HEMATOPOIESIS AND STEM CELLS

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

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Key Points

- 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.

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 (HSCs) in vivo and in vitro through suppressing the Wnt/β-catenin pathway,1-4 however, other studies found that β-catenin–independent Wnt signaling positively regulates HSC proliferation and self-renewal.5-7 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.5,9 Moreover, embryonic knockout of either Wnt5a or β-catenin (Cmbh1) impaired HSC self-renewal only in secondary, but not primary, bone marrow (BM) transplantsations (BMTs).10,11 β-catenin abundance can influence hematopoiesis, as forced overexpression of β-catenin in HSCs resulted in stem cell exhaustion.12 However, under normal circumstances, β- and γ-catenin are dispensable for normal adult hematopoiesis.13,14 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.15

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, whereas in others, such as the intestine, the stroma rather than the epithelium is the essential source of Wnts.16 In hematopoiesis, the source of Wnts is unclear. Some reports favor the BM niche as a source of Wnt ligands,3,6,17,18 whereas others support a major role for autocrine production.2,4,10 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.19-24 All Wnts are posttranslationally modified in the endoplasmic reticulum by addition of a palmitoleate moiety that is required both for their secretion and for binding to their cell-surface receptor Frizzled.25-28 Wnt palmitoleation is catalyzed by PORCN, a nonredundant membrane-bound O-acyl transferase. Genetic knockout or pharmacologic inhibition of Porcn therefore eliminates the activity, but not the expression, of all Wnts.29,30 Although 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 pharmacologic approach to investigate the role of hematopoietic Wnts in hematopoiesis, by knocking out Porcn in HSCs of mice using 3 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.


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The online version of this article contains a data supplement.

There is an Inside Blood Commentary on this article in this issue.

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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.\(^{26,31}\) Porcn\(^{lox}\) mice were backcrossed to C57BL/6 mice. Porcn\(^{lox}\) mice were crossed with Rosa-Cre\(^{ERT2}\),\(^{22}\) Mx1-Cre,\(^{33}\) and Vav-Cre mice.\(^{34}\) Age- and sex-matched mice were used in all experiments. For BMT, C57BL/6/Ly5.1 mice were used. Porcn genotyping, expression analysis, and primers was previously described.\(^{16,26,31}\) All mouse procedures were approved by the institutional care and use committee.

Inducible Porcn deletion and drug administration

Tamoxifen chow (80 mg tamoxifen/kg body weight assuming 20-g mice eat 3 g of 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 of Poly I:C every other day for 7 doses. Vehicle or C59 (50 mg/kg per day) was administered by gavage for 20 days as described previously.\(^{16}\)

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, eBioscience, or BioLegend. The antibodies used in our study were: Gr-1 (RC2C1), 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), Scal (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 BM cells from Porcn\(^{lox}\) or control marrow were analyzed using Diva (BD Pharmingen) and FlowJo (Tree Star) software.

BMT

For BMT, a total of 1 × 10\(^8\) BM cells from either control, Rosa-Cre\(^{ERT2}/Porcn\(^{lox}\)), or Mx1-Cre/Porcn\(^{lox}\) mice (CD45.2) were transplanted through tail vein injection into lethally irradiated CD45.1 congenic recipient mice. Samples collected 8 to 16 weeks after transplantation were analyzed by fluorescence-activated cell sorter (FACS) or secondary BMT.

Colony-forming assay

A total of 1 × 10\(^3\) BM cells were plated in the presence of Methocult M3434. Colonies were scored 2 weeks later. All assays were conducted in triplicate.

Proliferation assay

The Click-iT EdU Alexa Fluor 555 Imaging kit and 5-ethyl-2′-deoxyuridine (EdU) were from Life Technologies. Mice received 1.5 mg/kg EdU by intraperitoneal injection 24 hours prior to sacrifice. BM was scrapped to obtain 10 000 LSK (Lin−, Scal−, c-Kit+) cells on slides. Cells were fixed, washed, permeabilized, and stained based on the manufacturer’s instructions. Slides were mounted in fluorescent mounting medium with 4,6-diamidino-2-phenylindole (VectorShield) 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. The 2-tailed t test was performed in Excel for Mac 2011, version 14.3.2.

