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Review

Role of WNT signaling in normal and malignant hematopoiesis

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Summary. The WNT pathway is a powerful signaling pathway that plays a crucial role in cell fate determination, survival, proliferation and movement in variety of tissues. Abnormalities in the WNT signaling pathway have been implicated in a number of diseases, most notably cancer. Recent exciting evidence suggests that WNT signaling also plays an important role in hematopoietic stem cell self-renewal and progenitor development. In this review we discuss current state of knowledge on WNT signaling in hematopoiesis and extend our focus on aberrant WNT signaling in hematological malignancies.

Key words: WNT signaling, Hematopoiesis, Leukemia, Lymphoma

Introduction

WNT proteins are secreted, lipid-modified, glycoproteins that activate cell surface receptor-mediated signal transduction pathways to regulate a variety of cellular activities, including cell fate determination, proliferation, migration, polarity and gene expression (Moon et al., 2002). The first members of the WNT family were identified in Drosophila and mice. Wg1 was identified as a gene responsible for the defect in embryonic patterning resulting in the Wingless phenotype in Drosophila (wg1) (Sharma and Chopra, 1976), while int-1 was identified as a potential oncogene, activated by insertion of MMTV in mammalian cells (Nusse and Varmus, 1982). Upon further study, these were found to be homologous proteins and hence were named WNTs (Rijsewijk et al., 1987). There are 19 known WNT family members in mammals, which generally fall into two classes. Classical WNTs (WNTs -1, -3a, -8 and -8b) activate signaling through the canonical pathway involving ß-catenin. Non-classical WNTs (WNTs -4, -5a and -11) activate alternative non-canonical signaling pathways. Frizzleds (Fzd) are cell surface receptors for Wnt proteins that belong to a class of seven-pass transmembrane receptors (Bhanot et al., 1996). There are ten known members of the Fzd gene family in humans and nine in mice. Little is known about the specificity or affinity of Fzds for individual WNTs but there is likely to be some redundancy because there as twice as many WNTs as Fzds. Like WNTs, Fzds can be grouped according to their ability to activate canonical (Fzd-1, -7 and -8) or ß-catenin independent (Fzd-2, -3, -4 and -6) signaling pathways, however this is complicated by the formation of homo- and hetero-oligomers (Kaykas et al., 2004).

Disruption of a number of components of WNT signaling has been identified as key mediators in developmental defects and many types of cancers (Reya and Clevers, 2005). These include inactivating mutations in APC, axin, or conductin (axin 2) proteins, which reduce ß-catenin degradation and thereby constitutively activate TCF/ß-catenin driven transcription. Similarly, mutations in one of the serine/threonine-phosphorylation sites of ß-catenin lead directly to its stabilisation and transcription of target genes even in the absence of external WNT signals (Giles et al., 2003). Physiological, negative regulators of WNT signaling may also be inactivated in tumors (Giles et al., 2003).

As the volume of literature on WNTs is expanding rapidly, a few aspects of the current state of knowledge will be emphasised here. In this review we will focus on the role of WNT signaling in the regulation of normal hematopoiesis. This will be followed by exploring some emerging pieces of evidence on deregulation of WNT signaling in leukemogenesis. To learn more about a general role of WNT signaling in cancer development and other inherited disorders, readers are referred to recently published reviews (Giles et al., 2003; Logan and Nusse, 2004; Moon et al., 2004).

The WNT signaling pathways

WNT proteins activate at least three distinct intracellular signaling cascades: the WNT/ß-catenin

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pathway commonly referred to as the canonical pathway, the WNT/Ca^{2+} pathway and the WNT/planar cell polarity (PCP) pathway. All signaling through Fzd proteins is believed to be dependent on heterotrimeric GTP-binding proteins. This was initially assumed due to structural similarities between Fzds and other seven-transmembrane receptors. Later this was confirmed for the calcium-signaling pathway (Slusarski et al., 1997; Sheldahl et al., 1999) and evidence for this requirement in canonical signaling is emerging (Katanaev et al., 2005).

The canonical WNT/ß-catenin pathway

The best understood WNT signaling pathway – the WNT/ß-catenin pathway (Fig. 1) – has been characterised by a combination of genetic and biochemical studies. Acting through a core set of proteins that are highly conserved throughout the animal kingdom, this pathway regulates the ability of ß-catenin to activate the transcription of specific target genes (Prunier et al., 2004). This in turn regulates early embryonic patterning, epithelial-mesenchymal interactions and maintenance of stem cell compartments.

The key mediator of the pathway, ß-catenin, was first described for its role in cell adhesion (Ozawa et al., 1989). As a component of the adherens junctions, ß-catenin binds tightly to the cytoplasmic domain of type I cadherins and plays an essential role in the structural organisation and function of cadherins by linking them through α-catenin to the actin cytoskeleton. This adhesive function is based on a subcellular pool of ß-catenin that is membrane-associated and stable (Nelson and Nusse, 2004). In the absence of WNT signaling, the level of unbound ß-catenin is kept low through degradation. The serine/threonine kinase casein kinase Iα (CKIα) (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002) and glycogen synthase kinase (GSK-3ß) (Yost et al., 1996) phosphorylate excess ß-catenin targeting it for ubiquitination and degradation in the 26S proteosome. This occurs when the enzymes are bound to a scaffolding complex of axin and adenoma polyposis coli (APC) (Hart et al., 1998; Kishida et al., 1998), collectively known as the 'destruction complex'. Upon initiation of WNT signaling, WNTs bind to two receptor molecules, Fzd and lipoprotein receptor-related proteins 5 or 6 (LRP5/6) (Pinson et al., 2000; Tamai et al., 2000). More recently Ryk, a kinase dead receptor tyrosine kinase has been identified as being required for canonical WNT signaling in neurites (Lu et al., 2004b). The potential role for Ryk in the hematopoietic system is yet to be elucidated.

