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REVIEW

Hematopoietic stem cell fate decisions are regulated by Wnt antagonists: Comparisons and current controversies

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Wingless and int (Wnt) proteins are secreted proteins that are important for regulating hematopoietic stem cell self-renewal and differentiation in the bone marrow microenvironment in mice. The mechanisms by which Wnt signaling regulates these hematopoietic cell fate decisions are not fully understood. Secreted Wnt antagonists, which are expressed in bone and bone marrow stromal cells, either bind to Wnt ligands directly or block Wnt receptors and co-receptors to halt Wnt-mediated signal transduction in both osteolineage and hematopoietic cell types. Secreted frizzled related proteins-1 and -2, Wnt inhibitory factor-1, Dickkopf-1, and Sclerostin are Wnt antagonists that influence hematopoietic cell fate decisions in the bone marrow niche. In this review, we compare and contrast the roles of these Wnt antagonists and their effects on hematopoietic development in mice, and also discuss the clinical significance of targeting Wnt antagonists within the context of hematopoietic disease.

In the mouse, it is clear that critical relationships between hematopoietic stem cells (HSCs) and nonhematopoietic cells in bone and the bone marrow (BM) cavity exist [1]. External signals from the bone microenvironment initiate internal transcriptional programs in the HSC to mediate cell fate decisions, such as self-renewal, quiescence, differentiation, and cell death [2]. Within the past 15 years, wingless and int (Wnt) signaling has emerged as an important facilitator of cell fate decisions during hematopoietic differentiation in the BM. Wnt signaling is also critical for maintaining bone homeostasis, and experimental evidence shows that disruptions in the regulation of Wnt pathway genes in osteolineage cells can affect hematopoietic differentiation in a non–cell autonomous manner. As such, investigation of the mechanisms by which Wnt signaling is activated and deactivated during hematopoiesis has been an area of focused research efforts. Of note, naturally occurring soluble Wnt antagonist proteins have been identified in osteolineage cells that play functional roles in directing cell fate decisions in HSCs and committed hematopoietic lineages. In this review, we provide a brief overview of Wnt signaling and the role of Wnt signaling in cell fate decisions in HSCs and early hematopoietic progenitors, as well as a comprehensive comparison of the studies of Wnt antagonists' roles during hematopoiesis.

Canonical and noncanonical Wnt signaling

Wnts are secreted glycoproteins that range in size from 350 to 400 amino acids [3]. The Frizzled (FZD) proteins are a family of seven-pass transmembrane receptors to which Wnts bind. Signaling through FZD proteins can be mediated by G-proteins [4–7]. To date, 19 different Wnt ligands and 10 FZD receptors have been identified in humans and mice. The diversity of the Wnt and FZD protein families, their widespread tissue expression, and requirement during embryonic development, has challenged the field to identify common as well as unique functions for each Wnt and FZD protein [8,9]. The discovery that Wnts also utilize distinct co-receptor proteins that initiate canonical and

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noncanonical Wnt signaling pathways has added another level of complexity to the field. We present a broad summary of canonical and noncanonical Wnt signaling pathways and describe the roles of these two types of Wnt signaling pathways in HSCs and early hematopoietic progenitors.

Canonical Wnt signaling

The defining event that identifies the activation of canonical Wnt signaling is the translocation of β-catenin, a transcriptional coactivator, to the nucleus, where it binds to the classically canonical Wnt transcription factors, T-cell factor (TCF) and lymphoid enhancer binding factor (LEF) to initiate transcription of target genes. In the absence of Wnt ligand binding to FZD cell surface receptors, β-catenin is retained in the cytoplasm in a protein complex that consists of axis inhibition protein 1 (AXIN1), adenomatous polyposis coli complex, and the serine/threonine glycogen synthase kinase 3β (GSK3β) (Fig. 1A). In this inactivated state, β-catenin protein is phosphorylated by GSK3β, ubiquitinated, and proteolytically degraded.

In contrast, activation of canonical Wnt signaling results in rearrangement of cytoplasmic proteins in which Dishevelled (DSH, DVL), a scaffolding protein, is used to dock AXIN1 and GSK3β to the FZD co-receptors, LRP4, LRP5, or LRP6. The LRPs are phosphorylated by GSK3β, forming an activated FZD/LRP receptor complex. In the activated state, β-catenin is no longer phosphorylated by GSK3β, is not targeted for proteosomal degradation, and translocates to the nucleus to initiate gene transcription (Fig. 1B). This model has recently been challenged by the demonstration that dissociation of the cytoplasmic protein complex after Wnt signaling activation does not occur, and the authors suggest that newly synthesized,