Results

Total-body knockout of Porcn in adult mice

We crossed Porcn\(^{lox}\) with Rosa-Cre\(^{ERT2}\) mice to generate mice with widespread expression of a tamoxifen-sensitive Cre recombinease.\(^{31,32}\) Porcn is on the X chromosome. To induce Porcn inactivation, control (Rosa-Cre\(^{ERT2}/Porcn\(^{WT,WT}\)) or Porcn\(^{WT,Y}\)) and inducible Porcn knockout mice (Rosa-Cre\(^{ERT2}/Porcn\(^{lox,lox}\)) or Porcn\(^{lox,Y}\)) received tamoxifen chow for 3 weeks. The excised allele(s) are referred to collectively as Porcn\(^{lox}\). Consistent with the well-documented role of Wnt/b-catenin signaling in hair follicle formation,\(^{20}\) Rosa-Cre\(^{ERT2}/Porcn\(^{lox}\) mice showed progressive global alopecia beginning 5 weeks after starting tamoxifen chow (Figure 1B).\(^{22,35}\) Substantial excision of Porcn exon 3 was observed in skin samples (supplemental Figure 1B, available on the Blood Web site). Histologically, Porcn\(^{lox}\) mice exhibited an impaired skin structure with lack of hair follicles in the dermis and an increased number of cells in the epidermal layer (Figure 1C), similar to the phenotype seen after inactivation of either b-catenin or the Wnt transporter Wls in the skin.\(^{20,36}\) As expected, Porcn inactivation was accompanied by a marked reduction of Porcn messenger RNA (mRNA) in skin (Figure 1A). In addition, there was a decrease in Wnt/b-catenin signaling, shown by loss of b-catenin protein in hair follicles (supplemental Figure 1E) and downregulation of the Wnt/b-catenin target gene Axin2 (Figure 1A). These results confirm the Porcn\(^{lox}\) mouse as a useful tool to study the tissue-specific role of Wnt secretion.

The Porcn\(^{lox}\) mice started to lose body weight 4 weeks after tamoxifen treatment (supplemental Figure 1C). 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 BM, where we confirmed multiple Wnt genes are expressed (supplemental Figure 1A). To test whether hematopoiesis was impaired in the Rosa-Cre\(^{ERT2}/Porcn\(^{del}\), mice, we examined complete blood counts (CBCs) 4 to 5 weeks after starting tamoxifen chow. The CBCs of Porcn\(^{del}\) and Porcn\(^{WT}\) blood samples did not differ significantly in hemoglobin (Hg), erythrocytes (red blood cells [RBCs]), platelets, and total white blood counts (WBCs) (Figure 1D, supplemental Figure 1D). There was a modest increase in mature neutrophils as compared with controls (Figure 1D), which we suspect is secondary to the inflammation accompanying hair loss 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 polymerase chain reaction (PCR) from genomic DNA 2 weeks after stopping tamoxifen (supplemental Figure 1F).

We investigated the role of hematopoietic Wnt secretion in the maintenance of 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 quantitative reverse transcription PCR (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-HSCs, Lin–, Sca-1+, c-kit+, CD150+, CD48–), short-term HSCs (ST-HSCs, Lin–, Sca1+, c-Kit+, CD150–, CD48+), and hematopoietic progenitor cells (HPCs; Lin–, Sca1+, c-Kit+, CD150+, CD48+) in these mice (Figure 1F).37 Moreover, the number of common lymphoid progenitors (CLPs; CD127+, c-Kit+, Sca1+, Flik2+) and myeloid progenitors (common myeloid progenitor [CMP], granulocyte-monocyte progenitor [GMP], megakaryocyte-erythroid progenitor [MEP]) in PorcnDel mice were in the range observed in the PorcnWT mice (Figure 1G-H, supplemental Figure 1H).38 Consistent with peripheral blood findings, there was a greater frequency of granulocytes in the BM of PorcnDel mice (supplemental Figure 1G). Collectively, the global knockout of Porcn in adult Rosa-CreERT2 mice (while producing significant hair, weight, and neurologic effects) hematologically caused modestly increased numbers of colonies compared with controls (Figure 2A, supplemental Figure 1I). 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.

