Wnts are dispensable for differentiation and self-renewal of adult murine hematopoietic stem cells

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Wnt secretion can be genetically and pharmacologically blocked without effect on normal adult hematopoiesis.

The clinical use of upstream Wnt inhibitors is unlikely to cause significant hematopoietic toxicity.

Abstract

Wnt signaling controls early embryonic hematopoiesis and dysregulated β-catenin is implicated in leukemia. However, the role of Wnts and their source in adult hematopoiesis is still unclear, and is clinically important as upstream Wnt inhibitors enter clinical trials. We blocked Wnt secretion in hematopoietic lineages by targeting Porcn, a membrane-bound O-acyltransferase that is indispensable for the activity and secretion of all vertebrate Wnts. Surprisingly, deletion of Porcn in Rosa-CreER<sup>T2</sup>/Porcn<sup>Del</sup>, MXI-Cre/Porcn<sup>Del</sup>, and Vav-Cre/Porcn<sup>Del</sup> mice had no effects on proliferation, differentiation or self-renewal of adult hematopoietic stem cells. Targeting Wnt secretion in the bone marrow niche by treatment with a PORCN inhibitor, C59, similarly had no effect on hematopoiesis. These results exclude a role for hematopoietic PORCN-dependent Wnts in adult hematopoiesis. Clinical use of upstream Wnt inhibitors is not likely to be limited by effects on hematopoiesis.
**Introduction**

Wnt signaling plays a key role in proliferation and differentiation in development. Wnts also regulate adult stem cells in highly proliferative organs such as gut and skin. Wnt signaling has been implicated in hematopoiesis, but its precise role remains controversial. Wnts signal through β-catenin and additional pathways to regulate processes such as proliferation, fate commitment and cell migration. The diverse Wnt pathways interact in complex ways. Wnt5a was reported to inhibit the proliferation of hematopoietic stem cells (HSC) *in vivo* and *in vitro* through suppressing the Wnt/β-catenin pathway, however other studies found that β-catenin-independent Wnt signaling positively regulates HSC proliferation and self-renewal. Conversely, inhibition of the Wnt/β-catenin pathway by overexpression of Dkk1 and Wif1 in osteoblasts in the HSC niche impaired the reconstitution capacities of HSCs. However, this effect was prominent in secondary but not in primary transplanted recipient mice, a result difficult to reconcile with an effect of the niche. Moreover, embryonic knockout of either Wnt3a or β-catenin (*Ctnnb1*) impaired HSC self-renewal only in secondary, but not primary, bone marrow transplantations. β-catenin abundance can influence hematopoiesis, as forced overexpression of β-catenin in HSCs resulted in stem cell exhaustion. However, under normal circumstances, β- and γ-catenin are dispensable for normal adult hematopoiesis. These diverse and seemingly contradictory results emphasize the need to better elucidate the role of Wnt signaling in hematopoiesis. This need takes on increasing clinical relevance as small molecules and antibodies targeting Wnt signaling are now entering clinical trials.

To understand the role of Wnts in hematopoiesis, it is essential to know which Wnt ligands are functionally important, and which cells produce them. In some tissues, Wnts function via autocrine loops, while in others, such as the intestine, the stroma rather than the epithelium is the essential source of Wnts. In hematopoiesis the source of Wnts is unclear. Some reports favor the bone marrow (BM) niche as a source of Wnt ligands, while others support a major role for autocrine production. Identifying the source of functionally important Wnts presents an experimental challenge, because there are multiple Wnt ligands expressed in various hematopoietic and BM niche cells. Fortunately, new tools are available to better address the tissue specific functions of Wnts. All Wnts are post-translationally modified in the endoplastic reticulum by addition of a palmitoleate moiety that is required both for their secretion and for binding to their cell-surface receptor Frizzled. Wnt palmitoleation is catalyzed by PORCN, a non-redundant membrane-bound
O-acyl transferase. Genetic knockout or pharmacological inhibition of *Porcn* therefore eliminates the activity, but not the expression, of all Wnts. While embryonic knockout of *Porcn* is lethal, targeted knockout in specific tissues can provide important insights into Wnt biology.

In the current study, we used a genetic and pharmacological approach to investigate the role of hematopoietic Wnts in hematopoiesis, by knocking out *Porcn* in HSCs of mice using three different alleles expressing Cre recombinase. We find that hematopoietic production and secretion of Wnt is completely dispensable for the proliferation and differentiation of blood progenitors, as well as for HSC self-renewal. In addition, treatment with a highly active PORCN inhibitor, C59, that blocks Wnt secretion both from hematopoietic and stromal cells, had minimal effects on normal hematopoiesis. Thus, Wnts have an unexpectedly limited role in adult murine hematopoiesis.

**Methods**

**Mouse Strains**
 Generation and validation of the *Porcn* conditional null allele was described previously. *Porcn* mice were backcrossed to C57BL/6 mice. *Porcn* mice were crossed with *Rosa-CreER*⁴, Mx1-Cre, and Vav-Cre mice. Age and gender-matched mice were used in all experiments. For BM transplantation C57BL6/Ly5.1 mice were used. *Porcn* genotyping, expression analysis, and primers was previously described. All mouse procedures were approved by the Institutional Care and Use Committee (IACUC).