Activation of the receptor by WNTs leads to phosphorylation of dishevelled (Dsh) (Yanagawa et al., 1995), which through its association with axin, prevents GSK-3ß from phosphorylating ß-catenin (Itoh et al., 1998). This allows unphosphorylated ß-catenin to escape ubiquitination by ß-TrCP, and subsequent degradation by the proteosome (Aberle et al., 1997; Latres et al., 1999; Liu et al., 1999). This leads to the accumulation and nuclear translocation of ß-catenin (Tolwinski and Wieschaus, 2004), which associates with the LEF/TCF family of transcription factors (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). In the absence of WNT signaling, LEF/TCFs complex with Groucho and act as transcriptional repressors (Cavallo et al., 1998). Nuclear ß-catenin converts this co-repressor complex into a transcription activator complex, by displacement of Groucho and recruitment of the histone acetylase CBP/p300 (cyclic AMP response element-binding protein), resulting in transcription of WNT target genes (Hecht et al., 2000; Takemaru and Moon, 2000). Further interactions between the TCF-ß-catenin complex and chromatin, depend on two additional nuclear proteins, pygopus and Legless, first identified in *D. melanogaster* (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). The resultant transcriptional changes are the key read-outs of canonical WNT signaling and so far seventy-five different target genes have been identified including regulators of cellular proliferation, survival, developmental control and genes involved in tumorigenesis (refer to the following link for the most updated list of target genes http://www.stanford.edu/

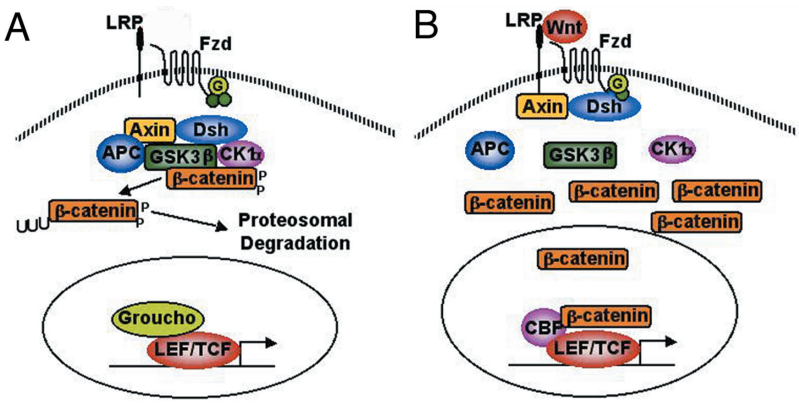

Fig. 1. Canonical WNT signaling. **A.** In the absence of WNT, ß-catenin levels are kept low by constant proteosomal degradation within the cytoplasm. Within the multi-molecular 'destruction complex', that contains adenomatous polyposis coli (APC) and axin, glycogen synthase 3ß (GSK-3ß) and casein kinase 1α (CK1α) phosphorylate ß-catenin. This leads to ubiquitination (U) and subsequent degradation. **B.** When WNT binds to frizzled (Fzd) and low density lipoprotein receptor related proteins 5 or 6 (LRP), dishevelled (Dsh) inactivates GSK-3ß. This results in the accumulation of ß-catenin in the cytoplasm and ultimately the nucleus where it displaces the transcription repressor Groucho. In association with cyclic AMP response element-binding protein (CBP) ß-catenin facilitates transcriptional activation of lymphocyte enhancer binding actor (LEF)/T cell factor (TCF) resulting in altered gene transcription.

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The WNT/Ca2+ pathway

Studies in zebrafish and *Xenopus* demonstrated that WNT proteins also induce the release of intracellular calcium (Fig. 2A). It appears that like the canonical pathway, signaling via the calcium pathway may be dependent on the presence of co-receptors, initially identified in *Xenopus* (Hikasa et al., 2002). The mammalian homologue, Ror2, interacts directly with Fzd2 and Fzd5 but not Fzd8 and with WNT5a but not WNT3a suggesting a role in non-canonical signaling (Oishi et al., 2003). This pathway involves activation and membrane association of phospholipase C (PLC) through hetero-trimeric GTP binding proteins (Slusarski et al., 1997). Here PLC hydrolyses membrane phospholipids to produce di-acyl glycerol (DAG), and inositol 1,4,5-triphosphate (IP3). IP3 induces the release of Ca^{2+} from the endoplasmic reticulum by association with the SERCA-ATPase pump and this in turn increases the expression and activity of calmodulin, and calmodulin kinases (Kuhl et al., 2000). Increased intracellular Ca^{2+} can activate protein kinase C (PKC), which can also be directly activated by DAG. Activation of PKC can influence a range of cellular functions including motility, apoptosis and differentiation, which in turn regulate processes such as morphogenesis. PKC can also regulate the expression of WNT5a resulting in a positive feedback loop (Jonsson et al., 1998; Sheldahl et al., 1999).

Further complexity of the pathway has recently been revealed with the discovery that WNT5a can also activate phosphodiesterase (PDE) via signaling through the G protein a subunit $G\alpha_{t2}$. This results in reduced levels of cyclic GMP, which has the potential to modulate cyclic nucleotide–gated ion channels, guanylylcyclases, and protein kinase G. The role of these factors in WNT signaling remains to be determined. However it appears that signaling through PDE may synergise with canonical WNT signaling by inhibiting protein kinase G, which phosphorylates ß-catenin independently of GSK3ß. Interestingly, PDE inhibitors have been proposed as anticancer agents (Li et al., 2001).

The WNT/planar cell polarity pathway

The third pathway activated by WNTs results in JNK activation and is involved in determining planar cell polarity (PCP) in *Drosophila*, hence its name (Fig. 2B). Signaling through this pathway occurs in the absence of the co-receptors LRP5/6 but in at least some settings requires Ror2 (Oishi et al., 2003). Signaling components of this pathway include Dsh, Van Gogh/strabisthmus, prickle, diego and flamingo/Starry night in the fly but a role for Dsh only has been confirmed in mammalian cells. These components are thought to act in a non-linear complex with Dsh. Although Dsh is required for both canonical and PCP signaling the DEP domain is required for non-canonical signaling, rather than the Dix and PDZ domains of the protein which are involved in canonical signaling (Axelrod et al., 1998). Signaling through the DEP domain activates the GTPases Rac and Rho which in turn regulate the activity of Rho-kinase (Rock) and c-Jun NH_2-terminal kinase (JNK) (Strutt et al., 1997; Boutros et al., 1998). In vertebrates, this route has been implicated in the regulation of morphogenetic movements such as convergent extension during gastrulation by directing asymmetric cytoskeletal organization and coordinated polarization of cells within the plane of epithelial sheets (Yamanaka et al., 2002; Strutt, 2003).