Luïs et al reported that Wnt3a-deficient HSCs could not successfully reconstitute the BM of WT lethally irradiated recipient mice after a secondary BMT, suggesting a role for WNT3A in the maintenance of embryonic HSCs.10 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 BM at 20 weeks. We confirmed that hematopoietic reconstitution was predominantly from PorcnDel rather than PorcnDel donor cells by genomic PCR of peripheral blood samples (supplemental Figure 2B). As with the colony-forming assays, this rules out the possibility that residual PorcnDel nonexcised HSCs were responsible for the successful primary BMT. The frequencies of donor neutrophils (CD45.2+, Mac1+, Gr1–), B lymphocytes (CD45.2+, CD19+, CD3e–), and
were treated with poly I:C every other day for 7 doses. WBCs were
PorcnWT
was
PorcnWT
primary recipient mice were similar regardless of whether the donor
Finally, a competitive reconstitution assay (supplemental Figure 2G-H)
Porcn
ulocyte and monocyte (Gr/M) (*
ulocyte (GEMM), granulocyte (Gr), monocyte (M), gran-
ulocyte and monocyte (Gr/M) (P < 0.05 and ** P < 0.01,
NS: P > 0.05, Mann-Whitney test). (B) Rosa-CreERT2
PorcnWM successfully reconstitutes sublethally irradi-
ated recipient mice. Analysis was performed 5 months after BMT. Representative gating for donor BM lympho-
cytes and neutrophils (CD45.2+) (n = 4 and 5 for PorcnWM and PorcnWM mice, respectively, 2 independent experi-
ments). (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-HSCs, and HPC
gating based on donor cells (CD45.2+) (right panel). Quan-
tification of donor LT-HSCs, and HPC frequency in
total BM cells from recipient mice (n = 4 and 5 for
PorcnWM and PorcnWM mice, respectively, 2 independent experi-
ments). (F) Representative myeloid progenitor
gating based on donor cells (left panel). Quantification of
donor myeloid progenitor frequency in total BM cells from
recipient mice.

To assess whether 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. PorcnDel, like PorcnWT, HSCs successfully reconstituted the
primary recipient mice (supplemental Figure 2D-F) and outcompeted
the residual PorcnWT donor cells (supplemental Figure 2C). We con-
clude that HSCs of the Rosa-CreERT2/PorcnWT mice are phenotypi-
cally and functionally normal in the absence of HSC Wnt secretion.
Finally, a competitive reconstitution assay (supplemental Figure 2G-H)
showed stable engraftment of both PorcnWT and PorcnWM HSCs.
The fraction of reconstitution from PorcnDel was slightly smaller than that
from PorcnWT 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-CreERT2 driver was unexpected. We considered the
possibility that although we had significantly reduced PORCN function
(e.g., as demonstrated by reduction of Porcn mRNA), subtotal excision
of Porcn in Rosa-CreERT2/PorcnWT mice left sufficient Wnt secretion
to maintain normal function. As a second test, we crossed PorcnDel
mice with Mxl-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 (supplemental Figure 3A). Extensive excision of
Porcn was confirmed in the peripheral blood (Figure 3B). Similar to
Rosa-CreERT2 mice, expression of Wnt target genes did not change in
the Mxl-Cre/PorcnDel mice (Figure 3C). The BM total and differential
cell counts, examined 4 months after poly I:C, remained in the normal
range (supplemental Figure 3B-D). In addition, the frequencies of
HSCs and progenitors were not affected (Figure 3D-E). 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 (supplemental
Figure 3E) 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 nondeleted 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 (CD45.2).
PorcnDel donor HSCs were capable of successful reconstitution of the
recipient (supplemental Figure 3G-H). We confirmed that recipient
hematopoiesis was by cells with deletion of Porcn exon 3 (supplemental
Figure 3F), ruling out the possibility that reconstitution was due to
survival of rare non-Porcn–deleted cells. To test the long-term pro-
genitor 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 >1 year with normal hematopoiesis, and BM analysis
showed successful reconstitution from donor PorcnDel cells (supple-
mental 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 in-
crease of granulopoiesis in the Mxl-Cre mice. Therefore, the increased
granulopoiesis in the Rosa-CreERT2/PorcnDel mice is likely to be
secondary to nonhematologic effects of decreased Wnt activity in other
organs such as skin, hair, and brain.
There was incomplete hematopoietic excision of Porcn in Mx1-Cre mice, as a faint floxed, nonexcised 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, Porcnfl 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/Porcnfl mice should exhibit impaired hematopoiesis. Again unexpectedly, Vav-Cre/Porcnfl 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-B). However, the BM expression of Axin2, c-Myc, and Cyclin D1 was not altered, indicated that hematopoietic Wnts are not controlling their expression (Figure 4B). WBCs, RBCs, and Hg were normal in the Vav-Cre/Porcnfl mice (supplemental Figure 4A-B). The frequencies of BM granulocytes and lymphocytes, and BM myeloid and CLP cell numbers were not altered in Vav-Cre/Porcnfl mice (Figure 4C-D, supplemental Figure 4C-E). Thymic cells had normal populations of double-negative, double-positive, CD4 T cells and CD8 T cells (supplemental Figure 4G).