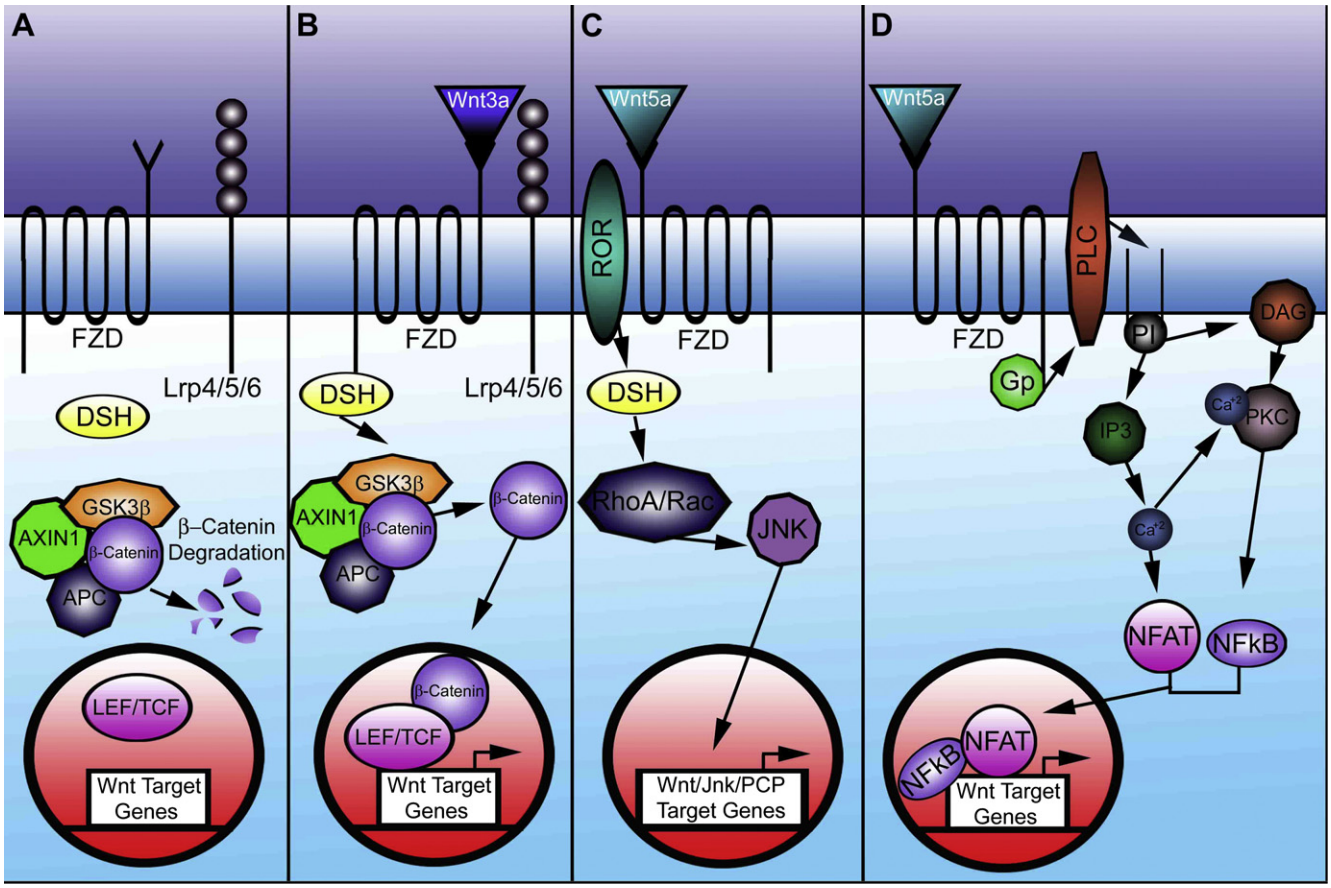

Figure 1. Canonical and noncanonical Wnt signaling pathways. (**A**) In canonical Wnt signaling, The FZD is inactive until Wnt ligands bind to FZD receptors and LRP4/5/6 co-receptors. In the absence of ligand binding, β-catenin is sequestered in the cytoplasm in a protein complex consisting of the AXIN1, GSK3β, and adenomatous polyposis coli (APC) complex. In the inactive state, GSK3β phosphorylates β-catenin, marking it for proteasomal degradation. (**B**) In the presence of Wnt ligand binding to FZD/LRP, DSH protein signals the AXIN1/GSK3β/APC complex to release β-catenin, preventing its degradation and instead, promotes its translocation to the nucleus. Migration of β-catenin to the nucleus results in the activation of TCF and LEF-1 transcription factors and up-regulation of Wnt target genes. (**C**) The WNT/JNK/PCP pathway is activated by Wnt binding to FZD/ROR receptor complexes, and involves DSH and RhoA/RAC family proteins, culminating in the activation of JNK family proteins. (**D**) The Wnt-Ca^{+2} pathway is also initiated by Wnt and FZD binding, but activates distinct downstream signaling mediators from the WNT/JNK/PCP pathway. In the Wnt-Ca^{2+} pathways, G-protein (Gp) signaling is activated, culminating in inositol triphosphate (IP3)-mediated release of intracellular Ca^{+2}, and the activation of protein kinase C (PKC) to trigger the translocation of nuclear factor of activated T cells (NFAT) and nuclear factor κB (NFκB) transcription factors to the nucleus.

nonphosphorylated β-catenin is the mediator of Wnt target gene transcription [10]. WNT3A is often used as the prototypical canonical Wnt ligand in hematopoietic studies, and LRP5 and LRP6 co-receptors are categorized as canonical Wnt signaling co-receptors [9]. LRP4 is expressed in osteoblasts [11], but is not expressed in hematopoietic cells [12]. It should also be noted that WNT3A can stimulate β-catenin–independent signaling in osteoblasts during bone formation [13], so when referring to Wnt signaling within the hematopoietic niche, the cellular context must be described clearly.

Noncanonical Wnt signaling

Both canonical and noncanonical Wnt signals can utilize FZD receptors. However, in contrast to canonical Wnt signaling, noncanonical Wnt signaling is defined as a set of alternative Wnt-activated pathways that do not require β-catenin and utilize a co-receptor that is distinct from LRP4, LRP5, or LRP6, or no co-receptor at all. Multiple pathways of noncanonical Wnt signaling have been described, but we will focus on the Wnt/JUN N-terminal kinase (JNK)/planar cell polarity (PCP) pathway and the Wnt-Ca^{+2} pathways in this review. These two noncanonical Wnt signaling pathways are involved in hematopoiesis and are the best understood noncanonical Wnt pathways in mice and humans [14] (Figs. 1C and 1D).

Wnt/JNK/PCP pathway. WNT5A is often described as a prototypical noncanonical Wnt ligand. WNT5A-mediated signaling is initiated by binding to FZD family receptors and uses the receptor tyrosine kinase–like orphan receptor (ROR) proteins as co-receptors. The ROR1 and ROR2 co-receptors are considered to be prototypical noncanonical Wnt receptors. Similar to LRP5 and LRP6, ROR1 and ROR2 are phosphorylated by GSK3β and associate with DSH proteins after WNT5A binding, to form a FZD/ROR-activated receptor complex [15] (Fig. 1C). In the noncanonical Wnt/JNK/PCP pathway, RhoA/RAC family proteins are up-regulated and JNK family members are ultimately activated [14].

Wnt-Ca^{2+} pathway. The Wnt-Ca^{+2} pathway can also be initiated via binding of WNT5A to a FZD/ROR complex, but in contrast to the Wnt/JNK/PCP pathway, G-protein mediated signaling is activated (Fig. 1D). G-protein signaling results in phospholipase C–mediated cleavage of phosphatidylinositol, which produces diacylglycerol and inositol 1,4,5,-triphosphate. Diacylglycerol activates protein kinase C, and inositol 1,4,5,-triphosphate binds to gated calcium channels to release internal stores of Ca^{+2} [16–18]. The Wnt-Ca^{+2} pathway culminates in the activation the expression of nuclear factor of activated T cells and nuclear factor κB, transcriptional co-activators that translocate from the cytoplasm to the nucleus and up-regulate noncanonical Wnt target genes. Some of these targets suppress canonical Wnt signaling [14,19].

Wnt signaling in hematopoietic development

Canonical Wnt signaling in HSCs

The hierarchy of blood cell lineages stemming from the HSC and their cell surface marker profiles in the mouse have been reviewed extensively elsewhere [20]. The hematopoietic lineages that are discussed in this article are summarized in Figure 2. The role of canonical Wnt signaling as a positive or negative regulator of HSCs and committed progenitors is somewhat controversial. It is widely accepted that crosstalk between cells of the bone microenvironment and hematopoietic cells affects each other's behavior [21–23]. Tissue-specific knockout mice have been utilized to target canonical Wnt signaling in hematopoietic cells and in cells of the bone microenvironment, on which HSCs are dependent.

Canonical Wnt signaling in the BM stroma is required for efficient support of hematopoietic progenitors. When β-catenin–deficient BM stromal cells were co-cultured with wild-type (WT) hematopoietic cells, high levels of apoptosis in the hematopoietic cells were observed after short-term culture [24]. In the same study, it was reported that the number of hematopoietic colony-forming units were decreased when derived from hematopoietic progenitors cocultured with β-catenin–deficient stroma, which suggested that canonical Wnt signaling in the stroma was required for support of hematopoietic progenitors. β-catenin deficiency in the BM stroma also resulted in fewer osteoblasts in vivo. These changes did not appear to affect the functional engraftment and differentiation of WT HSCs that developed in the β-catenin–deficient environment. These data suggested that canonical Wnt signaling was important for development of the niche cells, but was not necessary for HSC function [24].

In contrast, studies of hematopoietic cell–autonomous roles of canonical Wnt signaling have demonstrated its ability to regulate HSC self-renewal. Retroviral overexpression of β-catenin in HSCs clearly resulted in HSC expansion and inhibited HSC differentiation in vitro and in vivo. In addition, direct stimulation of HSCs with purified canonical WNT3A ligand resulted in the activation of TCF/LEF reporter genes and induced HSC proliferation [25]. Conversely, inhibition of canonical Wnt signaling in HSCs via overexpression of AXIN1 resulted in decreased HSC proliferation. Given these results, it was surprising that inducible Mx-Cre–mediated conditional deletion of β-catenin in mice did not alter the frequency, differentiation capacity, or function of hematopoietic stem cells, progenitor cells, or lymphoid lineage cells [26,27]. However, later studies using constitutively active Vav-Cre–mediated deletion of β-catenin, which deletes "floxed" genes in