Several extracellular and intracellular proteins can negatively regulate WNT signaling. Dickkopfs (Dkks) and secreted frizzled related proteins (sFRPs) are two families of extracellular factors that antagonise WNTs. Dkks limit the availability of LRP5/6 co-receptors to WNTs by sequestering LRP5/6 into complexes with Kremen (Krm) and promoting their internalisation to lyosomes (Mao et al., 2002). Dkks can therefore be expected to specifically block canonical signaling requiring the presence of LRP co-receptors. In contrast, sFRPs bind directly to WNTs and prevent their

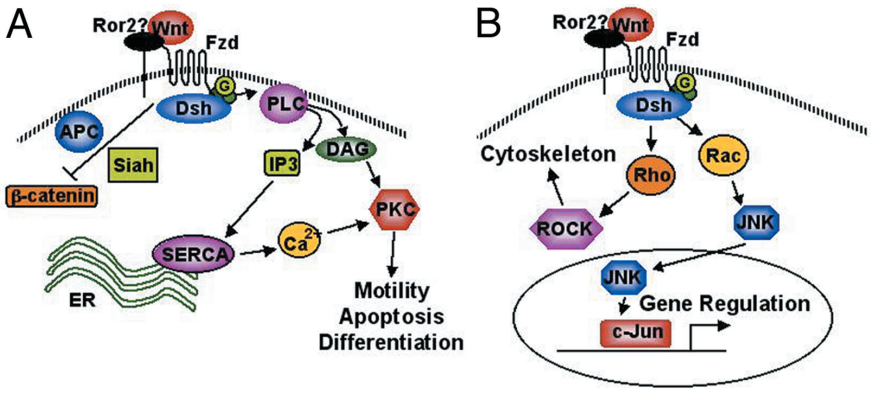

Fig. 2. Non-canonical WNT signaling. **A.** The WNT/Ca^{2+} pathway is activated when WNT binds to Fzd, possibly in association with co-receptors such as Ror2. Activation of phospholipase C (PLC) results in the production of lipid signaling mediators inositol-3 phosphate (IP3) and daicylglycerol (DAG). These induce the release of intracellular calcium (Ca^{2+}) and activation of protein kinase C (PKC) which in turn regulate cytoskeletal function, apoptosis and differentiation. This pathway can inhibit the ß-catenin pathway, by APC and Siah. **B.** The planar cell polarity pathway signals through the small GTPases Rho and Rac and modulates cytoskeletal function and gene transcription via activation of the MAP kinase JNK and the transcription factor c-Jun.

association with receptors (Wang et al., 1997) and have the potential to inhibit both canonical and non-canonical signaling. Finally, activation of the non-canonical WNT/Ca^{2+} can suppress canonical WNT signals during axis formation in *Xenopus* embryos (Kuhl et al., 2001).

WNT signaling in hematopoietic cells

In mammals, the earliest stages of hematopoietic development arise from the mesoderm and are first detected in the region destined to form the aorta-gonad-mesonephros (AGM) (Dzierzak, 2002). *Wnt* genes, notably Wnt-3, -3a, -5a, -5b and -8, are expressed in the primitive streak, an area that contributes to the AGM region (Takada et al., 1994; Bouillet et al., 1996). WNT-3 is highly expressed in differentiating murine embryonic stem (ES) cells and can enhance the hematopoietic commitment of these cells (Lako et al., 2001) suggesting that various WNT family members have the potential to regulate proliferation and cell fate in hematopoietic progenitors.

In the adult, hematopoietic cells develop within the bone marrow in intimate association with a highly organized three-dimensional microenvironment that consists of variety of cell types, matrix components and supportive factors thought to define a 'niche' critical for the homeostatic maintenance of stem cell populations (Taichman, 2005). The best-characterised stem cell niche is the osteoblastic niche where spindle-shaped N-caherin positive osteoblasts (SNO cells) maintain the quiescence of HSC. Actively dividing HSC are found in the vascular niche, suggesting that HSC may migrate from the osteoblast niche to the vascular niche during the process of proliferation and maturation (Suda et al., 2005). Hematopoietic tissues express a number of WNT family members including -2b, -3a, -5a and -10b and their receptors Fzd-3, -4, -5 and -7 (Austin et al., 1997; Van Den Berg et al., 1998; Reya et al., 2000). WNTs are produced by the hematopoietic cells themselves as well as by non-hematopoietic components of the bone marrow such as stromal cells, which produce WNT-5a and WNT-3 (Chiba et al., 2004). The more primitive $CD34^+$ cells also express WNT-5a (Van Den Berg et al., 1998). This provides the opportunity for both autocrine and paracrine stimulation of HSC by WNTs within the bone marrow (Fig. 3).

Initially a role for WNT signaling in hematopoiesis was implied from experiments where stromal cells, transfected with a number of different WNTs, demonstrated an enhanced ability to support the proliferation of and maintain the primitive phenotype of HSC in both murine and human systems (Austin et al., 1997; Van Den Berg et al., 1998). WNT-5a and -10b were also found to synergise with Kit ligand (KL) to promote the growth and inhibit the differentiation of murine hematopoietic progenitors (Austin et al., 1997). Of more biological relevance is the observation that WNT-5a is expressed in stromal cell lines that support HSCs but absent from stromal cell lines that do not, suggesting that it might play an important role in HSCs self-renewal in vivo (Hackney et al., 2002). Indeed, WNT-5a administration significantly increased HSC engraftment in non-obese diabetic severe-combined immunodeficiency (NOD/SCID) mice xenografted with human $CD34^+$ HSC (Murdoch et al., 2003). The contribution of WNT proteins to self-renewal was further supported in experiments where transduction of highly purified HSCs with constitutively activated ß-catenin enhanced self-renewal in vitro and reconstitution in vivo (Reya et al., 2003). However, the use of Bcl-2 transgenic mice for these experiments raised questions regarding the relevance of this data to normal hematopoiesis. This was clarified by the recapitulation of these observations following exposure of wildtype HSCs to purified WNT-3a (Willert et al., 2003). Taken together these data demonstrate that WNTs provide proliferative and self-renewal signals for HSC but they do not demonstrate that WNT signaling occurs within the normal BM or that it is required for normal hematopoiesis. The former was demonstrated by reporter gene activity in transplanted HSC within the bone marrow (Reya et al., 2003) while the later was revealed using HSC engineered to over-express axin a negative regulator of WNT signaling. Cells over expressing axin demonstrated inhibition of HSC growth in vitro and reduced reconstitution in vivo (Reya et al., 2003).

