulate ITGA4 expression *in vivo* (*Online Supplementary Figure S6D*).

Reduced phosphorylation of SMAD1 upon *BMPR-II* deletion

In order 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 SMAD 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,[26,32-34] cells responding transcriptionally to BMP through SMAD were measured by green fluorescent protein (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). In order 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).



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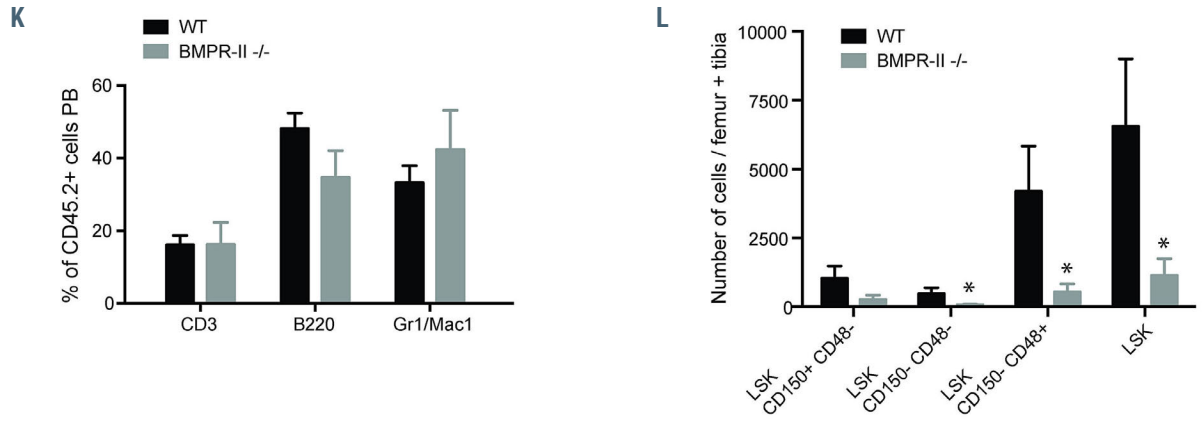

Figure 3. Reduced self-renewal capacity of hematopoietic stem cells upon loss of BMPR-II. (A) Competitive transplantation. (B) Representative fluorescence activated cell sorting (FACS) plots of peripheral blood (PB) showing CD45.1 competitor *vs.* CD45.2 donor contribution, and lineage distribution within the CD45.2 subset of cells. T: T cells; B: B cells; M: myeloid cells. (C) CD45.2 donor contribution in PB of primary recipients at 16 weeks post transplant. Mean engraftment 50.6%±6.2 for wild-type (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^{+} Long-term hematopoietic stem cells (LT-HSC) in BM at 16 weeks post transplantation in primary, secondary, and tertiary recipients (n=4-7). (I) Transplantation of sorted LT-HSC 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*<0.05; ***P*<0.01; †*P*=0.078.

Loss of *BMPR-II* results in a reduction of p38

As p38 has been implicated downstream of BMP 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 a 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 (*Online Supplementary Figure S7A* and *B*). In order 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 (*Online Supplementary Figure S7C*). Finally, we assessed expression levels of BMP type-I and other type-II receptors in sorted WT and *BMPR-II*$^{-/-}$ primitive hematopoietic cells (LSK CD48-) to determine whether *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 (*Online Supplementary Figure S7D to E*).

Deficiency of *BMPR-II* results in up-regulation of TJP1 in long-term hematopoietic stem cells

In order to further explore underlying mechanisms behind the *BMPR-II*$^{-/-}$ phenotype, we performed microarray analysis on highly purified LT-HSC (LSK-CD150^{+}CD48^{-}CD9^{hi})[35] from adult mice. The analysis generated a number of differentially expressed genes (*Online Supplementary Figures S8* and *S4B*) and enriched gene sets (*Online Supplementary Figure S4A*). Selected 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 *TJP1* in *BMPR-II*$^{-/-}$ LT-HSC (Figure 5F).

In order to further investigate whether the reduction in LSK cell numbers in *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 (*Online Supplementary Figure S7C*). We also found no significant differences in expression of Alpk or microRNA 15a/23b/27a, which were other hits in the array (*Online Supplementary Figure S7F* and *G*).