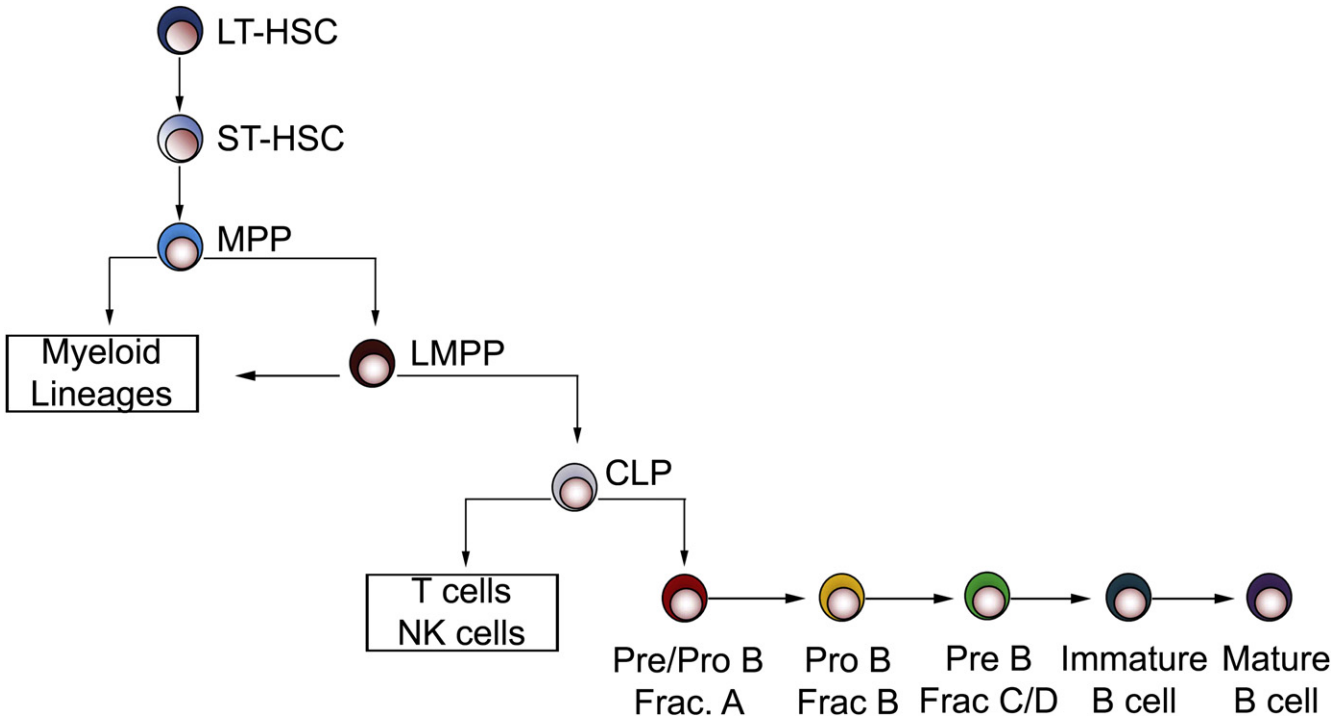

Figure 2. Hematopoietic lineage hierarchy. A simplified schematic of the hematopoietic cell hierarchy in the adult mouse is shown and includes only the cell types that are discussed in the text. The abbreviations for the distinct cell types and their identifying cell surface marker profile as listed as follows: LT-HSC (long-term hematopoietic stem cell) = lineage (Lin)$^-$ c-Kit$^+$ Sca-1$^+$ FLK2$^-$ CD34$^-$ CD150$^+$ CD41$^-$ IL7Rα; ST-HSC (short-term hematopoietic stem cell) = Lin$^-$ c-Kit$^+$ Sca-1$^+$ FLK2$^-$CD34$^+$ CD150$^+$ CD41$^-$ IL7Rα$^-$; MPP (multipotent progenitor) = Lin$^-$ c-Kit$^+$ Sca-1$^+$ FLK2$^+$ CD34$^+$ CD150$^+$ CD41$^-$ IL7Rα$^-$; LMPP (lymphoid primed multipotent progenitors) = Lin- c-Kit$^+$ Sca-1$^+$ FLK2$^+$; CLP-Lin$^-$ c-Kit^{lo} Sca-1$^{lo/int}$ FLK2$^+$ CD34$^+$ IL7Rα$^+$ CD16/32$^-$ CD27$^+$); B-cell precursors (includes pre/pro-B [Fraction A], proB [Fraction B], and preB [Fractions C/D] = Lin$^-$ CD19$^-$ B220$^+$ IgM$^-$ IgD$^-$; immature B cells (Lin$^-$ CD19$^+$ B220$^+$ IgM$^+$ IgD$^-$; recirculating mature B cells (Lin$^-$ CD19$^+$ B220$^+$ IgM$^+$ IgD$^+$). LT-HSC, ST-HSC, MPP, and B cells rely on Wnt signaling during their development. Please refer to main text for more details.

hematopoietic cells beginning at the fetal stage, resulted in a loss of HSC self-renewal capacity [28]. Stimulation of HSCs with WNT3A before transplantation detrimentally affected their long-term engraftment ability [29], and other studies showed no defects in hematopoiesis in double β-catenin/γ-catenin knockout mice [26,27]. These data clearly demonstrated that the catenins (and, by inference, canonical Wnt signaling) were not absolutely essential for hematopoiesis, and suggested other unidentified proteins or pathways that could have functionally compensated for their loss. For example, Jeannet et al. reported no alterations of canonical Wnt signaling in their double β-catenin/γ-catenin knockout mice, but noted the expression of a truncated β-catenin protein that did not interact with TCF1, but possibly could function to mediate alternative Wnt signaling pathways [26]. In support of this idea, treatment of HSCs with a soluble portion of the FZD receptor blocked their proliferation [25]. Because FZD receptors are used by both canonical and noncanonical Wnt pathways (Fig. 1), it is possible that both pathways were blocked in this study. As such, it is also important to review the current state of knowledge on the role of noncanonical Wnt signaling in hematopoiesis.

Noncanonical Wnt signaling in HSCs

The importance of Wnt signaling for HSC fate decisions was first shown in vitro by the observation of the effect of a noncanonical Wnt ligand, WNT5A. The addition of soluble WNT5A to murine HSC cultures induced their proliferation [30,31]. This role for WNT5A has been confirmed more recently in mice [32], and extended to the expansion of human cord blood progenitors [33] and hematopoietic progenitors derived from human embryonic stem cells [34]. Furthermore, other studies have shown that WNT5A positively regulates HSC repopulation ability by maintaining HSC quiescence, and that WNT5A acts as an antagonist of canonical Wnt signaling in HSCs (described in more detail in the section, "Noncanonical Wnt Ligand, WNT5A"). The specific noncanonical Wnt pathway that is utilized by WNT5A in mouse HSCs is not completely understood, and finding consensus on this topic may be hampered due to the promiscuity in their use of co-receptors. For example, as mentioned, ROR1 and ROR2 are often referred to as prototypical noncanonical Wnt co-receptors. In HSCs, WNT5A may bind to FZD4, but WNT5A is likely not to utilize ROR2 because ROR2 (and ROR1) is not expressed in HSCs [32,35].

The lack of ROR2 expression in HSCs rules out the Wnt/JNK/PCP pathway in this case, suggesting that another alternative noncanonical pathway is involved (Fig. 1). Analysis of ROR1 and ROR2 knockout mice [36] is required to confirm whether they are dispensable for hematopoiesis.

Recently, the role of another noncanonical Wnt ligand, WNT4, on HSC fate decisions has been identified. WNT4 overexpression in lineage^{neg} Sca1^{+} c-kit^{+} (LSK) fetal liver cells (which include HSCs and lymphoid-primed multipotent progenitors) resulted in the expansion of lymphoid-primed multipotent progenitors in a JNK-dependent fashion [35] and appeared to favor a quiescent state in the hematopoietic progenitors, as assessed by cell cycle analysis [37]. Conversely, haploinsufficiency of WNT4 in vivo resulted in a reduction of LSK, lymphoid primed multipotent progenitors, and multipotent progenitors populations [35,37]. Therefore, a functional linkage has been made between FZD6, WNT4, JNK, and expression of PCP pathway target genes in early hematopoietic stem and progenitor cells.

WNT11 is a noncanonical Wnt ligand that activates the Wnt/Ca^{2+} pathway. Cultures of mouse and human embryonic stem cells with WNT11 promotes the induction of hemogenic and hematopoietic precursors [34,38] that up-regulate FZD7 expression. After hematopoietic commitment, these WNT11-stimulated precursors could then be induced to proliferate in the presence of the canonical Wnt ligand, WNT3A, similar to adult mouse HSCs [25]. WNT16, another noncanonical Wnt ligand, was shown to be necessary for embryonic HSC specification in zebrafish (*Danio rerio*) [39]. WNT16 is also expressed in early hematopoietic precursors derived from mouse embryonic stem cells, as well as in hematopoietic tissues in the mouse embryo [40], but whether WNT16 is essential for mammalian hematopoiesis must still be confirmed experimentally. Taken together, these studies demonstrate that the induction of embryonic hematopoiesis is regulated by noncanonical and canonical Wnt signaling pathways in a temporal manner.