**Inducible Porcn deletion and drug administration**
 Tamoxifen chow (80 mg tamoxifen/kg BW assuming 20 gram mice eat 3 gram chow per day, Harlan Laboratories (TD.110403)) was made available for 5 days followed by normal chow for 2 days, for 3 consecutive weeks, before resuming normal chow. Where indicated, Mx1-cre mice were injected with 800 μg Poly I:C every other day for 7 doses. Vehicle or C59 (50 mg/kg/day) were administered by gavage for 20 days as described previously.

**Flow cytometry**
 Peripheral blood from the facial vein was analyzed with a HemaVet. Single-cell suspensions from BM, blood, spleen, and thymus were analyzed by flow cytometry. Monoclonal antibodies conjugated with various dyes including allophycocyanin (APC),
APC–Cy7, phycoerythrin (PE), PE–CY7, eFluor 450 or fluorescein isothiocyanate obtained from BD Pharmingen (BD), eBioscience or BioLegend. The antibodies used in our study were: Gr-1 (8C5), CD3 (KT31.1), Mac-1/CD11b (M1/70), B220 (RA3-6B2), CD19 (1D3), TER119 (TER-119), CD4 (GK1.5), CD8 (53-6.7), c-Kit (2B8), Sca1 (E13-161-7), CD16/32 (2.4G3), CD48 (HM48-1), CD150 (TC15-12F12.2), CD45.2, CD45.1 (A20), CD127 (A7R34), and Flk2 (A2F10). Stained cells were examined with an LSRII flow cytometer (BD Biosciences) and sorted by FACSAria. Propidium iodide staining was performed to exclude dead cells from analysis. Identical numbers of total bone marrow cells from Porcn\textsuperscript{Del} or control marrow were analyzed using Diva (BD) and FlowJo (Tree Star) software.

**Bone Marrow Transplantation (BMT)**

For BMT, a total of 1×10\(^6\) bone marrow cells from either control, Rosa-Cre\textsuperscript{ERT2}/Porcn\textsuperscript{Del} mice or MX1-Cre/Porcn\textsuperscript{Del} mice (CD45.2) were transplanted through tail vein injection into lethally irradiated CD45.1 congenic recipient mice. Samples collected 8-16 weeks after transplantation were analyzed by FACS or secondary BMT.

**Colony forming Assay**

1 x 10\(^4\) BM cells were plated in the presence of Methocult M3434. Colonies were scored two weeks later. All assays were conducted in triplicate.

**Proliferation assay**

Click-iT EdU Alexa Fluor 555 Imaging Kit and EdU, were from Life Technologies (Cat. #C10338 and #A10044, respectively). Mice received 1.5 mg/kg EdU by IP injection 24 hours prior to sacrifice. BM was sorted to obtain 10,000 LSK (Lin-, Sca1+, c-Kit+) cells on slides. Cells were fixed, washed, permeabilized, and stained based on the manufacturer’s instructions. Slides were mounted in fluorescent mounting media with DAPI (VectaShield, Cat. #H-1200) and images obtained with a LSM710 Carl Zeiss confocal Microscope. ImageJ software was used for analyzing the images. The percentage of proliferative (EdU+) LSK cells was determined by counting 1500 LSK cells per mouse.

**Statistical analysis**

Data were analyzed using Prism 5 software, ImageJ and Excel. Two-tailed T-test was performed in Excel for Mac 2011 version 14.3.2.
Results

Total body knock out of Porcn in adult mice

We crossed Porcn<sup>fl</sup>ox with Rosa-CreER<sup>T2</sup> mice to generate mice with widespread expression of a tamoxifen-sensitive Cre recombinase. Porcn is on the X chromosome. To induce Porcn inactivation, control (Rosa-CreER<sup>T2</sup>/Porcn<sup>WT,WT</sup> or Porcn<sup>WT,Y</sup>) and inducible Porcn knockout mice (Rosa-CreER<sup>T2</sup>/Porcn<sup>fl</sup>ox<sup>fl</sup>ox or Porcn<sup>fl</sup>ox<sup>Y</sup>) received tamoxifen chow for 3 weeks. The excised allele(s) are referred to collectively as Porcn<sup>Del</sup>. Consistent with the well-documented role of Wnt/β-catenin signaling in hair follicle formation, Rosa-CreER<sup>T2</sup>/Porcn<sup>Del</sup> mice showed progressive global alopecia beginning 5 weeks after starting tamoxifen chow (Figure 1B). Substantial excision of Porcn exon 3 was observed in skin samples (Figure S1.B). Histologically, Porcn<sup>Del</sup> mice exhibited an impaired skin structure with lack of hair follicles in the dermis and an increased number of cells in epidermal layer (Figure 1C), similar to the phenotype seen after inactivation of either β-catenin or the Wnt transporter Wls in the skin. As expected, Porcn inactivation was accompanied by a marked reduction of Porcn mRNA in skin (Figure 1A). In addition, there was a decrease in Wnt/β-catenin signaling, shown by loss of β-catenin protein in hair follicles (Figure S1E) and down-regulation of the Wnt/β-catenin target gene Axin2 (Figure 1A). These results confirm the Porcn<sup>fl</sup>ox mouse as a useful tool to study the tissue specific role of Wnt secretion.