Although it is now reasonably clear that WNT signaling plays a significant role in normal hematopoiesis the mechanisms underlying the effects of WNTs remains obscure. Activation of WNT signaling in HSCs led to elevated levels of Notch 1 and HoxB4, genes previously implicated in self-renewal of HSCs (Reya et al., 2003). This raises the possibility that WNT

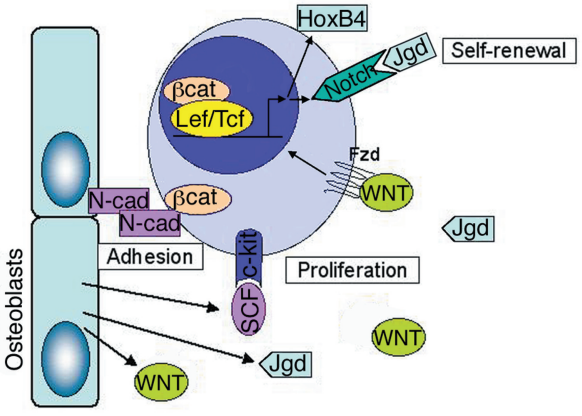

Fig. 3. WNT signaling in the BM. Within the bone marrow microenvironment stromal cells, including osteoblasts provide adhesive support, which is in part mediated by N-cadherin (N-cad). These cells also provide soluble factors including stem cell factor (SCF), WNTs (WNT) and jagged (Jgd). SCF and WNT signalling can synergise to induce hematopoietic stem cell proliferation, while the binding of Jgd to Notch enhances self-renewal. WNT signalling also results in increased expression of Notch and HoxB4, which could further enhance the self-renewal capacity of the hematopoietic stem cells.

signaling exerts its influence by activating the HoxB4 (Sauvageau et al., 1995; Thorsteinsdottir et al., 1999) and/or Notch1 (Karanu et al., 2000; Stier et al., 2002) signaling pathways in HSCs. It was recently confirmed that Notch signaling is required for WNT-mediated maintenance of undifferentiated HSCs but not for their survival and cell cycle entry (Duncan et al., 2005). These data support a model in which WNT signaling integrates with Notch signaling in HSCs, with WNT enhancing proliferation and survival and Notch preventing differentiation. Cumulatively, these studies suggest that WNT signaling can contribute to HSC and progenitor cell expansion and self-renewal (Fig. 3).

Although there is no doubt that the canonical pathway is activated in HSC (Reya et al., 2003), the often equivalent effects of WNTs that are thought to act through the canonical pathway (such as WNT-3a) and those that can act through non-canonical pathways (for example, WNT-5a), means that other WNT-mediated pathways may also be important for HSC function in vivo. Indeed, one group has reported that conditional knock-out of ß-catenin in bone marrow progenitors does not impair self-renew or reconstitution of all hematopoietic lineages under competitive transplant conditions (Cobas et al., 2004). These data surprisingly excluded an essential role for ß-catenin during hematopoiesis. One scenario for such contrasting results is that plakoglobin (γ-catenin), a close relative of ß-catenin whose degradation is also enhanced by axin, is able to compensate for ß-catenin in hematopoietic cells, and thus restore WNT signaling under strong selective pressure. In murine hematopoietic progenitors, induction of plakoglobin is accompanied by transactivation of LEF/TCF transcription factors, which in turn lead to enhanced proliferation and survival (Muller-Tidow et al., 2004). Alternatively it could indicate that non-canonical WNT signaling is sufficient for these activities, although the data from the axin deficient animals would argue against this. It would be of interest to know whether these cells still activate WNT reporter gene constructs in vivo. Studies looking at the roles of other molecules involved in WNT signaling should clarify the mechanisms involved. Finally there is the complexity of the HSC niche to be considered with other factors such as Notch and Flt-3L potentially crosstalking to WNT signaling pathways (Duncan et al., 2005; Tickenbrock et al., 2005)

Clearly these studies indicate that HSCs have active WNT signaling which promotes expansion and self-renewal both in vitro and in vivo. However many important questions remain unanswered. Which cells are the primary source of WNT proteins in the bone marrow microenvironment under physiological conditions? Are these sources an important component of the BM stem cell niche and how might WNT factors synergise/interact with other elements of self-renewal? Together these studies have provided us with fascinating glimpses of mechanisms that are bound to have important implications in the area of HSC biology/stem cell therapy.

WNT signaling and the bone marrow micro-environment

The experiments described above have demonstrated that WNT proteins stimulate survival and proliferation of hematopoietic stem cells and progenitors. However most studies have not used purified *WNT* proteins in isolation but either conditioned medium from Wnt transduced lines, direct contact with such lines or stimulation/inhibition of WNT signaling in vivo. This raises questions regarding the response of non-hematopoietic cells to WNTs and how this may in turn regulate hematopoiesis. How WNT proteins might modulate/regulate hematopoiesis in the context of this microenvironment is a very important question that remains to be addressed. A scant number of reports suggest that WNT proteins may be regulating hematopoiesis by affecting hematopoietic-supporting stromal cells. It would be interesting to speculate that WNT proteins secreted by the hematopoietic cells bind to Fzds on stromal cells, transduce signals and regulate the function of stromal cells. Indeed murine stromal cell line, ST-2, and primary stromal cells respond to canonical WNT signaling by up-regulating ß-catenin, altered morphology and extended growth (Yamane et al., 2001). Using WNT-3a conditioned media in stromal cultures resulted in a dramatic decrease in the number of B-lineage and myeloid lineage cells, while this effect was not seen under stromal-free conditions. This suggests that WNT-3a was mediating its effect via the stroma (Yamane et al., 2001). In another study with a similar approach, gene transfer of WNT-3 into human stromal cells drastically reduced their ability to support the formation of cobblestone areas by $CD34^+$ cells. However, WNT-3 did not recapitulate these effects in the absence of stroma (Chiba et al., 2004). It is certainly hard to interpret these results due to the complex nature of the model system. Although the functional significance of these findings is lacking at the moment, in vivo assays in mouse models could provide greater insight into stromal mediated effects of WNTs on hematopoiesis.