TJP1 knockdown partly rescues the *BMPR-II* knockout phenotype

In order to evaluate the contribution of *TJP1* up-regulation to the observed *BMPR-II*$^{-/-}$ phenotype, *TJP1* knockdown was performed using shRNA lentiviral vectors in ckit-enriched BM cells from *BMPR-II*$^{-/-}$ and WT mice. Transduced cells were transplanted to WT recipients. Using shRNA-C knockdown of *TJP1* was achieved to at least 0.51-fold level (compared to un-transduced cells) (*Online Supplementary Figure 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 (*Online Supplementary Figure S9B*).

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 HSC, although this did not reach significance (Figure 6B). In hierarchically lower populations no similar effect on engraftment was seen (Figure 6C and 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.[5-7] Studies performed *in vitro* indicate that BMP signaling continues to function in the regulation of HSC beyond development.[19,20,36] However, *SMAD1* and *SMAD5* are dispensable for adult HSC function in mice, leading to the conclusion that BMP play a limited role, if any, in adult HSC regulation *in vivo*.[21,22] The SMAD circuitry is undoubtedly the best characterized pathway downstream of BMP, but the lack of HSC phenotype in mice deficient of *SMAD1* and *SMAD5* does not automatically rule out a role for BMP signaling in adult HSC, as non-SMAD pathways can also be activated by BMP.[24,25] The fact that *BMPR-II* is highly expressed in LT-HSC has left a gap in knowledge between the BMP circuitry and its function in adult HSC *in vivo*.[23]