Canonical and noncanonical Wnt signaling in B-cell development

The role of Wnt signaling in the hematopoietic hierarchy downstream of the HSC is highly context-dependent. It has been observed that the same Wnt ligand can elicit opposite cellular responses in HSCs and more committed hematopoietic lineages. In addition to regulating HSC fate decisions, Wnt signaling can regulate B-cell development and survival. There is still controversy concerning B-cell development and Wnt signaling, most notably, the question of whether or not Wnt signaling is absolutely essential for B-cell development. To address this controversial topic, we have highlighted some of the studies that investigated B-cell development in relation to both canonical and noncanonical Wnt signaling.

Several groups have demonstrated that canonical Wnt signaling influences B-cell differentiation in the BM, starting at the earliest B-cell precursors (Fig. 2). LEF-1 can promote the activity of the *Rag2* gene promoter for immunoglobulin (Ig) gene rearrangement [41], and B-cell receptor stimulation resulted in up-regulation of β-catenin activity in vitro [42]. However, canonical Wnt signaling is not absolutely essential for normal B-cell maturation. For example, *Lef-1*$^{-/-}$ mice displayed decreased proliferation and increased apoptosis of developing pro-B cells, but at later stages of differentiation, *Lef-1*$^{-/-}$ B cells were relatively normal [43], and loss of β-catenin did not affect B-cell lymphopoiesis [44]. In marked contrast to the loss-of-function studies, constitutive tissue-specific (Mx-Cre–initiated) activation of β-catenin in BM cells resulted in a block in B-cell development at the pro-B, pre-B and immature stages; but, similar to the β-catenin$^{-/-}$ mouse, circulating mature B cells were still present [45]. B-cell–specific deletion of β-catenin (using CD19-Cre) led to no major changes in B-cell development, but subtle differences in B-cell functions were observed. Specifically, after lipopolysaccharide stimulation, Ig class switching to IgG3 and IgG1 isotypes was increased, and the total number of plasma cells generated was decreased in β-catenin-deficient mice. However, T-dependent and T-independent B-cell responses to recall antigens were not impaired in the absence of β-catenin [46]. Deletion of the FZD9 receptor implicated Wnt signaling in the control of B-cell development and differentiation in the BM in a cell-autonomous fashion [47]. In these mice, B-cell development was blocked at the pre-B cell stage (Fig. 2), but whether this defect was due to aberrant canonical or noncanonical Wnt signaling was not directly addressed. Again, mature B-cell development in the periphery was not affected.

Loss of WNT5A signaling through the Wnt-Ca^{+2} pathway resulted in the formation of B-cell lymphomas, implying that noncanonical Wnt signaling normally down-regulates proliferation of B-cell precursors [48]. This inhibitory role of WNT5A is in direct contrast to its ability to activate proliferation in HSCs in vitro [30,31], as described in the previous section. Paradoxically, WNT5A has been shown to be a direct antagonist of canonical Wnt signaling in HSCs [29], and the observation of uncontrolled B-cell proliferation in *Wnt5a*-deficient mice is consistent with this role.

Therefore, based on the experimental evidence in the literature, it appears that Wnt signaling is important, but not absolutely essential for B-cell development in the BM. The seemingly opposite functions of WNT5A may reflect temporal differences in the expression of FZD family in fetal liver HSCs as compared to adult hematopoietic tissues [40,49]. In addition, perhaps quantitatively different threshold levels of Wnt signaling regulate stage-specific B-cell development and B-cell fate decisions, and

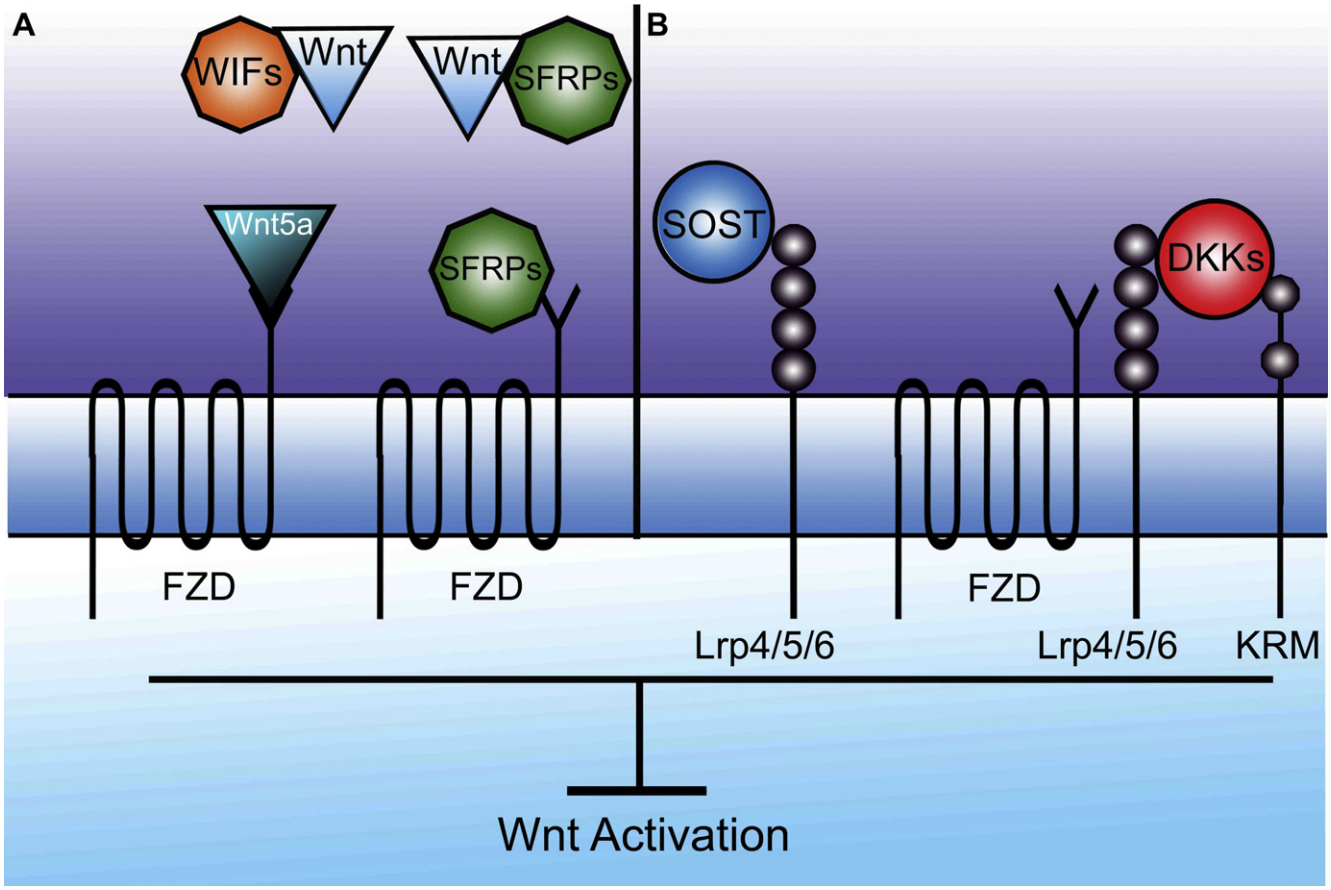

Figure 3. Wnt antagonists. Wnt antagonists use a variety of mechanisms to block Wnt signaling transduction. (**A**) SFRPs can bind both Wnt and FZD receptors to halt the Wnt activation, whereas WIF-1 can only bind to Wnt proteins directly. Wnt5a can bind directly to FZD receptors to inhibit Wnt signaling. (**B**) DKKs and SOST bind to LRP family members and are FZD co-receptors. DKKs require another co-receptor, Kremen (KRM), to fully block Wnt signaling. It is not known if SOST requires a similar co-receptor.

understanding how Wnt signaling is quantitatively controlled could add clarity to the field [50]. To achieve this understanding, we advocate that the role of naturally occurring Wnt antagonists in hematopoiesis should be included in the ongoing scientific discussion.