The Porcn<sup>Del</sup> mice started to lose body weight 4 weeks after tamoxifen treatment (Figure S1C). Additionally, they developed signs of neurologic impairment including altered gait and poor grooming. These mice survived 5 to 7 weeks after tamoxifen administration, when they were sacrificed due to weight loss. Necropsy did not reveal additional specific pathology beyond loss of body fat.

Normal hematopoiesis after total body Porcn inactivation

We had anticipated that Porcn deletion would affect multiple tissues including the bone marrow, where we confirmed multiple Wnt genes are expressed (Figure S1A). To test if hematopoiesis was impaired in the Rosa-CreER<sup>T2</sup>/Porcn<sup>Del</sup> mice, we examined complete blood counts (CBCs) 4-5 weeks after starting tamoxifen chow. The CBCs of Porcn<sup>Del</sup> and Porcn<sup>WT</sup> blood samples did not differ significantly in hemoglobin (Hg), erythrocytes (RBC), platelets (PLT), and total white blood counts (WBC) (Figure 1D, S1D). There was a modest increase in mature neutrophils as compared to controls (Figure 1D), which we suspect is secondary to the inflammation accompanying hair and weight loss. This unexpected lack of
effect on hematopoiesis was not due to poor excision or selection against the deleted allele, as we confirmed near-total Porcn deletion in peripheral blood cells by PCR from genomic DNA 2 weeks after stopping tamoxifen (Figure S1F).

We investigated the role of hematopoietic Wnt secretion in the maintenance of hematopoietic stem cells (HSCs) and in the proliferation of progenitor cells. Similar to the peripheral blood, the BM of the PorcnDel mice showed substantially reduced expression of Porcn by qRT-PCR, but expression of Wnt target genes such as Axin2, c-myc, and Cyclin D1 was not altered (Figure 1E). In addition, there were no changes in the number of long term HSCs (LT-HSC, Lin-, Sca-1+, c-kit+, CD150+, CD48-), short term HSC (ST-HSC, Lin-, Sca1+, c-Kit+, CD150-, CD48-), and hematopoietic progenitor cells (HPC, Lin-, Sca1+, c-Kit+, CD150-, CD48+) in these mice (Figure 1F). Moreover, the number of common lymphoid progenitors (CLPs, CD127+, c-Kit+, Sca1+, Flk2+) and myeloid progenitors (CMP: common myeloid progenitor, GMP: granulocyte-monocyte progenitor, MEP: megakaryocyte-erythroid progenitor) in PorcnDel mice were in the range observed in the PorcnWT mice (Figure 1G, S1H, 1H). Consistent with peripheral blood findings, there was a greater frequency of granulocytes in the BM of PorcnDel mice (Figure S1G). Collectively, the global knockout of Porcn in adult Rosa-CreERT2 mice, while producing significant hair, weight and neurologic effects, hematologically caused only a modest increase in granulocytes.

We asked if cell-autonomous Wnt production was important in the differentiation of hematopoietic progenitor cells using colony forming assays. Unexpectedly, both PorcnDel LSK (Lin-, Sca1+, c-Kit+) and BM cells were able to form colonies of all lineages and showed modestly increased numbers of colonies compared to controls (Figure 2A, S1I). The increase in total colony numbers from PorcnDel cells was due to an increase in granulocyte and mixed granulocyte/monocyte colonies, consistent with peripheral blood findings. Importantly, colony formation was not the result of selection for rare cells not undergoing Porcn excision by the Rosa-CreERT2 driver, as we confirmed that the colonies from PorcnDel mice arose from PorcnDel cells.

Luis et al. reported that Wnt3a deficient HSCs could not successfully reconstitute the BM of WT lethally irradiated recipient mice after a secondary bone marrow transplantation (BMT), suggesting a role for WNT3A in the maintenance of embryonic HSCs. To investigate adult HSC function after inhibition of Wnt secretion, BM from Rosa-CreERT2/PorcnDel mice (CD45.2) was transplanted into PorcnWT irradiated recipient mice (CD45.1). PorcnDel BM fully reconstituted the recipients, as determined by CBC and FACS analysis of peripheral blood at 8 weeks, and FACS analysis of bone marrow at 20 weeks. We
confirmed that hematopoietic reconstitution was predominantly from Porcn\textsuperscript{Del} rather than Porcn\textsuperscript{flox} donor cells by genomic PCR of peripheral blood samples (Figure S2B). As with the colony forming assays, this rules out the possibility that residual Porcn\textsuperscript{flox} non-excised HSCs were responsible for the successful primary BMT. The frequencies of donor neutrophils (CD45.2+, Mac1+, Gr1+), B- (CD45.2+, CD19+, CD3e-) and T- lymphocytes (CD45.2+, CD19-, CD3e+) in the total BM cells of the primary recipient mice were similar regardless of whether the donor was Porcn\textsuperscript{WT} or Porcn\textsuperscript{Del} (Figure 2B-D, S2A). Interestingly, the frequencies of donor LSKs, LT-HSCs, HPCs, and myeloid progenitors (CMP, GMP, MEP) indicated a normal reconstitution of the BM from both Rosa-CreER\textsuperscript{T2}/Porcn\textsuperscript{Del} and Porcn\textsuperscript{WT} mice (Figure 2E, 2F).