Osteoblasts were recently identified as important constituents of the bone marrow 'stem cell niche' capable of maintaining long-term HSC activity and self-renewal (Calvi et al., 2003; Zhang et al., 2003). The WNT signaling pathway plays an important role in bone formation and several studies demonstrate that WNT proteins stimulate osteoblast precursor growth and early events in osteoblast differentiation (Bradbury et al., 1994; Gong et al., 2001; Rawadi et al., 2003). Transgenic mice over-expressing *Wnt-10b* from an adipocyte-specific promoter exhibit increased bone volume, strength and more trabecular bone mass (Bennett et al., 2003). Accordingly, *Wnt-10b* deficient mice have less bone mass and fewer trabeculi (Bennett et al., 2003). More recently Ror2 was found to

negatively regulate WNT3a but potentiate WNT1 signaling in osteoblasts. Considering that osteoblasts play a significant role in the hematopoietic stem cell niche, regulation of osteoblast function can be anticipated to modulate hematopoiesis. In vitro studies indicate that osteoblasts may be a potential source of WNT factors as they express several WNT family members including WNT-1, -4, -7b and -14 (Kato et al., 2002; Zhang et al., 2004). Bone morphogenic protein (BMP)-2 treatment of a mesenchymal cell lines C3H10T1/2 induced expression of WNT-1 and WNT-3a, which might play a role in initiating paracrine WNT signaling (Rawadi et al., 2003). Osteoblasts have a crucial role in the maintenance of hematopoietic stem cells and B-cell development. Whether osteoblasts are an important source of WNT proteins in the hematopoietic stem cell niche is yet to be confirmed.

WNT signaling and lymphopoiesis

Evidence that WNT proteins can influence lymphopoiesis have come from both gain and loss of function approaches in mice and cultured mammalian cells. There is an extensive amount of literature underscoring a definitive role of WNT signaling in T cell development however relatively less is known in B cell development. Readers are referred to recent reviews by Staal et al and Van de Wetering et al for detail analysis of WNT signaling in T lymphopoiesis (van de Wetering et al., 2002; Staal and Clevers, 2005).

T-lymphopoiesis

The first evidence for the role of WNT cascade in lymphopoiesis was established from gene knockout studies of *Tcf1* and *Lef1* in mice (van Genderen et al., 1994; Verbeek et al., 1995). TCF1 is the first definitive T cell marker expressed in the most immature $CD4^-CD8^-$ negative T cells (DN1) compartment of developing T cells in fetal thymus (Hattori et al., 1996). Two different *Tcf1* knockout mice have been generated; with one carrying an in-frame deletion resulting in reduced TCF1 mRNA levels whereas the other model carries an exon seven deletion resulting in complete knockout. In these mice, a dose dependent effect of TCF1 on the thymus is apparent, with the absence of TCF1 resulting in a dramatic reduction in thymocyte number, which is exacerbated with increasing age. This appears to be primarily due to a T cell intrinsic impairment in cell proliferation, although an increase in apoptotic cell death of more mature $CD4^+CD8^+$ thymocytes has also been detected (Schilham et al., 1998; Ioannidis et al., 2001). There is little evidence for a role for TCF1 or indeed canonical WNT signaling in mature T cells with $Tcf^{-/-}$ mice being fully immuno-competent, suggesting that WNT signaling is essential for maintenance for early thymocyte progenitors but is dispensable in more mature T cells (Schilham et al., 1998; Prieve and Waterman, 1999).

LEF1 expression coincides with that of TCF1 in T lineage cells but LEF1 deficient mice display no overt abnormalities in the T cell compartment (van Genderen et al., 1994). Interestingly $Tcf1^{-/-}/Lef1^{-/-}$ mice, which are embryonic lethal, display a more severe defect in T cell development, that is characterised by a complete block at the immature CD8 single positive stage as well as the impairment of DN thymocyte subsets. The defect is T cell autonomous since $Tcf1^{-/-}/Lef1^{-/-}$ fetal liver cells fail to reconstitute the thymus in lethally irradiated host (Okamura et al., 1998; Held et al., 2003). These data clearly imply redundancy between the two genes as the double knockout phenotype is more severe than those observed in either $Tcf1^{-/-}$ or $Lef1^{-/-}$ alone.

Since ß-catenin knockout mice are early embryonic lethal (Haegel et al., 1995; Huelsken et al., 2000), Sen and co-workers used an inducible gene targeting approach to delete ß-catenin specifically in T-lineage cells. This resulted in impairment of T cell development at the T cell receptor (TCR) ß-chain checkpoint (Xu et al., 2003). Interestingly, this phenotype differs from that observed in *Tcf1* deficient mice. Conditional ablation of ß-catenin in BM progenitors did not impair reconstitution of T lymphopoiesis in competitive chimera experiments although redundancy with γ-catenin is a possible explanation for this finding (Cobas et al., 2004). In more mature T cells, expression of constitutively active ß-catenin permits developing T cells to by-pass pre-TCR signals (Gounari et al., 2001) and WNT signaling can enhance the survival of these cells (Ioannidis et al., 2001).

A role for WNT signaling in T lymphopoiesis is also suggested by the inhibitory effects of sFRPs, which block WNT binding to cell expressed Fzd on T cell development in fetal thymus organ cultures (Staal et al., 2001). This is further supported by the reduction in thymic cellularity in axin-transgenic mice (Hsu et al., 2001). The WNT proteins responsible for activating LEF/TCF are not known. However over-expression of WNT-1 and WNT-4, which are normally expressed in the murine thymus (Staal et al., 2001) in fetal thymocytes resulted in increased cell numbers in suspension culture (Staal et al., 2001) In contrast WNT1 and WNT4 knockout mice displayed decreased thymic cellularity, which was attributed to reduced proliferation of immature thymocytes (Mulroy et al., 2002). There is little evidence relating to the receptors involved, with Fzd-9 being expressed by thymocytes, but the $Fzd\text{-}9^{-/-}$ animals having only a mild thymic phenotype related to atrophy in older animals (Ranheim et al., 2005). All of these studies suggest that WNT proteins are important factors that deliver proliferative and possibly survival signals to developing T cells within the thymus however the details of the specific WNT and Fzd proteins involved are not clear.