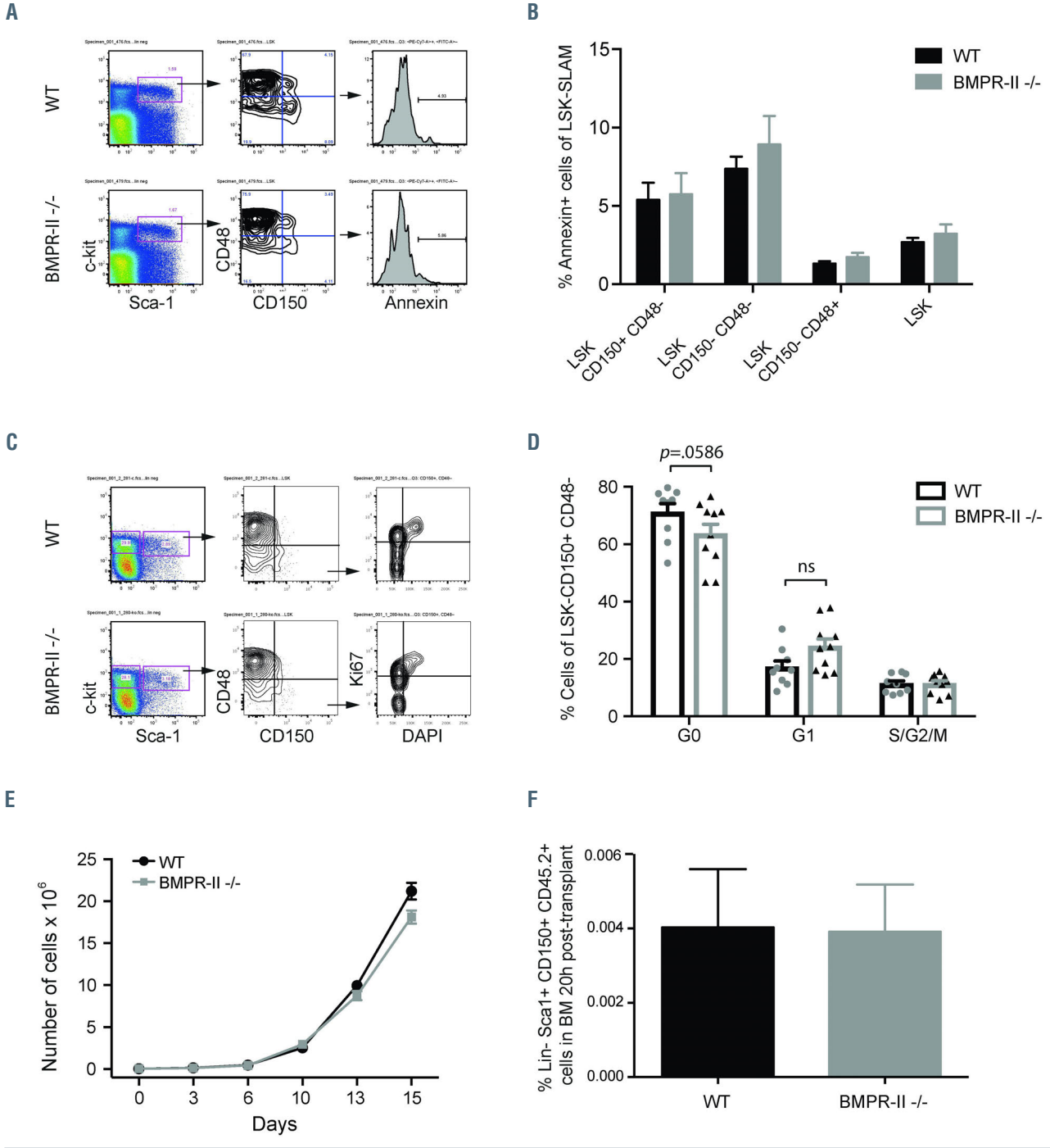

Figure 4. BMPR-II deficient mice exhibit normal apoptosis and cell cycle parameters of primitive hematopoietic cells. (A) Representative fluorescence activated cell sorting (FACS) plots of LSK-SLAM/Annexin V stain of bone marrow (BM). (B) Percentage of Annexin V$^+$ cells within indicated LSK-SLAM subsets 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 percent within long-term hematopoietic stem cells (LT-HSC) (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).