To assess if loss of Wnt secretion reduced the functional frequency of HSCs and progenitors, BM samples from the primary recipient mice were transplanted into secondary lethally irradiated mice. Porcn\textsuperscript{Del}, like Porcn\textsuperscript{WT} HSCs successfully reconstituted the secondary recipient mice (Figure S2D-2F) and outcompeted the residual Porcn\textsuperscript{flox} donor cells (Figure S2C). We conclude that HSCs of the Rosa-CreER\textsuperscript{T2}/Porcn\textsuperscript{Del} mice are phenotypically and functionally normal in the absence of HSC Wnt secretion. Finally, a competitive reconstitution assay (Figure S2G, S2H) showed stable engraftment of both Porcn\textsuperscript{WT} and Porcn\textsuperscript{Del} HSCs. The fraction of reconstitution from Porcn\textsuperscript{Del} was slightly smaller than that from Porcn\textsuperscript{WT} which may be technical, or may reflect a modest depletion of HSC after global Porcn deletion.

**Normal hematopoiesis following Porcn inactivation in hematopoietic cells.**

The lack of significant effect on hematopoiesis after excision of Porcn with the Rosa-CreER\textsuperscript{T2} driver was unexpected. We considered the possibility that while we had significantly reduced PORCN function (e.g. as demonstrated by reduction of Porcn mRNA), subtotal excision of Porcn in Rosa-CreER\textsuperscript{T2}/Porcn\textsuperscript{Del} mice left sufficient Wnt secretion to maintain normal function. As a second test, we crossed Porcn\textsuperscript{flox} mice with Mx1-Cre mice to get more complete and specific excision of Porcn in HSC after induction of Cre expression in adult mice. Mice were treated with poly I:C every other day for 7 doses. WBCs were suppressed as expected after poly I:C injection and recovered normally after 15 days (Figure 3A). In addition, RBCs and platelets remained in the normal range (Figure S3A). Extensive excision of Porcn was confirmed in the peripheral blood (Figure 3B). Similar to Rosa-CreER\textsuperscript{T2} mice, expression of Wnt target genes did not change in the Mx1-Cre/Porcn\textsuperscript{Del} mice (Figure 3C). The BM total and differential cell counts, examined 4 months after poly I:C, remained in the
normal range (Figures S3B-S3D). In addition, the frequencies of HSCs and progenitors were not affected (Figure 3D, 3E). Consistent with the results from Rosa-CreERT2 mice, Mxl-Cre/PorcnDel BM cells produced both myeloid and erythroid colonies similar to controls in methylcellulose colony forming assays (Figure 3F). Quantitative real time PCR on genomic DNA from BM, blood and hematopoietic colonies confirmed a near total excision of Porcn (Figure S3E) in Mxl-Cre/PorcnDel mice. Again, the deletion of Porcn in the colonies confirmed that there was no strong selection for colony formation from rare non-deleted cells. To test the self-renewal ability of Mxl-Cre/PorcnDel HSCs, BM from PorcnDel and PorcnWT CD45.2 mice were transplanted into lethally irradiated recipient mice (CD45.1). PorcnDel donor HSCs were capable of successful reconstitution of the recipient (Figure S3G, S3H). We confirmed recipient hematopoiesis was by cells with deletion of Porcn exon 3 (Figure S3F), ruling out the possibility that reconstitution was due to survival of rare non-Porcn-deleted cells. To test the long term progenitor function of PorcnDel HSCs, the primary recipient mice were sacrificed 6 months following BMT and their BM was subsequently transplanted into secondary recipient mice. The secondary recipient mice survived more than one year with normal hematopoiesis, and BM analysis showed successful reconstitution from donor PorcnDel cells (Supplementary table 1). These results strongly suggest that hematopoietic Wnts are fully dispensable for maintenance of HSCs.

In contrast to the Rosa-CreERT2/PorcnDel mice, there was no increase of granulopoiesis in the Mxl-Cre mice. Therefore, the increased granulopoiesis in the Rosa-CreERT2/PorcnDel mice is likely to be secondary to non-hematologic effects of decreased Wnt activity in other organs such as skin, hair, and brain.

There was incomplete hematopoietic excision of Porcn in Mxl-Cre mice, as a faint floxed, non-excised band was seen after genomic PCR of blood and BM samples. Importantly, these Wnt-competent cells were not selected for in the transplantation and colony forming assays.