B-lymphopoiesis

Less is known about the role of WNT signaling in B

lymphopoiesis with the most compelling evidence supporting a significant role revealed by B cell defects detected in the Lef1$^{-/-}$ mouse. These mice demonstrate a significant reduction in B220+ cells in the fetal liver and perinatal bone marrow due to increased apoptosis and concomitant decrease in the number of cycling cells (Reya et al., 2000). However LEF1 is not required for normal B cell maturation as mature B cells are present in these animals. This is further supported by the restriction of LEF1 expression to the pro- and pre-B cell compartment, with expression being undetectable once cells differentiate into IgM positive immature and mature B cells (Reya et al., 2000). In wild type animals, pro-B cells respond to WNT-3a with enhanced proliferation, which is associated with stabilisation and nuclear translocation of ß-catenin (Reya et al., 2000). Interestingly, in animals transplanted with HSCs expressing constitutively activated ß-catenin, exhibited the highest proportion of chimerism in B-lineage cells with approximately 58% of cells being of donor origin compared to 19 and 15% of T and myeloid lineage cells respectively. One may speculate that activation of WNT signaling in HSCs preferentially leads to B-lineage commitment but this would need to be substantiated, particularly since these experiments were performed on a bcl-2 transgenic background (Reya et al., 2003).

Although the receptor for WNT proteins involved in B lymphopoiesis are not known, *Fzd-9* gene knockout mice have revealed an unexpected role of this gene in lymphoid development and maturation (Ranheim et al., 2005). Fzd-9 knockout mice exhibited a profound defect in B cell development due to a reduction in cell numbers at the pro/pre-B cell stage of maturation, which became more pronounced with age. The absence of an accumulation of more immature cells and the presence of relatively normal levels of mature circulating B cells suggests that there is not a block in maturation but rather an inhibition of expansion at the pro/pre-B cell stage, similar to the situation observed in Lef1$^{-/-}$ animals. The reduction in cell numbers in the B cell progenitor fraction appears to be mainly due to extrinsic factors as Fzd-9 null bone marrow cells compete successfully with WT cells to produce pro/pre-B cells in WT animals. This suggests that alterations in the microenvironmental B cell progenitor niche may be responsible for the reduced expansion of early B cell progenitors. Indeed, Yamane et al. demonstrated that B lymphopoiesis can be regulated independently of myelopoiesis by stromal cells, depending on the mechanism used to activate WNT signaling (Yamane et al., 2001). Conditional inactivation of osteoblasts in bone marrow of mice results in a complete loss of B lymphopoiesis indicating that osteoblasts are the primary source of factors capable of maintaining B cell development (Visnjic et al., 2004). Although osteoblasts and indeed other components of the stem cell niche are known to respond to WNT proteins, whether they express Fzd-9 is not known (Yamane et al., 2001; Bennett et al., 2005). Surprisingly, Fzd-9 null bone marrow cells fail to completely reconstitute the mature B cell compartment in competitive transplant experiments, suggesting an intrinsic role for Fzd-9 in the expansion of mature B cells, possibly in the periphery. This is consistent with the high level of expression of Fzd-9 on recirculating mature B cells in the bone marrow. Binding of WNT-2 to Fzd-9 is known to promote the growth of 293T cells by activating canonical WNT signaling and it is tempting to speculate that a similar effect of WNT-2 or another WNT protein may occur in mature peripheral B cells (Karasawa et al., 2002).

In an attempt to characterize genes involved in the commitment of progenitor cells to the B lineage, a serial analysis of gene expression revealed high expression of WNT-16 in these cells (Muschen et al., 2002). At present the role of WNT-16 in normal B cell development is unknown. These results clearly point to an important role of WNT signaling in regulation of normal B cell progenitor development. Given the dependence of normal B cell progenitors on bone marrow stromal derived molecules, it is quite reasonable to speculate that WNT proteins are providing important self-renewal cues to B cell progenitors undergoing a proliferative burst at the pre-B stage after successful Ig rearrangements.

WNT signaling in hematological malignancies

The WNT/ß-catenin signaling pathway is most notably perturbed in cancers. Mutations in various components of the pathway can frequently be found in variety of cancers including breast, colon, hepatic, pancreatic, lung, prostate, gastrointestinal, ovarian, medulloblastoma and melanoma (Giles et al., 2003). The involvement of the WNT pathway in regulation of hematopoietic progenitor/stem cell growth and self-renewal, in combination with its oncogenic potential in other cell types suggests that it might be deregulated in haematological malignancies. This speculation has recently been supported by experimental evidence suggesting that aberrant WNT signaling leads to oncogenic growth in both lymphoid and myeloid malignancies. A summary of alterations in WNT signalling components identified in hematological malignancies is summaried in Table 1.

Lymphoid malignancies

In T cell leukemia/lymphoma the tumor suppressor gene APC was methylated in approximately half of the cases examined with a greater proportion of the acute form of the disease being affected (Yang et al., 2005). Unfortunately the studies on the expression of APC were not performed so whether the observed methylation impacts on expression is yet to be confirmed, but the malignant T cell line ST1, lacks APC expression and demethylation with 5-azacytidine restored expression of this gene. In T lineage acute lymphoblastic leukemia APC was only methylated in 2 of 9 cases examined (Yang et al., 2006). However, it is interesting to

speculate whether a similar hyper-methylation of APC could be responsible for the observed over expression of ß-catenin in a number of T-ALL (Chung et al., 2002). In Jurkat T cells, inhibition of WNT signaling by over-expressing dominant negative forms of ß-catenin or TCF led to reduced proliferation and clonogenecity suggesting constitutive signaling though the pathway may be promoting proliferation and survival. Fas-mediated apoptosis is also potentiated by proteolysis of ß-catenin suggesting that this protein plays an important

Table 1. Defects in WNT signalling and their implication for haematological malignancies.