Regulation of hematopoiesis by naturally occurring Wnt antagonists

Wnt signaling is tightly modulated by a multitude of soluble Wnt antagonists that vary in both function and structure. Several Wnt antagonists have been described that inhibit canonical Wnt signaling, but few studies to determine if and how these Wnt antagonists affect noncanonical Wnt signaling have been performed. The noncanonical Wnt ligands themselves have been suggested to be canonical Wnt signaling antagonists. Other Wnt antagonists, such as the Secreted Frizzled Related Proteins (SFRPs) and Wnt inhibitory factor-1 (WIF-1), bind to Wnt ligands directly and inhibit canonical Wnt signaling by preventing their interaction with FZD receptors (Fig. 3A). In addition, another class of Wnt antagonists, such as Dickkopf (DKK) family proteins and Sclerostin (SOST), bind to Wnt co-receptors LRP4, LRP5 and LRP6 and down-regulate Wnt signaling (Fig. 3B). Below, we review the documented roles of these Wnt antagonists on hematopoiesis. A summary of these studies is provided in Table 1, and a scheme of the localization of these Wnt antagonists in the hematopoietic niches of the bone is shown in Figure 4.

Noncanonical Wnt ligand, WNT5A

As described, canonical WNT3A-mediated signaling in HSCs induces active cell cycling and activation of β-catenin. Nemeth et al. demonstrated that noncanonical Wnt ligands may be natural antagonists to WNT3A-mediated HSC fate decisions [29]. In the presence of exogenous WNT5A, WNT3A-induced HSC proliferation was reduced, apoptosis of hematopoietic cells was increased, and these effects were connected with a decrease in β-catenin activation in HSCs. However, the purpose of this antagonism was postulated to have an alternative function: the induction of

Table 1. Wnt antagonist mouse models that display altered hematopoiesis

	Model	Is the Wnt antagonist expressed by hematopoietic cells?	Effect on HSC/ HSPC numbers	Defect in HSC quiescence?	Alterations to peripheral blood cells?	Alterations to lymphoid populations?	Alterations to bone structure?	Non-cell autonomous effect on hematopoiesis?	Reference
Loss-of-function	*Sfrp-1*$^{-/-}$	No	None	Yes	Yes, increased B220^{+} B cells after secondary transplant	Yes, B and T cell defects	Yes	Yes	[56,57]
	Sfrp-2$^{-/-}$	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	[56]
	Sost$^{-/-}$	No	None	Unknown	No	Yes, reduced B cells	Yes	Yes	[12]
Gain-of-function	SFRP1 treatment in vitro	No	Decrease*	Unknown	NA	NA	NA	Yes	[56,108]
	SFRP2 treatment in vitro	Unknown	Increase*	Unknown	NA	NA	Yes	Yes	[56,112]
	Wif-1 Tg	No	Increase†	Yes	No	No	No	Yes	[80]
	Dkk-1 Tg	Yes	None	Yes	No	No	Yes	Yes	[89]
	Sost-Tg	No	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	[97]

NA = not applicable.
*as measured by HSC engraftment after in vivo transplantation.
†as measured by flow cytometry of BM derived HSCs.

a quiescent HSC pool. WNT5A stimulation led to a notable increase in HSCs in the G_0 state, and improved their engraftment efficiency after transplantation. Studies of B-cell development in vitro showed that B-cell lymphopoiesis was increased in *Wnt5a*-overexpressing stromal cocultures, as compared to *Wnt3a*-overexpressing cultures [51]. Although this study did not show a direct antagonistic role of WNT5A on WNT3A-mediated responses in B cells, it clearly demonstrated a differential response in lymphoid progenitors to distinct Wnt ligands. Exactly how WNT5A antagonizes canonical Wnt signaling during hematopoietic differentiation is not known, but it has been hypothesized that competition of WNT5A and WNT3A ligands for FZD receptors at the cell surface is one possibility [15]. In addition, it is also possible that specific FZD receptors are used for noncanonical Wnt signaling, such as FZD8, which is required for the maintenance of quiescent HSCs [52].

SFRPs

SFRPs bind to Wnt ligands directly. As such, SFRPs can block both canonical and noncanonical Wnt signaling pathways [53] (Fig. 3A). Currently, five SFRPs have been identified. All SFRPs proteins contain a cysteine-rich domain that is homologous to that of FZD transmembrane receptors [54]. SFRP-1, SFRP-2, and SFRP-5 contain netrin-like domains (NTR), which are distinct from the NTR domains in SFRP-3 and SFRP-4 [54]. The NTR is thought to bind Wnt proteins, although conclusive studies in mammalian models are lacking [55]. To date, only SFRP-1 and SFRP-2 have been reported to influence hematopoiesis [56,57], and we will review these studies.

Most Wnt antagonists are expressed and secreted by osteolineage cells (i.e., mesenchymal stem cells, bone marrow stromal cells, osteoblasts, and osteocytes) (Table 1 and Fig. 4). Osteolineage cell-derived Wnt antagonists can influence the cell fates in neighboring osteolineage cells and cells of the hematopoietic lineage within the bone (reviewed in [58]). Wnt signaling is critical for bone homeostasis [58], and disruption of this balance can result in an abnormal hematopoietic niche [12, 89]. In particular, up-regulation of Wnt signaling is required for the maturation of mesenchymal stem cells in the BM to immature osteoblast, as well as for osteoblast mineralization [59]. Down-regulation of Wnt signaling in mineralized osteoblasts induces their terminal differentiation into highly calcified, bone-embedded osteocytes [59]. Osteoblasts, perivascular CXCL12-abundant reticular cells, endothelial cells, and nestin^{+} mesenchymal stem cells are all considered to be hematopoietic niche cells that can support HSC self-renewal and other aspects of hematopoietic differentiation [60–66]. In addition, osteoblasts and BM stromal cells that remain close to the endosteum support B-cell development, via the release of interleukin-7, CXCL12 (stromal cell derived factor–1), and stem cell factor, all