**Murine hematopoietic Wnts are dispensable for adult hematopoiesis.**

To achieve a complete inhibition of Wnt secretion from hematopoietic cells, Porcnfloxflox mice were bred with Vav-Cre mice, with constitutive expression of Cre in all hematopoietic lineages from early in development. If hematopoietic Wnts are essential at any point after Vav expression begins, Vav-Cre/PorcnDel mice should exhibit impaired hematopoiesis. Again unexpectedly, Vav-Cre/PorcnDel mice were developmentally normal, fertile, and did not show any gross phenotypic abnormalities.
Complete deletion of *Porcn* exon 3 was confirmed in blood and BM samples (Figure 4A, 4B). However, the BM expression of *Axin2*, *c-Myc* and *Cyclin D1* were not altered, indicated that hematopoietic Wnts are not controlling their expression (Figure 4B). WBCs, RBCs and Hb were normal in the *Vav-Cre/Porcn* Del mice (Figure S4A, S4B). The frequencies of BM granulocytes and lymphocytes, and BM myeloid and common lymphoid progenitor cell numbers were not altered in *Vav-Cre/Porcn* Del mice (Figure 4C-D, S4C-E). Thymic cells had normal populations of double negative (DN), double positive (DP), CD4 T-cells and CD8 T-cells (Figure S4G).

Florian et al. suggested that aging of HSCs is driven by a shift from Wnt/β-catenin signaling to β-catenin independent Wnt signaling due to increased expression of WNT5A in aged LT-HSC. To evaluate the intrinsic effect of Wnt signaling in HSC aging, the total number of LT-HSCs, ST-HSCs, and HPCs in both aged and young mice was compared. We found no significant differences between *Vav-Cre/Porcn* WT and *Porcn* Del mice at any age (Figure 4E, 4F). In addition, the proliferation rate of aged *Vav-Cre/Porcn* Del LSK cells was similar to aged *Vav-Cre/Porcn* WT LSK cells (Figure S4F). Thus we find no evidence for a role of Wnt ligands in regulating proliferation and differentiation of young or aged HSCs.

**Porcn inhibition had minimal effects on adult murine hematopoiesis.**

We considered the possibility that stromal rather than hematopoietic Wnts regulated hematopoiesis. Deletion of *Porcn* from the stroma using the *Rosa-CreER* T2 driver gave no hematopoietic phenotype, but stromal excision of *Porcn* might have been incomplete. As a second approach to inhibit Wnt secretion in both hematopoietic and BM niche cells, we used the PORCN inhibitor C59. We previously reported that as little as 5-10 mg/kg (mpk) daily C59 for 20 days suppressed the growth of MMTV-WNT1-driven mouse mammary tumors, with significant downregulation of Wnt target genes. PORCN inhibition did not result in significant toxicity at effective doses, including no obvious small intestine or BM toxicity. Substantially higher dose C59 (50 mpk daily for up to 20 days) blocked Wnt signaling in the small intestine, as evidenced by decreased stem cell proliferation and downregulation of Wnt target genes. We saw no effect of that dose on WBCs, RBCs, platelets, and neutrophils (Figure S5A-S5D). The C59 was active, as expression of *Axin2* in intestine samples of C59 treated mice was significantly reduced. Again consistent with a lack of pre-existing Wnt signaling, C59 did not alter the expression of *Axin2* and *c-Myc* in the BM of the same mice (Figure 5A). Expression of *Cyclin D1* was modestly but non-significantly reduced in BM samples of C59 treated mice (Figure 5A). Importantly, the frequency of myeloid progenitors,
including CMP, GMP and MEP, did not differ between C59 and vehicle-treated mice (Figure 5G). Furthermore, FACS analysis showed no changes in LT-HSCs, ST-HSCs, and HPCs numbers, while the CLP cell numbers were non-significantly increased in C59 treated mice (Figure 5C, 5D, S5E). Thus, pharmacologic inhibition of Wnt secretion from stroma and hematopoietic cells in adult mice had minimal effects on normal hematopoiesis.

Discussion

In this study, we genetically and pharmacologically blocked Wnt secretion by targeting the Wnt O-acyltransferase, Porcn, in the hematopoietic system. We find that hematopoietic and stromal production of Wnts is dispensable for the maintenance, proliferation and differentiation of adult HSCs. Porcn excision using three different Cre drivers consistently produced no overt hematopoietic phenotype. Targeting PORCN in the stem cell niche either by the PORCN inhibitor C59, or by excision in Rosa-CreER\textsuperscript{T2}/Porcn\textsuperscript{Del} mice similarly had minimal effects on adult hematopoiesis. We conclude that hematopoietic Wnts are dispensable in adult hematopoiesis and that Wnts from the niche play a limited, if any, role in normal adult hematopoiesis. Thus, therapeutic targeting of Wnt secretion by PORCN inhibitors could be beneficial for the patients with high Wnt diseases without immediate toxicity on HSCs and blood cell production.