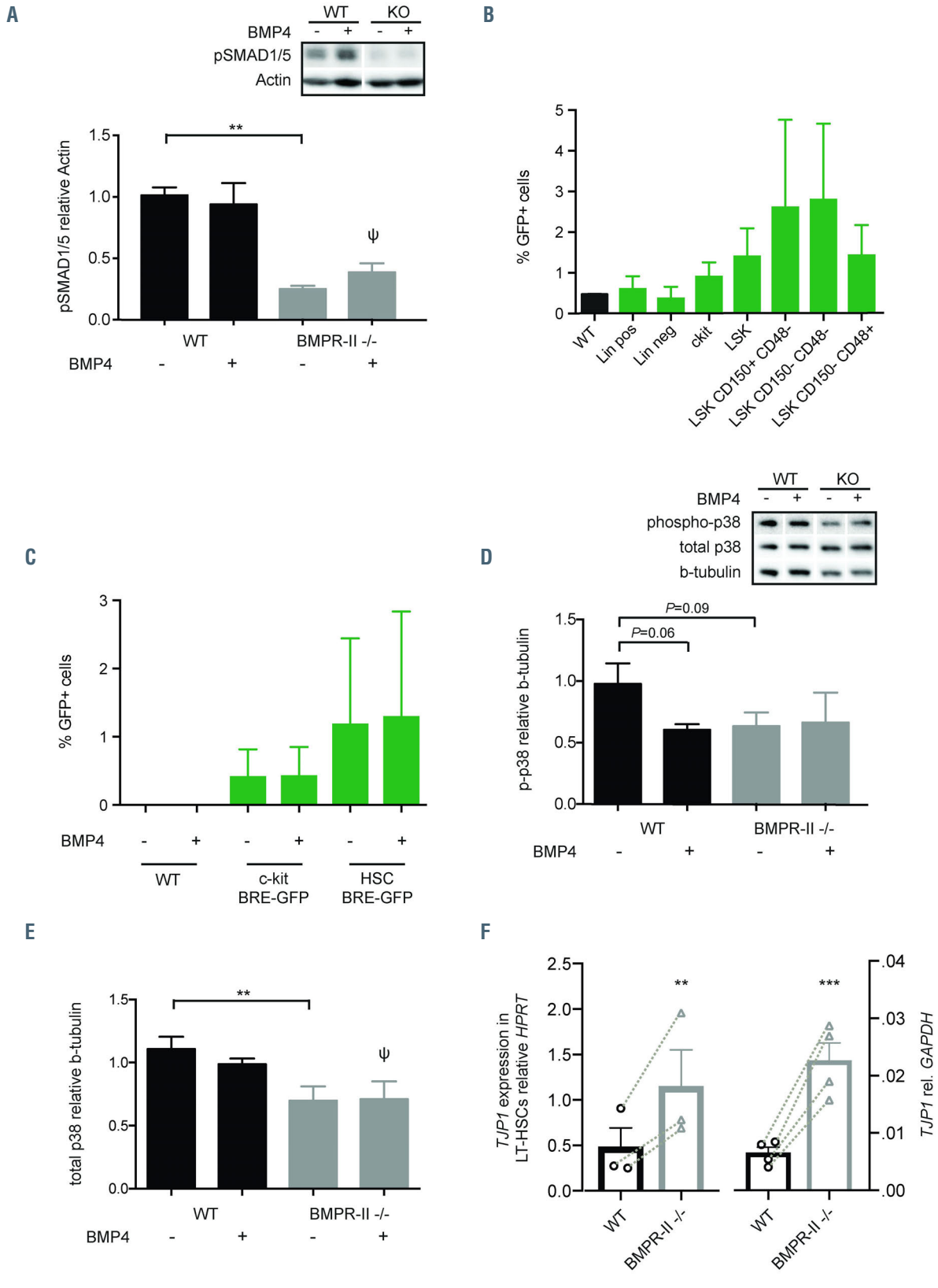

Figure 5. BMPR-II deficient cells have reduced p38 levels and up-regulation of TJP1. (A) Western blot analysis of SMAD1/5 phosphorylation in WT and BMPR-II deficient c-kit$^+$ cells, with and without BMP4 stimulation *in vitro* (n=3). ψP<0.05 compared to wild-type (WT) stimulated with BMP4 and P<0.01 compared to WT (without BMP4). (B) Western blot analysis of phospho-p38 in WT and BMPR-II$^{-/-}$ cells cultured over night with or without BMP4 (n=3).(C) Green fluorescent protein-positive (GFP$^+$) cells in BM from BRE-GFP reporter mice by flow cytometry analysis (n=3). (D) Western blot analysis of total p38 in WT and BMPR-II$^{-/-}$ cells cultured over night with or without BMP4 (n=3). ψP<0.05 compared to WT stimulated with BMP4 and P<0.01 compared to WT (without BMP4). (E) GFP$^+$ cells following over night *in vitro* culture with or without BMP4 (n=3). (F) Two separate quantitative polymerase chain reaction analyses of *TJP1* expression in WT and BMPR-II deficient LT-HSC (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<0.01; ***P<0.001.