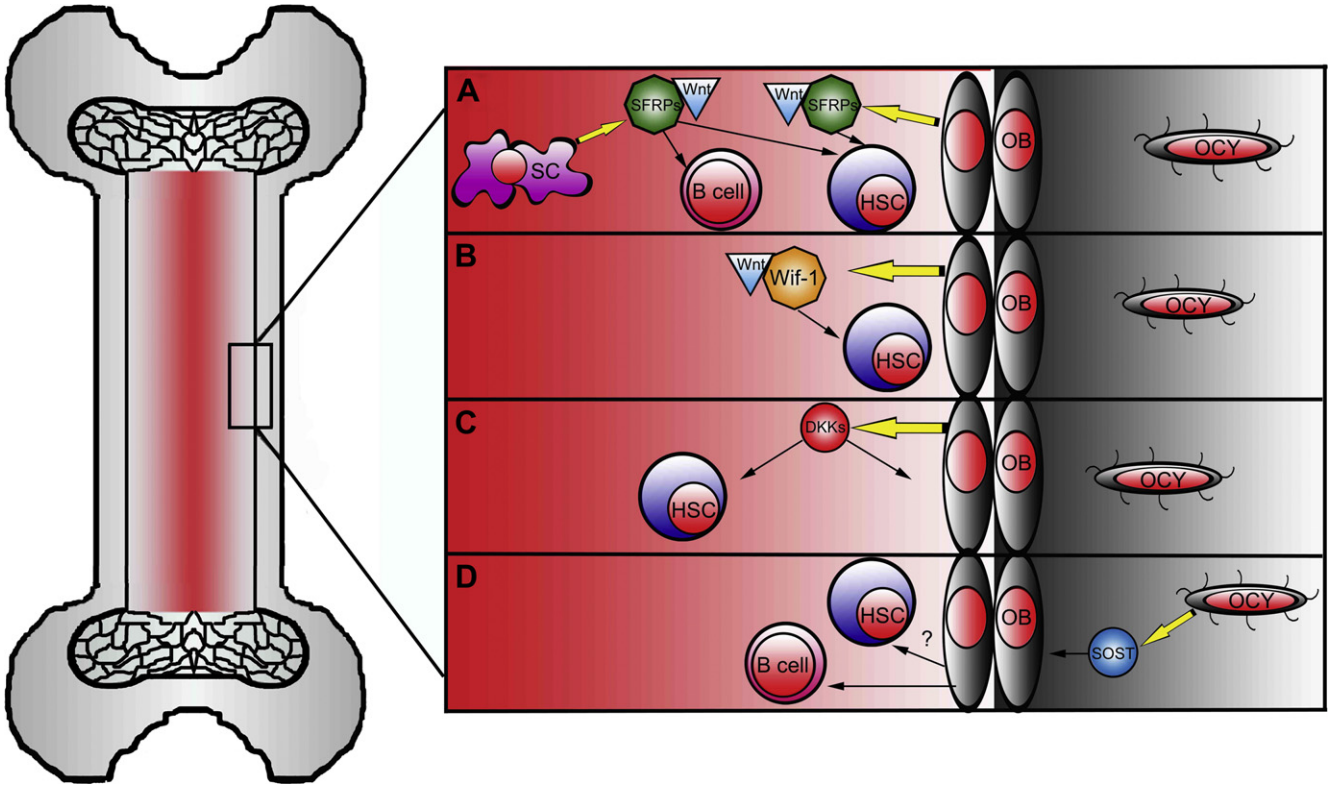

Figure 4. Wnt antagonists in the hematopoietic niches of the bone. A cartoon of the long bone is depicted, in which the BM cavity, osteoblasts (OB), BM stromal cells (SC), osteocytes (OCY) and HSCs are shown, as well as the proposed mechanism by which each Wnt antagonist type acts to affect hematopoiesis in the bone. Yellow arrows indicate the cellular source of each Wnt antagonist, and the black arrows symbolize the influence of the Wnt antagonists on other cell types in the bone. (**A**) SFRP; (**B**) WIF-1; (**C**) DKK-1; (**D**) SOST.

of which are required for B-cell progenitors to mature into naïve B cells in the BM [63]. As we will review, changes in the expression of Wnt antagonists in osteoblasts and osteocytes can indirectly affect hematopoiesis.

SFRP-1 is expressed by BM stromal cells and osteoblasts (Fig. 4A). *Sfrp-1*$^{-/-}$ mice display increased trabecular bone formation and osteoblast numbers due to reduced apoptosis in osteoblast and osteocyte populations [67]. As the osteoblast has been identified as a HSC niche cell, one might have expected that HSCs would be increased in *Sfrp-1*$^{-/-}$ mice. In agreement with this, long-term (LT)-HSCs (as defined by the LSK CD34^{-} Flk2^{-} profile; Fig. 2) were significantly increased in *Sfrp1*$^{-/-}$ mice. Cell cycle analysis revealed a higher proportion of LSK HSCs in the G_0/G_1 phase in *Sfrp-1*$^{-/-}$ mice, consistent with a state of quiescence. This quiescent state is typical of stem cells when they are in contact with their niche [68]. *Sfrp-1*$^{-/-}$ LSK HSCs also contained lower levels of phosphorylated β-catenin and higher levels of nuclear β-catenin, which indicated that canonical Wnt signaling was hyperactive in HSCs in the absence of *Sfrp1*. In contrast, no difference in the number of JNK^{+} cells was observed, suggesting that the absence of *Sfrp1* did not affect the noncanonical Wnt/JNK/PCP signaling pathway.

BM transplantation (BMT) assays (also known as hematopoietic cell transplantation assays) are utilized to determine intrinsic or extrinsic effects of gene ablation on HSC engraftment and differentiation. Serial BMT studies revealed an interesting cell-extrinsic effect of *Sfrp-1* deficiency on HSCs. Serial BMT assays demonstrated that the maintenance of self-renewing HSCs was negatively affected in the *Sfrp1*$^{-/-}$ bone microenvironment [57]. That is, transplantation of WT BM into lethally irradiated *Sfrp1*$^{-/-}$ primary hosts initially revealed no significant difference in the numbers of LSK HSCs and multipotent progenitor compared to WT control hosts. However, when the BM cells from WT→*Sfrp1*$^{-/-}$ chimeras were subsequently transplanted into secondary WT irradiated hosts, the absolute numbers of engrafted LSK HSCs and multipotent progenitor were significantly lower. No difference in the engraftment of WT and *Sfrp-1*$^{-/-}$ HSCs transplanted into WT recipients was observed. These results suggested that in the primary *Sfrp-1*−deficient microenvironment, WT HSCs experienced an increase in Wnt signaling and retreated to a quiescent state. Upon secondary transfer to a WT recipient, the HSCs that developed in the *Sfrp1*-deficient environment were then "released" from the quiescent state,

which resulted in active HSC cycling and differentiation at the expense of self-renewal. In support of this idea, increased numbers of $B220^+$ B cells in the BM, spleen, and peripheral blood were observed in secondary recipients.

Contradicting these studies, treatment of hematopoietic progenitors with exogenous SFRP-1 protein before transplantation also resulted in decreased HSC engraftment efficiency [56], similar to the HSC phenotype observed in the *Sfrp1*-deficient mice. Moreover, the addition of SFRP-1 to WT HSCs also adversely affected hematopoietic differentiation, as measured by colony-forming unit assays. These results appear to be the opposite effect of *Sfrp1* loss-of-function on hematopoietic differentiation in vivo. Taken together, these studies demonstrate that SFRP-1 is an important mediator of early hematopoietic cell fate decisions, particularly those of LT-HSCs and early stage progenitors. The discrepancies between the results of the independent studies still need to be resolved.

Redundancy between SFRP-1 and SFRP-2 is evident during embryonic development, but this redundancy does not appear to exist for adult hematopoiesis [69,70]. In contrast to the results with SFRP-1, LT-HSCs treated with SFRP-2 ex vivo led to an increase in HSC engraftment capacity, as measured by serial BMT assays [56]. *Sfrp-2*$^{-/-}$ mice are viable, but the analyses of HSC numbers and cell dynamics in these mice have not been reported. Whether *Sfrp-2* expression is up-regulated in BM stromal cells or osteoblasts in the *Sfrp-1*$^{-/-}$ mice has not been reported. Given the observations that SFRP-1 and SFRP-2 mediate opposite effects on HSCs in vitro, it is possible that these two proteins also serve distinct functions during hematopoiesis in vivo. It has been hypothesized that as osteoblasts mature and progress to the terminally differentiated osteocyte fate, that the expression of genes involved in hematopoietic support decreases [71]. SFRP-2 secretion from osteoblasts increases as a consequence of their mineralization [72]. The secretion of SFRP-2, in turn, could influence hematopoietic cell fate at the endosteum, the area where mature osteoblasts reside in the bone. Based on the published literature, secreted SFRP-2 from mineralized osteoblasts could inhibit hematopoietic differentiation by increasing the proliferation of self-renewing HSCs [56]. In line with this, our laboratory has cocultured mouse HSCs with mineralizing osteoblasts, and observed a decrease in hematopoietic differentiation compared to cocultures with nonmineralizing osteoblasts. We have observed that SFRP-2 is up-regulated by mineralizing osteoblasts, but we have not observed any increases in HSCs in our cocultures (Cain et al., unpublished data). In summary, the SFRPs produced by BM stromal cells and osteoblasts definitely can influence the behavior of their neighboring hematopoietic cells, but in somewhat perplexing and reciprocal manners.