Wnts have long been proposed to play a role in self-renewal of HSC, but the data is generally indirect. Studies that implicate Wnt signaling in the maintenance of HSC have generally targeted downstream proteins such as \(\beta\)-catenin, APC, and GSK3 present in the signal-receiving cells.\textsuperscript{11,41-43} However, these proteins regulate, and can be regulated by, additional signaling pathways, so phenotypes arising from their mutation cannot prove a role for Wnt proteins. For example, \(\beta\)-catenin plays a role in stabilizing cadherin/actin interactions at the membrane, APC regulates chromosomal segregation and DNA methyltransferase expression independent of \(\beta\)-catenin, and GSK3 is downstream in diverse pathways such as insulin and hedgehog signaling and may regulate the stability of many proteins besides \(\beta\)-catenin.\textsuperscript{44-50} Abnormal \(\beta\)-catenin signaling has been implicated in CML and AML.\textsuperscript{51,52} Our data are not inconsistent with studies that demonstrate stabilization of \(\beta\)-catenin in leukemia occurs from diverse downstream events such as inactivation of GSK3 and increased translation of \(\beta\)-catenin mRNA rather than an increase in Wnt ligand expression.\textsuperscript{53,54} There are few studies that focus specifically on the role of Wnt ligands in the self-renewal of HSCs and they mostly examined fetal rather than adult hematopoiesis. Wnts may have different
roles in embryonic versus adult hematopoiesis, as embryonic knockout of Wnt3a and Wnt4 impaired the self-renewal function of HSC.\textsuperscript{10,55} This contrasts with the lack of requirement for hematopoietic or stromal Wnt production in our study in adult hematopoiesis. While we did not overtly stress BM function, we subjected mice to serial bone marrow transplantation, and assessed HSC function in \textit{in vitro} culture. These assays, which can be considered stressors of HSC function, also did not demonstrate any role for PORCN in adult hematopoiesis.

Both stromal niche and hematopoietic cells express various Wnt genes and so both sources were proposed to be involved in different steps of hematopoiesis.\textsuperscript{56,57} One limitation of this study is that we did not directly demonstrate that PORCN knockout or inhibition blocked Wnt secretion from these specific hematopoietic or stromal cells. However, multiple lines of evidence demonstrate that non-palmitoleated Wnts are inactive in all other cell types tested. It is important to note that PORCN inhibition will not alter the abundance of Wnt mRNA nor Wnt protein, but will, by inhibiting Wnt modification, block Wnt travel to the cell surface, and the ability of Wnts to interact with its receptors. Our results exclude cell-autonomous requirements for Wnts in murine adult hematopoiesis. Pharmacological targeting of PORCN was also well tolerated, excluding a major role for stromal Wnts. Possibly, longer-term or more complete inhibition of stromal Wnt secretion will reveal differences in HSCs that are not apparent in 20 days of treatment.

Florian et al. proposed that a shift from canonical to non-canonical Wnt signaling is involved in aging of HSCs and suggested a role for hematopoietic Wnt5a in this process.\textsuperscript{4} However, we did not observed any defect in the proliferation or frequencies of HSCs in either young and aged $\text{Porcn}^{\text{Del}}$ mice and our data exclude an intrinsic source of hematopoietic Wnts for normal hematopoiesis. We confirmed that several Wnt ligands are highly expressed in murine BM cells, but our data indicate they do not play a significant role in normal hematopoiesis. We speculate that these Wnts, instead might play a role in bone anabolism or vasculogenesis.\textsuperscript{58,59}

$\beta$-catenin-dependent and independent Wnt signaling might reciprocally regulate hematopoiesis. For instance, the pattern of Wnt gene expression is distinctive in hematopoietic tissues. $\text{Wnt3a}$ is only expressed in hematopoietic cells, while the $\beta$-catenin-independent $\text{Wnt5a}$ is expressed in both hematopoietic and stromal cells. $\beta$-catenin independent Wnts could antagonize Wnt/$\beta$-catenin signaling in HSCs and enhance their repopulation capacity.\textsuperscript{17} However, inhibition of Wnt/$\beta$-catenin signaling is also reported to impair hematopoiesis in mice.\textsuperscript{8,9,11,60} In the current study, we suppressed both $\beta$-catenin-dependent and independent Wnts in all hematopoietic cells, but we observed intact
hematopoiesis. It is possible that there is a balance between these pathways in hematopoietic cells that control precise hematopoiesis. Consequently, disruption of each Wnt/β-catenin dependent or β-catenin independent pathway individually could affect hematopoiesis. In contrast, targeting all Wnt pathways together might have a balanced and hence limited effect on hematopoiesis. This is a reassuring finding as novel agents that pharmacologically inhibit PORCN function enter clinical trials.39,40
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Authorship contributions

ZK, AN, AK, Edison, DGT and DMV designed the experiments and analyzed the data, ZK, AN, AK, and E performed the research, and ZK and DMV wrote the paper.

Disclosure of Conflicts of Interest

The authors have no conflict of interest to report.
References


Figure Legends

Figure 1. *Porcn* inactivation blocks Wnt signaling and caused alopecia in adult mice, but did not impair hematopoiesis.

A) Inactivation of *Porcn* down-regulated expression of Wnt/β-catenin target gene (*Axin2*) in skin samples of *Rosa-CreER<sup>T2/Porcn<sup>Del* mice. Expression was normalized to *PGK*. The mean values of *Porcn<sup>WT* samples were set to 1 within each group. (n=4 mice in each group, two independent experiments, * p<0.05, Mann-Whitney test).