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-HSCs. 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/PorcnWT and Porcnfl mice at any age (Figure 4E-F). In addition, the proliferation rate of aged Vav-Cre/Porcnfl LSK cells was similar to aged Vav-Cre/PorcnWT LSK cells (supplemental Figure 4F). 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-CreER2 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 to 10 mg/kg 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 mg/kg...
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.\(^1\) We saw no effect of that dose on WBCs, RBCs, platelets, and neutrophils (supplemental Figure 5A-D). The C59 was active, as expression of Axin2 and c-Myc, and Cyclin D1 was not downregulated in Porcn inactivated BM samples. No expression of Porcn was detected in BM samples of Vav-Cre/Porcn\(^{Del}\) mice \((n = 7\) per group [3 independent experiments, \(P < .05\), Mann-Whitney test]). (C) Similar myeloid progenitor cell numbers were observed in Vav-Cre/ Porcn\(^{WT}\) and Porcn\(^{Del}\) mice. Graphs represent the CMP, MEP, and GMP cell numbers from 1 leg \((n = 6\) per group, 3 independent experiments, \(P > .05\) Mann-Whitney test). (D) The CLP cell numbers (1 leg) were not significantly different in Vav-Cre/Porcn\(^{WT}\) and Porcn\(^{Del}\) mice \((n = 6\) mice per group, 3 independent experiments, \(P > .05\) Mann-Whitney test). (E) The LT-HSC, ST-HSC, HPC numbers (1 leg) were not significantly different in 1-year-old Vav-Cre/Porcn\(^{WT}\) and Porcn\(^{Del}\) mice \((n = 6\) mice per group, 2 independent experiments, \(P > .05\) Mann-Whitney test). (F) The LT-HSC, ST-HSC, HPC numbers (1 leg) were not significantly different in 6- to 8-week-old Vav-Cre/ Porcn\(^{WT}\) and Porcn\(^{Del}\) mice \((n = 4\) mice per group, \(P > .05\) Mann-Whitney test).

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 3 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-Cre\(^{ER}\)/Porcn\(^{Del}\) 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 HSCs, but the data are generally indirect. Studies that implicate Wnt signaling in the maintenance of HSCs have generally targeted downstream proteins such as \(\beta\)-catenin, adenomatous polyposis coli (APC), and GSK3 present in the signal-receiving cells.\(^{11,41-43}\) 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 β-catenin. Abnormal β-catenin signaling has been implicated in chronic myelogenous leukemia and acute myelogenous leukemia. Our data are not inconsistent with studies that demonstrate that stabilization of β-catenin in leukemia occurs from diverse downstream events such as inactivation of GSK3 and increased translation of β-catenin mRNA rather than an increase in Wnt ligand expression. 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 vs adult hematopoiesis, as embryonic knockout of Wnt3a and Wnt4 impaired the self-renewal function of HSCs. This contrasts with the lack of requirement for hematopoietic or stromal Wnt production in our study in adult hematopoiesis. Although we did not overtly stress BM function, we subjected mice to serial BMT, and assessed HSC function 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. 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 nonpalmitoylated 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. Pharmacologic 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 noncanonical Wnt signaling is involved in aging of HSCs and suggested a role for hematopoietic Wnt5a in this process. However, we did not observe any defect in the proliferation or frequencies of HSCs in either young and aged Porcn−/− 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.

β-catenin–dependent and β-catenin–independent Wnt signaling might reciprocally regulate hematopoiesis. For instance, the pattern of Wnt gene expression is distinctive in hematopoietic tissues. Wnt3a is only expressed in hematopoietic cells, whereas the β-catenin–independent Wnt5a is expressed in both hematopoietic and stromal cells. β-catenin–independent Wnts could antagonize Wnt/β-catenin signaling in HSCs and enhance their repopulation capacity. However, inhibition of Wnt/β-catenin signaling is also reported to impair hematopoiesis in mice. In the current study, we suppressed both β-catenin–dependent and β-catenin–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 Wnt/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.

Acknowledgments

The authors thank Dr Tim Chan Hon Man, Hui Si Kwok, and Jamal Aliyev for technical support.

This work was supported by grants to D.M.V. and D.G.T. by the National Research Foundation Singapore under its STAR Award.
Authorship

Contribution: Z.K., A.N., A.K., Edison, D.G.T., and D.M.V. designed the experiments and analyzed the data; Z.K., A.N., A.K., and Edison performed the research; and Z.K. and D.M.V. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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