Wnt inhibitory factor

Like the SFRPs, Wnt inhibitory factor (WIF) family proteins bind to Wnt ligands directly, so they could block both canonical and noncanonical Wnt signaling [73] (Fig. 3A). WIF-1 was first characterized in *Xenopus* and is highly conserved from *Drosophila* to humans, but only one WIF has been identified in mammals to date [74]. WIF-1 contains a unique Wnt inhibitory factor domain that lacks the cysteine-rich domain and NTR domain that is found in SFRPs [75,76]. This WIF domain is also present in the Ryk proteins, which are another family of putative Wnt transmembrane receptors [77,78]. In contrast to SFRPs, WIF-1 lacks the capacity to bind to FZD proteins [76]. WIF-1 is expressed by mature osteoblasts [79], and its role in hematopoiesis is only beginning to be elucidated.

Recently, Schaniel et al. showed that in Col2.3-*Wif-1* transgenic (*Wif-1*Tg) mice in which *Wif-1* was overexpressed in osteoblasts, no profound effect on thr bone architecture was evident. However, increased percentages of LT-HSCs were observed compared to WT control mice, and *Wif-1*Tg LT-HSCs were less quiescent and actively cycling [80]. When *Wif-1*Tg mice and control mice were injected with weekly doses of 5-fluorouracil, which eliminates proliferating cells, *Wif-1*Tg mice died by 12 weeks due to hematopoietic failure, whereas all control mice survived. This result indicated that *Wif-1*Tg HSCs were more proliferative than WT HSCs, and/or were unable to self-renew after treatment. In serial BMT assays, WT → *Wif-1*Tg BM chimeras displayed some enhanced HSC engraftment in primary hosts (at the lowest dose of donor cells), but exhaustion of HSCs from WT → *Wif-1*Tg chimeras was observed in secondary WT recipients. These data definitively demonstrated a non–cell autonomous effect of *Wif-1* in osteoblasts on HSC fate. Canonical Wnt signaling in *Wif-1*Tg HSCs was significantly higher than controls, and autocrine expression of *Wnt3a* was dramatically increased, which could have led to overactive cycling of the HSCs. In line with this, *Jagged-1*, *Cxcl12*, and *N-cadherin*, which are important HSC self-renewal and maintenance factors, were all up-regulated in *Wif-1*Tg osteoblasts [81].

Even though one was a gain-of-function model and the other a loss-of-function model, the HSCs in the *Wif-1*Tg and the *Sfrp1*$^{-/-}$ mice displayed similar phenotypes in lack of quiescence and increased HSC cycling behavior after secondary transplantation, as well as clear activation of canonical Wnt signaling in the HSCs. For the *Wif-1*Tg, the increased activation of Wnt signaling in HSCs is surprising, because it would be expected that the increased levels of the Wnt antagonist in the osteoblast would have inhibited β-catenin activation in the HSCs. Immunoprecipitation studies have shown direct binding of recombinant WIF-1 to both WNT3A and WNT5A [73]. Therefore, it is possible that WIF-1 sequestration of WNT5A could promote canonical WNT3A-mediated signaling in LT-

HSCs and their proliferation; but this requires further experimentation. Reciprocal *Wif-1* loss-of-function studies, such as the analysis of osteoblast-specific conditional *Wif-1* knockout mice, might help to determine how WIF-1 regulates cell fate decisions in LT-HSCs and their differentiated progeny.

DKK family proteins

There are four members in the DKK family of proteins, DKK-1, DKK-2, DKK-3, and DKK-4. The mode of Wnt antagonism by DKK is somewhat distinct from SFRPs and WIFs, as the DKK proteins directly bind to the LRP4, LRP5 and LRP6 co-receptors [11,82–84] (Fig. 3B). In addition, Kremen (KRM) proteins are receptors for DKKs that are utilized to further effectively block canonical Wnt signaling [85] (Fig. 3B). DKK proteins contain a unique cysteine-rich domain (Cys1) and also contain a colipase domain (Cys2 domain) that may have a role in binding to LRPs and KRMs [11,86]. Inhibition of Wnt signaling by DKKs occurs by the prevention of the formation of the Wnt receptor complex: binding of DKK proteins to LRPs and KRMs form a cell-surface protein complex that is quickly internalized. *Dkk-1*$^{-/-}$ mice are embryonic lethal and display head and limb defects. *Dkk-1*$^{+/-}$ and *Krm1*$^{-/-}$ *Krm2*$^{-/-}$ double-mutant mice are viable and have increased bone mass in part to an increased number of osteoblasts [87,88]. Conversely, reduced trabecular bone mass was observed in transgenic mice in which *Dkk-1* was overexpressed in osteoblasts [80,89]. To date, DKK-1 is the only DKK family protein that has been analyzed for its influence on the HSCs and the HSC niche [89,90] (Fig. 4C).

HSCs in *Dkk-1*Tg mice showed dysregulated in HSC quiescence, similar to *Wif-1*Tg mice [80,89]. β-catenin levels were severely reduced in both LT-HSCs and ST-HSCs in *Dkk-1*Tg mice, showing that the high levels of DKK1 secreted from endosteal osteoblasts could antagonize Wnt signaling in HSCs. HSCs from *Dkk-1*Tg mice formed fewer colony-forming units in vitro. Fewer recipients of *Dkk-1*Tg BM survived after transplantation, and this lethality was explained by exhaustion of *Dkk-1*Tg HSCs due to reduced quiescence. Similar to what occurs in the *Wif-1*Tg and *Sfrp1*$^{-/-}$ mice [57,80], serial transplantation of BM from primary WT→*Dkk-1*Tg chimeras back into secondary and tertiary WT recipients revealed persistent HSC hyperproliferation, even though the HSCs were no longer in a *Dkk-1*Tg microenvironment in the latter recipients. It was postulated that this persistent nonquiescent phenotype in the transplanted HSCs resulted from permanent epigenetic programming in the HSCs when they are exposed to the *Dkk-1*Tg bone microenvironment [89], but more detailed analysis is required to confirm this.

*Dkk-1*Tg mice did not display differences in the frequencies of HSCs or myeloid/erythroid progenitors, but there was a significant increase in common lymphoid progenitors (CLPs) in the BM [89]. Clear alterations of the bone microenvironment and Wnt signaling occur with changes in *Dkk-1* levels in vivo [88,91], which could also affect B-cell development from CLP in the BM. However, whether or not B-lymphocyte development is affected in the *Dkk-1* loss-of-function or gain-of-function mouse models has not been reported.

Sclerostin

Sclerostin (SOST) is a secreted protein that is encoded by the *Sost* gene, and is mainly restricted to mature osteocytes in its expression [92] (Fig. 4D). Initial studies described SOST's role in the regulation of bone development as a bone morphogenic protein (BMP) antagonist, but later, it was shown that SOST was a more potent antagonist for Wnt signaling [93]. SOST down-regulates Wnt signaling in osteoblasts, controlling their proliferation and differentiation [94]. SOST binds directly to the LRP4, LRP5, and LRP6 co-receptors, effectively down-regulates Wnt signaling in osteoblasts, which controls their proliferation and halts their differentiation [11,92–94] (Fig. 3B). There still exists some controversy as to whether or not SOST requires a co-receptor (similar to DKK-1 co-receptor KRM), although no such co-receptor has been identified [95].

The best known function of the *Sost* gene is to negatively reduce bone mass. In murine models, overexpression of the *Sost* gene results in marked defects in bone development and an osteoporotic phenotype [96,97]. Conversely, in *Sost*$^{-/-}$ mice, osteoblasts and osteocytes are increased seven-fold, resulting in osteopetrosis [98,99]. Our laboratory has characterized the hematopoietic phenotype in *Sost*$^{-/-}$ mice and observed that *Sost*$^{-/-}$ mice contain bones with severely reduced BM cavities that contain hematopoietic abnormalities [12]. Previous descriptions of other osteopetrotic mouse models have reported transient increases in HSC numbers [100]. However, in the *Sost*$^{-/-}$ mouse, in which osteopetrotic bones are evident, the frequencies and absolute numbers of LSK HSCs, CLP, and myeloid and erythroid progenitors were not affected. Primary transplantation of *Sost*$^{-/-}$ BM into lethally irradiated WT recipients, as well as competitive co-transplantation of WT and *Sost*$^{-/-}$ BM into WT recipients revealed no functional disadvantages of *Sost*$^{-/-}$ BM cell in their engraftment ([12] and Cain et al., unpublished data). However, similar to that observed in the *Wif-1*Tg, *Sfrp1*$^{-/-}$, and *Dkk-1*-Tg mice, serial transplantation of *Sost*$^{-/-}$ HSCs might reveal alterations in HSC quiescence and long-term renewing capacity. These studies are currently ongoing.