B) Tamoxifen supplementation in chow for 3 weeks caused progressive alopecia in mice. Photo taken 7 weeks after start of tamoxifen.

C) Loss of hair follicles in skin after *Porcn* inactivation. H&E stain. Scale bar = 200 μm

D) CBC after deletion of *Porcn* (5-6 weeks after tamoxifen chow administration). Total white blood and lymphocyte counts did not differ significantly between *Rosa-CreER<sup>T2/Porcn<sup>WT* and *Porcn<sup>Del* mice (n=5) (p<0.05, NS, not significant, Mann-Whitney test), while neutrophils were modestly increased (representative data of four independent experiments, p=0.019, Mann-Whitney test).

E) *Porcn* was down-regulated in BM of *Porcn* inactivated mice 4 weeks after tamoxifen chow administration. Expression assessed by qRT-PCR was normalized to *PGK and β-actin*. Individual expression was normalized to mean of respective *Porcn<sup>WT* samples within the same group. (n=6, two independent experiments, *p<0.01, Mann-Whitney test).

F) The total number of LT-HSC (Lin-, Sca1+,c-Kit+, CD150+, CD48-), ST-HSC (Lin-, Sca1+,c-Kit+, CD150-, CD48-), and HPC (Lin-, Sca1+,c-Kit+, CD150-, CD48+) did not differ between *Rosa-CreER<sup>T2/Porcn<sup>WT* and *Porcn<sup>Del* mice (right panel). Representative flow cytometry data is shown in left panel (n=6, cell number per one leg, two independent experiments, p>0.05, Mann-Whitney test).

G) Total common lymphoid progenitor (CLP) cells (one leg) were similar in *Rosa-CreER<sup>T2/Porcn<sup>WT* and *Porcn<sup>Del* mice 4 weeks after tamoxifen. (n=6 in each group, p>0.05, Mann-Whitney test).

H) Total number of myeloid progenitor cells (one leg) were similar in *Rosa-CreER<sup>T2/Porcn<sup>WT* and *Porcn<sup>Del* mice (middle and right panels) (n=6 mice, four independent experiments, p>0.05, Mann-Whitney test). Left panel showed the representative gating for Common Myeloid Progenitor (CMP), Megakaryocyte-Erythrocyte Progenitor (MEP), and Granulocyte-Monocyte Progenitor (GMP).
Figure 2. Successful BM reconstitution from *Porcn*<sup>Del</sup> HSCs

A) *Rosa-CreER<sup>TE2</sup>*/*Porcn<sup>Del</sup>* BM cells formed hematopoietic progenitor colonies in culture. *Porcn<sup>Del</sup>* (n=5 wells, 2 mice) were compared to *Porcn*<sup>WT</sup> donors (n=4 wells, 2 mice). A mild increase in granulopoiesis was observed. Total: all types of colonies including GEMM: Granulocyte, Erythrocyte, Monocyte, Megakaryocyte colonies, Gr: Granulocyte colonies, M: Monocyte colonies, Gr/M: Granulocyte and Monocyte colonies (*p< 0.05 and **p<0.01. NS: p>0.05, Mann-Whitney test.)

B) *Rosa-CreER<sup>TE2</sup>*/*Porcn<sup>Del</sup>* BM successfully reconstitutes sub-lethally irradiated recipient mice. Analysis was performed 5 months after BM transplantation (BMT). Representative gating for donor BM Lymphocytes and Neutrophils (CD45.2+). (n=4 and 5 for *Porcn*<sup>WT</sup> and *Porcn*<sup>Del</sup> mice respectively, two independent experiments)

C) Quantification of donor neutrophil frequency in total BM cells from recipient mice.

D) Quantification of donor lymphocyte frequency in total BM cells from recipient mice.

E) Representative LT-HSC, and HPC gating based on donor cells (CD45.2+)(right panel). Quantification of donor LT-HSC, and HPC frequency in total BM cells from recipient mice (n=4 and 5 for *Porcn*<sup>WT</sup> and *Porcn*<sup>Del</sup> mice respectively, two independent experiments).

F) Representative myeloid progenitor gating based on donor cells (left panel). Quantification of donor myeloid progenitor frequency in total BM cells from recipient mice.

Figure 3. Normal function of adult HSC after inducible deletion of *Porcn* in *Mx1-Cre* mice.

A) Normal white blood counts (WBC) after Cre-mediated inactivation of *Porcn* in *MX1-Cre/Porcn<sup>flox</sup>* mice. The poly I:C induced transient leukopenia was similar in *Porcn*<sup>Del</sup> and *Porcn*<sup>WT</sup> mice (three independent experiments, p>0.05 Mann-Whitney test.)

B) Cre expression leads to *Porcn* deletion in blood samples of *Mx1-Cre/Porcn<sup>Del</sup>* mice as assessed by PCR of gDNA (Del: excised *Porcn* allele, FL: floxed allele, WT: wildtype allele). Each lane represents an individual mouse.