WNT Signalling Component	Alteration	Malignancy	Impact/Comment	References
APC	Gene methylation	Adult T leukemia/ lymphoma	Detected in 50% of cases. Greater in the acute cases.	(Yang et al., 2005; Yang et al., 2006)
ß-catenin	Increased expression	T-ALL	Down regulation of ß-catenin results in Increased apoptosis and decreased proliferation.	(Chung et al., 2002)
	Increased expression	AML	Active WNT canonical signalling	(Serinsoz et al., 2004; Simon et al., 2005)
	Increased expression	CML	Increased self renewal	(Jamieson et al., 2004)
γ-catenin	Activation resulting from AML1-ETO, PML-RARA or PLZF-RARA	AML	Increased proliferation and self renewal	(Muller-Tidow et al., 2004; Zheng et al., 2004)
WNT1	Increased expression	AML	Unknown	(Simon et al., 2005)
	Exogenous	MM	Increased migration	(Qiang et al., 2005)
WNT2b	Increased expression	AML	Unknown	(Simon et al., 2005)
	Increased expression	MM	Unknown	(Qiang et al., 2005)
Wnt3	Increased expression	CLL	Unknown	(Lu et al., 2004a)
WNT3a	Exogenous	MM	Increased proliferation and migration	(Derksen et al., 2004; Qiang et al., 2005)
WNT4	Exogenous	MM	Increased migration	(Qiang et al., 2005)
WNT5a	Decreased expression	ALL	Unknown	(Liang et al., 2003)
	Increased expression	MM	Unknown	(Qiang et al., 2005)
Wnt5b	Increased expression	CLL	Unknown	(Lu et al., 2004a)
Wnt6	Increased expression	CLL	Unknown	(Lu et al., 2004a)
WNT7a	Increased expression	MM	Unknown	(Qiang et al., 2005)
Wnt10a	Increased expression	CLL	Unknown	(Lu et al., 2004a)
WNT10b	Increased expression	MM	Unknown	(Qiang et al., 2005)
WNT11	Increased expression	MM	Unknown	(Qiang et al., 2005)
WNT13	Increased expression	MM	Unknown	(Qiang et al., 2005)
WNT14	Increased expression	CLL	Unknown	(Lu et al., 2004a)
Wnt16	Increased expression	CLL	Unknown	(Lu et al., 2004a)
WNT16b	Increased expression as a result of t(1;19)	B-ALL	Blockade of WNT16b results in Increased apoptosis and reduced proliferation	(McWhirter et al., 1999; Ross et al., 2003; Mazieres et al., 2005)
Fzd-3	Increased expression	CLL	Unknown	(Lu et al., 2004a)
Fzd-4	Increased expression as a result of Flt-3 mutations	AML	Present in 30% of patients. Effect unknown.	(Tickenbrock et al., 2005)
Dkk1	Increased expression	MM with mature phenotype	Associated with focal bone lesions	(Tian et al., 2003)
Dkk3	Gene methylation	ALL	↑Responsiveness to WNTs	(Roman-Gomez et al., 2004)
sFzd2	Increased expression	Plasmablastic MM	Associated with focal bone lesions	(Oshima et al., 2005)
BCL9	Increased expression as a result of t(1;14)	Pre-B ALL	Unknown	(Willis et al., 1998)
	Increased expression	Non-Hodgkin's lymphoma	Unknown	(Lestou et al., 2003)
	Increased expression	MM	Unknown	(Sawyer et al., 2005)

role in promoting leukemic cell survival. Expression of oncogenic E2A-Pbx1 resulting from the t(1;19) translocation in a subset of B-lineage acute lymphoblastic leukemias causes overexpression of WNT-16b (McWhirter et al., 1999; Ross et al., 2003). In functional studies, repression of WNT-16b expression either by neutralizing antibody or RNA interference induced apoptosis in E2A-Pbx positive cell lines (Mazieres et al., 2005). Interestingly, in response to these treatments, the mRNA expression of a number of downstream target genes including cyclin D1 and survivin was also modulated suggesting constitutive signaling of the canonical pathway. These data strongly suggest that autocrine stimulation of this pathway contributes to the development of leukemia in manner similar to loss of regulation of cytokine signaling pathways. This possibility is further strengthened by observation that Dkk-3, a secreted antagonist of WNT signaling, is methylated in one third of the acute lymphoblastic leukemia cells potentially making them more responsive to WNT signaling (Roman-Gomez et al., 2004). We have detected expression of various WNT and Fzd genes in a panel of pre-B ALL cell lines and patient samples that respond to WNT-3a stimulation by stabilising ß-catenin and augmenting proliferation and survival under serum-free conditions (N.I. Khan and L.J. Bendall, manuscript submitted). Together these results suggest that pre-B ALL cells express the necessary components of the WNT signaling machinery and may exhibit aberrant signaling by way of autocrine stimulation or epigenetic inactivation of antagonists of the pathway.

BCL9 is over-expressed in pre-B ALL cells bearing the t(1;14)(q21;q32) translocation compared to the very low levels of this gene in EBV transformed normal B cells (Willis et al., 1998). BCL9 was later identified as the mammalian orthologue of *D. melanogaster* Legless, a component of the WNT signaling pathway that is required for transcriptional activation of WNT target genes by ß-catenin (Kramps et al., 2002). Instability of the region around BCL9 on chromosome 1 is common in B cell malignancies and amplification of the BCL9 gene has been observed in 38 of 44 patients with multiple myeloma and 6 of 10 cases of Non-Hodgkin's lymphoma (Lestou et al., 2003; Sawyer et al., 2005). However the effect of these gene rearrangement on the level of protein expression has not been examined and the over expression of BCL9 alone has only marginal effects in TCF reporter assays. Although it is possible that the over expression of BCL9 may result in enhanced responsiveness to WNT signaling the potential role for over-expression of BCL9 in the aetiology of B cell malignancies still remains theoretical.

The B cells of patients with chronic lymphocytic leukemia (CLL) also over-express various WNT family members, LEF1 and Fzd-3 in comparison to their normal counterparts (Lu et al., 2004a). However at present it is unclear whether these components have a functional role in the aetiology of CLL. In contrast, cells from patients with low-grade non-Hodgkin's lymphoma, another malignancy of mature B cells, do not over express LEF1 but demonstrate the very low to undetectable levels of LEF1 as observed in normal mature B cells (Howe and Bromidge, 2006). Deregulation of a number of genes involved in Wnt signaling, were detected in cells from patients with Mantle cell lymphoma by microarray analysis (Rizzatti et al., 2005). However while some of these changes would be expected to enhance Wnt signaling, such as increased expression of TCF1, LRP5 and Fzd7 and decreased expression of Dkk1, other changes including the increased expression of APC and axin1 would be expected to antagonise Wnt signaling. This makes the overall interpretation of these findings difficult.