In contrast to their normal frequencies of HSCs, *Sost*$^{-/-}$ mice displayed a marked reduction in BM B-cell development, starting at the pro-B cell stage (Fraction B). Reciprocal WT→*Sost*$^{-/-}$ BM chimeras revealed a clear, non–cell autonomous effect of the *Sost*$^{-/-}$ bone microenvironment on B-cell development [12]. We verified that *Sost* is not expressed in any CD45^{+} hematopoietic cell in the BM. However, faint *Sost* expression has been observed in

the BM cavity by fluorescent reporter activity, and the nature of the cells that are responsible for this expression is unclear. There was an observable (but not statistically significant) reduction of stem cell factor, and a significant decrease in CXCL12 in the BM cells of *Sost*$^{-/-}$ mice, consistent with the B-cell phenotype. Interestingly, expression analysis of Wnt target genes in the *Sost*$^{-/-}$ B cells did not reveal any differences in expression of *Lef-1* and *cyclin D1*, which indicated that the dysregulation of Wnt signaling in the *Sost*$^{-/-}$ mice was not directly occurring in the B lymphocytes. These data support a model by which SOST normally regulates Wnt signaling in the osteoblast and other stromal cells, which in turn, down-regulates genes in those cells that are important for B-cell differentiation (Fig. 4D). Alternatively, it is possible that noncanonical Wnt signaling could be enhanced in the B cells in the absence of *Sost*, and affects their fate. In support of this idea, overexpression of *Wnt5a* resulted in decreased B-cell proliferation, a phenotype similar to the *Sost*$^{-/-}$ mice, however, decreased *cyclin D1* expression was observed, which is the opposite of what was reported in the *Sost*$^{-/-}$ mice [48]). Further analysis of B-cell function in *Sost*$^{-/-}$ mice and analysis of hematopoiesis in gain-of-function models (e.g., *Sost*Tg mice) could further illuminate SOST's role in these processes.

Wnt antagonists in hematological disease

It is possible that the down-regulation of Wnt antagonist protein expression could promote hyperactive Wnt signaling in the context of hematological malignancies. In line with this, the dysregulated expression of several Wnt antagonist genes has been observed in some hematopoietic cancers. Compared to hematopoietic cells from healthy controls, hematopoietic cells from patients with acute myeloid leukemia (AML) displayed hypermethylation of *Sfrp-1*, *Sfrp-2, Wif-1, and Dkk-1* promoters (among others) [101–103]. The hypermethylation observed in AML patients directly correlated with decreased gene expression levels, as measured by quantitative reverse transcription polymerase chain reaction [103]. However, as most Wnt antagonist proteins are not expressed by normal hematopoietic cells, hypermethylation of Wnt antagonist genes might only serve as a biomarker for AML, and might not represent any real functional advantage to AML cells. In line with this, Wnt signaling in AML cells was unresponsive to DKK-1 produced by osteoblasts [104]. In multiple myeloma (MM) cells, evidence of increased production of SFRP-3, SOST, and DKK-1 has been observed, which was suggested to contribute to the formation of osteolytic lesions in the bones of these patients [105–107]. However, Wnt signaling in the osteoprogenitor cells was not affected by the MM cells [106,107]. Further investigations are required to determine whether or not dysregulated Wnt antagonist expression intrinsically promotes AML and MM cell proliferation.

Precise control of the expression of specific Wnt antagonists could be an effective way of controlling bone diseases, such as osteoporosis. We and others have reported that alterations to the bone and BM stromal microenvironment due to changes in Wnt antagonist expression can have unintended consequences to hematopoiesis and the developing immune system [12,56,57,80,89,90,108]. Although antibody-based therapies that target SOST and DKK-1 have been successful in the clinical trials for the treatment of osteoporosis, any short-term or long-term effects of these treatments on the immune system cells of osteoporosis patients still remains to be determined. Given their clear role in hematopoietic cell fate decisions, we can expect that a more complete understanding of Wnt antagonists will assist in deciphering the role in hematological diseases.

Unresolved questions

Many general questions as to how Wnt antagonists regulate Wnt signaling remain, and more specific questions about how the diverse groups of Wnt antagonists are involved in the control of Wnt signaling "strength" in HSCs and committed hematopoietic lineages require further investigation. It is well established that the Wnt proteins form morphogen gradients that control cell fate decisions in a quantitative, highly conserved fashion across species and in many organs. The effects of the presence of Wnt antagonists on Wnt morphogen gradients in the hematopoietic niche, and correlations between lower and higher ranges of Wnt signaling thresholds and particular cell fates could be very insightful in dissecting the molecular mechanisms by which Wnt antagonists control hematopoietic cell fate decisions. Bat-Gal [109] and TOPGAL [110] transgenic mice have been useful in determining the effects of Wnt antagonists on canonical Wnt signaling during hematopoiesis. However, not all Wnt antagonist knockout and transgenic mice have been crossed to the Bat-Gal or TOPGAL mice. Given the non–cell autonomous effects of most Wnt antagonists on hematopoietic cell fate, it may be more efficient to perform BMT of hematopoietic cells from Bat-Gal or TOPGAL reporter mice into mutant Wnt antagonist recipients. This type of study would allow for quantification of the levels of Wnt signaling specifically in the hematopoietic cells that develop in Wnt-antagonist–altered bone environments. More recently, a novel β-catenin reporter mouse, in which quantitative measurements of Wnt signaling at single-cell resolution can be made, revealed new spatial and temporal information on the activation of Wnt signaling during embryonic development [111]. This mouse could potentially be used for dynamic imaging of Wnt signaling during hematopoiesis in BM chimeras.

Despite their utility, the Bat-Gal and TOPGAL reporter mice are utilized to quantify canonical Wnt signaling, and no equivalent reporter strain has yet been derived to quantify noncanonical Wnt signaling. Given the known diversity of noncanonical Wnt signaling pathways and no obvious

common downstream target among them, the creation of such a mouse strain will be very challenging. Instead, perhaps quantitative genomic analyses of the expression patterns of all Wnt family proteins in osteoblasts, other niche cells, and hematopoietic cells that develop in mutant Wnt antagonist mice could reveal some of these noncanonical targets. Microarray, quantitative polymerase chain reaction arrays, as well as modern next-generation sequencing approaches, could be utilized for this purpose. Such approaches might also reveal how the expression of other Wnt antagonists changes in specific Wnt antagonist knockout mice. For example, in *Sost*$^{-/-}$ mice, *Dkk-1* is up-regulated [12]. These types of analyses could reveal cell-specific functional redundancies among the distinct Wnt antagonists in HSCs, as compared to committed hematopoietic lineages. In addition, compensatory mechanisms to overcome Wnt antagonism, such as the up-regulation of WNT3A in Wif-1-Tg mice and differential expression of Wnt pathway genes in distinct gain-of-function and loss-of-function Wnt antagonist mouse models could be uncovered.

Conclusions

Wnt signaling is clearly important in normal hematopoiesis and hematological disease, and Wnt antagonists have emerged as important regulators of Wnt signaling during these processes. Despite the expansion of the scientific knowledge in the field of Wnt antagonist biology and the clear evidence for non–cell autonomous effects of Wnt antagonists on hematopoietic cells in the BM, the mechanistic understanding of how Wnt antagonists regulate hematopoiesis is still incomplete. Determination of possible functional redundancies of each Wnt antagonist type at distinct stages of hematopoietic development have the potential to reveal specific and unique roles of each Wnt antagonist family member on hematopoietic cell fate decisions and differentiation within the stem cell niches in the bone.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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