C) Wnt target genes were not down-regulated in PBMCs from *Mx1-Cre/Porcn<sup>Del</sup>* mice. Expression of *Axin2*, and *c-Myc* were normalized to *PGK* and *HPRT*. (n=5 mice in each group, p>0.05, Mann-Whitney test)

D) Myeloid progenitors frequency in *Mx1-Cre/Porcn*<sup>WT</sup> and *Mx1-Cre/Porcn*<sup>Del</sup> did not differ significantly (n=4 per group, two independent experiments).

E) LT-HSC, ST-HSC, and HPC frequency in *Mx1-Cre/Porcn*<sup>WT</sup> and *Mx1-Cre/Porcn*<sup>Del</sup> mice did not significantly differ (n=4 per group, two independent experiments).
F) Differentiation ability of Porcn deleted HSC did not differ from control in colony forming assay. Quantification of total number of colonies (CFU-GM, BFU-E, CFU-GEMM) from 3 wells in each group.

Figure 4. Normal hematopoiesis after Vav-Cre mediated complete inhibition of Wnt secretion from hematopoietic cells.

A) Complete excision of Porcn in blood samples of Vav-Cre/PorcnDel mice was assessed by PCR of gDNA (Del: excised Porcn allele, FL: floxed allele, WT: wildtype allele). Each lane represents an individual mouse from eight independent experiments.

B) Expression of Wnt target genes Axin2, c-Myc, and Cyclin D1 was not down-regulated in Porcn inactivated BM samples. No expression of Porcn was detected in BM samples of Vav-Cre/PorcnDel mice (n=7 per group, (three independent experiments, *p<0.05, NS p>0.05, Mann-Whitney test).

C) Similar myeloid progenitor cell numbers were observed in Vav-Cre/PorcnWT and PorcnDel mice. Graphs represent the CMP, MEP, and GMP cell numbers from one leg (n=6 per group, three independent experiments, NS p>0.05 Mann-Whitney test).

D) The common lymphoid progenitor cell numbers (one leg) were not significantly different in Vav-Cre/PorcnWT and PorcnDel mice (n=6 mice per group, three independent experiments, p>0.05 Mann-Whitney test).

E) The LT-HSCs, ST-HSCs, HPCs numbers (one leg) were not significantly different in one-year-old Vav-Cre/PorcnWT and PorcnDel mice (n=6 mice per group, two independent experiments, p>0.05 Mann-Whitney test).

F) The LT-HSCs, ST-HSCs, HPCs numbers (one leg) were not significantly different in 6- to 8-week-old Vav-Cre/PorcnWT and PorcnDel mice (n=4 mice per group, p>0.05 Mann-Whitney test).

Figure 5. Inhibition of Wnt secretion by Porcn inhibitor in BM niche had limited effect on hematopoiesis.

A) Expression of Wnt target gene Axin2 was significantly down-regulated in intestine samples of mice treated with 50 mpk for 20 days (two independent experiments, p= 0.007, Mann-Whitney test). However, expression of Axin2, c-myc, and Cyclin D1 was not significantly altered in BM samples of these C59 treated mice. (n=7, two independent experiments, p>0.05, Mann-Whitney test).

B) The myeloid progenitor cells (one leg) were not different in vehicle and C59 treated mice (n=7, three independent experiments, p>0.05, Mann-Whitney test).
C) C59 treatment did not alter the total LT-HSC, ST-HSC, and HPC numbers (one leg). Representative flow cytometer data is shown in the left. The HSC numbers were graphed in the right. (n=7, three independent experiments, p>0.05, Mann-Whitney test).

D) The common lymphoid progenitor cell numbers (one leg) were not significantly different in vehicle and C59 treated mice (n=7, two independent experiments, p>0.05, Mann-Whitney test).
Figure 1
Figure 2
Figure 3

Porcn Del Porcn WT

H2O FLWT

Mx1-Cre mice

Day 0 Del Del Del Del WT WT WT WT

Day 15

Day 30

Day 45

WBC Counts x 10^3/μl

5

10

15

Mx1-Cre A

Del Del FL WT

Mice blood samples

Porcn

C

Relative Expression in blood

Axin2 Del Del WT WT

c-myc Del Del Del Del

D

E

CD48 FITC

CD150 PE

Mx1-Cre

Porcn WT Porcn Del

% of total BM

F

Colony Counts

0 10 20

Porcn WT Del

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Figure 4
Figure 5

A

Normalized expression

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<td>CycD1</td>
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Intestine

BM samples

C

CD48 FITC

CD150 PE

Veh C59

C59

LT-HSC (x10^3 Cells)

ST-HSC (x10^3 Cells)

HPC (x10^3 Cells)

Veh C59

C59

D

CLP (x10^6 Cells)

Veh C59

NS NS NS

0.5 1.5 0

2 1

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Wnts are dispensable for differentiation and self-renewal of adult murine hematopoietic stem cells

Zahra Kabiri, Akihiko Numata, Akira Kawasaki, Edison Blank, Daniel G. Tenen and David M. Virshup

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