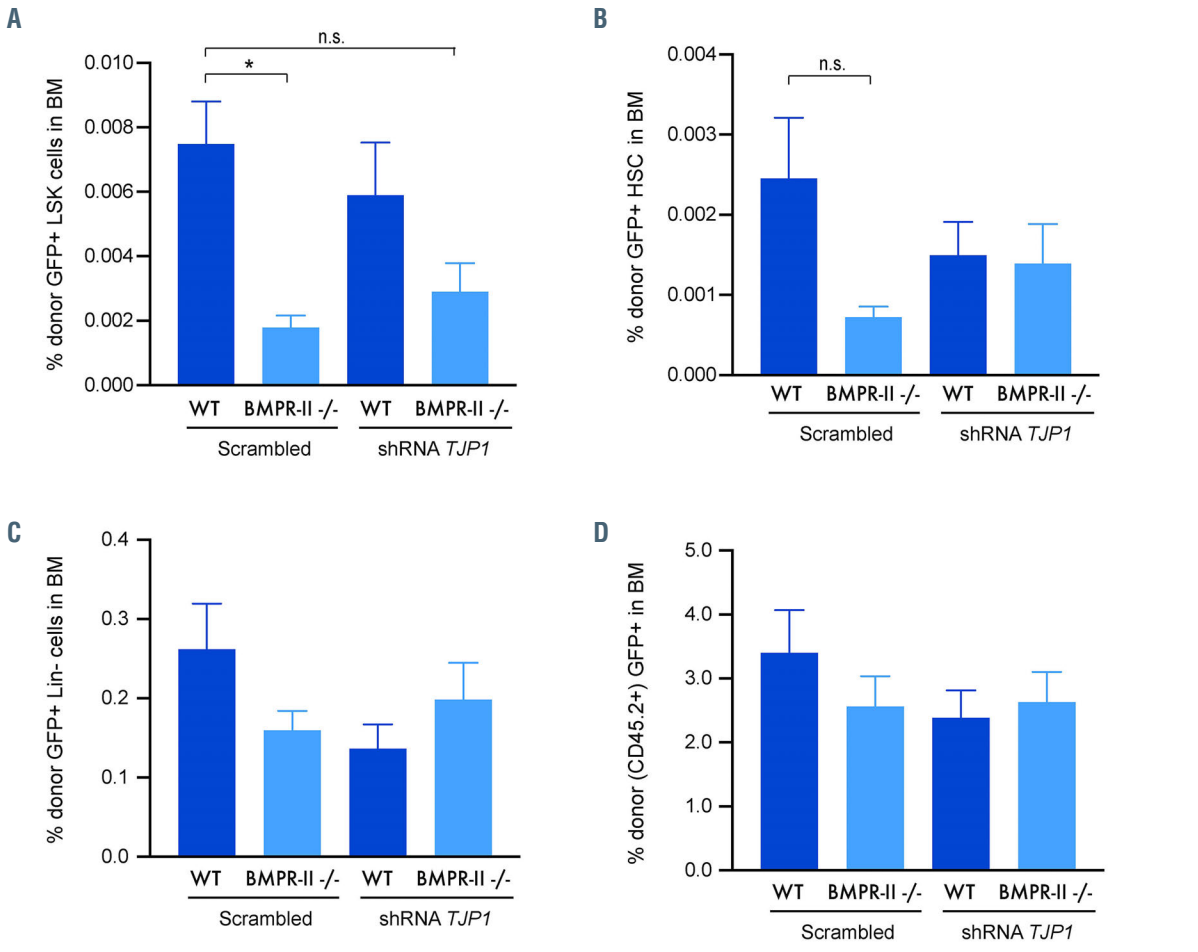

Figure 6. *TJP1* knockdown in BMPR-II deficient cells increases primitive cell engraftment. Engraftment of green fluorescent protein-positive (GFP$^+$) cells percent in bone marrow following transplantation of transduced cells in (A) CD45.2$^+$ (donor) GFP$^+$ LSK population, (B) CD45.2$^+$ GFP$^+$ HSC (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 (α=0.01) and removed; this did not alter outcomes of statistical analyses. *P<0.05.

Here we aimed to elucidate the endogenous role of BMP signaling in adult murine HSC, by conditional deletion of *BMPR-II* specifically in hematopoietic cells. Unlike deletion of *SMAD1* and *SMAD5*, we report here that BMPR-II is essential for self-renewal of adult HSC. It is likely that this non-SMAD signal in HSC 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-HSC and that we find no significant change in expression levels of other BMP receptors in primitive hematopoietic cells from *BMPR-II*$^{-/-}$ 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 HSC.

In this study we show that BMPR-II deficient HSC fail to efficiently generate additional HSC upon transplantation, thus causing a significant reduction in hematopoietic regeneration following serial BM transplantation. Loss of BMPR-II did not affect homing capacity of HSC to the BM, suggesting that the reduced regenerative capacity observed upon transplantation derives from compromised self-renewal ability of LT-HSC. 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-HSC. Furthermore, as cell cycle distribution among LT-HSC 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 BMP. Instead, LT-HSC 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 HSC *in vitro*.[23]

By investigating the transcriptional activity of the SMAD pathway, our data reveals that a majority of hematopoietic cells fail to respond transcriptionally to BMP and thus do not employ SMAD-dependent transcriptional response, despite phosphorylation of SMAD. We hypothesize that other regulatory mechanisms limit

the ability of the SMAD pathway to engage transcriptionally in response to BMP stimulation and that BMP 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 HSPC cultured in the presence of BMP4 *in vitro*.[20] 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 5 days of continuous BMP4 exposure. We assayed p38 after 30 minutes of BMP4 stimulation, a time point to measure 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-HSC. TJP1 has previously been linked to regulation of self-renewal in embryonic stem cells where loss of TJP1 results in increased self-renewal.[37] Expression of *TJP1* is shared between HSC, ES cells, and neural stem cells, indicative of a universal role for TJP1 in self-renewal of stem cells.[38] Additionally, *TJP1* is downregulated in a multipotent hematopoietic cell line upon differentiation.[39] Taken together, these data substantiate the link between TJP1 and HSC self-renewal. Contrary to what is seen in hematopoietic cells *in vitro*,[39] 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.[37] 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.

In order to increase the therapeutic applicability of HSC, more detailed information is required regarding mechanisms controlling fate options such as self-renewal. In human hematopoiesis BMP have been shown to have an important role in adhesion to stroma, differentiation potential and *ex vivo* maintenance.[19,20,36] 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.

Disclosure

No conflicts of interest to disclose.

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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