In multiple myeloma, a malignancy of more mature plasma cells, the vast majority of Fzds, co-receptors and WNTs are expressed (Qiang et al., 2003, 2005; Derksen et al., 2004). Multiple myeloma cells can respond to WNT-3a with activation of both canonical and the non-canonical WNT/Rho pathway (Qiang et al., 2003, 2005; Derksen et al., 2004). Exogenous WNT-3a stimulation augmented proliferation in absence of serum (Derksen et al., 2004). In contrast activation of signaling through Rho proteins was associated with altered cell morphology and motility and led to increased invasiveness of multiple myeloma cell lines in migration assays (Qiang et al., 2005). This suggests that Wnt signaling could enhance both the local proliferation and the spread of multiple myeloma cells throughout the bone marrow. Bone lesions are a feature of multiple myeloma and patients with focal bone lesions and a mature phenotype are reported to have high levels of the WNT antagonist, Dkk-1, in the bone marrow, plasma and peripheral blood (Tian et al., 2003). Dkk-1 was recently identified as a direct transcriptional target of the canonical WNT pathway in colorectal cancer cells (Gonzalez-Sancho et al., 2005). Multiple myeloma patients with an immature plasmablastic phenotype do not secrete Dkk-1 but other Wnt antagonists, primarily sFRP-2 (Oshima et al., 2005). It has been suggested that Dkk-1 and sFRP-2 may contribute to osteolytic lesions in multiple myeloma by suppressing normal osteoblast function (Tian et al., 2003; Oshima et al., 2005) with sFRP-2 being able to block BMP-2 induced osteoblast differentiation in vitro (Oshima et al., 2005). The inhibition of Wnt signaling within the bone marrow has the additional potential of inhibiting normal hematopoiesis and promoting the emigration of malignant cells from heavily infiltrated regions to areas of uninvolved marrow by inhibiting the local chemoattractant effect of WNTs.

Although these is accumulating evidence that canonical WNT signaling may be involved in the process of leukemogenesis a study by Liang et al demonstrated that WNT-5a-mediated activation of the non-canonical Ca^{++} pathway suppressed B-cell proliferation and that this could occur in an autocrine fashion (Liang et al., 2003). The authors observed that

Wnt-5a hemizygous knockout mice developed myeloid leukemias and B cell lymphomas spontaneously and analysis of primary human pre-B ALL revealed loss of WNT-5a expression in majority of cases examined in this study. These results demonstrate a novel tumor-suppressor function of WNT-5a in hematological malignancies mediated by the non-canonical WNT pathway. It also suggests that a delicate balance between canonical and non-canonical pathways maybe an essential mechanism for controlling dysregulated cell renewal and oncogenesis.

Myeloid malignancies

Several lines of evidence provide support for a role for the WNT signaling pathway in acute myeloid leukemia (AML). AML cells have significantly higher levels of ß-catenin mRNA and protein than normal hematopoietic progenitors (Serinsoz et al., 2004), and the presence of ß-catenin in nuclear fractions and associated LEF/TCF reporter activity indicates active WNT signaling (Simon et al., 2005).

No mutations have been found in ß-catenin or APC genes that would normally result in constitutive activation of WNT signaling in myeloid leukemias. Rather AML cases exhibit aberrant expression of LEF1, WNT-1 and -2b transcripts, which suggest autocrine expression of WNT signaling similar to lymphoid malignancies (Simon et al., 2005). The notion of active WNT signaling in myeloid leukemias is additionally supported by experimental evidence from a myeloid cell line which overexpressed Fzd-4 as a result of expression of oncogenic Flt3 mutation carrying an internal tandem repeat (ITD) (Tickenbrock et al., 2005). Analysis of AML patient samples carrying de novo activating mutations in Flt3 demonstrated active WNT/ß-catenin signaling which led to elevated levels of c-myc, a known downstream target gene. Given that up to 30% of AML patients carry activating mutations in Flt-3 (Kiyoi et al., 1998; Kondo et al., 1999), these data provide a novel mechanistic link between the most prominent mutation class in myeloid leukemias and aberrant WNT activation, thus providing a useful drug target. The possibility that AML cells are dependant on WNT signaling for oncogenesis is supported by studies in which cells transfected with various AML-associated translocation products (AML1-ETO, PML-RARA or PLZF-RARA) induced activation of plakoglobin (γ-catenin), a relative of ß-catenin (Muller-Tidow et al., 2004; Zheng et al., 2004). The induction of γ-catenin was accompanied by transactivation of LEF/TCF transcription factors, which in turn led to enhancement in proliferation and survival of murine hematopoietic progenitor cells (Muller-Tidow et al., 2004). Additionally transplantation of γ-catenin transduced HSCs into mice resulted in the development of AML like symptoms (Zheng et al., 2004). These reports clearly point to likely contribution of ß-catenin and γ-catenin in WNT signaling in myeloid leukemias, although development of mouse models with aberrant WNT signaling causing leukemias is awaited.

Perhaps the best evidence for the most conclusive role of aberrant WNT signaling in leukemogenesis comes from chronic myeloid leukemia (CML) in blast crisis (Jamieson et al., 2004). In this report, Weissman and colleagues, demonstrated elevated levels of nuclear ß-catenin levels in the granulocyte-macrophage progenitor (GMP) pool from patients with CML in blast crisis and in imatinib-resistant disease. Furthermore these progenitors had enhanced self-renewal ability in myeloid colony assays that was abrogated by enforced expression of axin. Although at present the exact mechanism of activated ß-catenin in CML remains unclear, mutations in components of the WNT signaling machinery cannot be ruled out. Also this data provides strong evidence for the role of ß-catenin driven self-renewal in leukemogenesis of CML.

Results from all these studies indicate that high expression of ß-catenin is a common denominator in myeloid leukemic cells; although the exact mechanism of this is not entirely clear. Unlike solid malignancies with activating mutations in components of the WNT pathway it is likely that in leukemias there is autocrine stimulation of the WNT pathway that leads to oncogenesis. Detection of activated ß-catenin may be a useful marker to predict disease progression, risk of relapse or development of imatinib resistance in a select group of patients.

Conclusion

WNT signaling provides proliferative signals for the most immature progenitor cells in both the B- and T-cell lineages, as well as self-renewal of HSCs and aberrant WNT activation is common to leukemias. However information is still scarce at the moment and there are still many questions that remain unanswered, such as does WNT signaling have a role in normal myeloid development in the bone marrow. Another question concerns the full detail of the role of this pathway in B cell development and how canonical and non-canonical pathways may be interacting in the BM stromal cells. It is also unclear which WNTs and Fzds are functionally involved in any of these processes. We are likely to see more studies evaluating large clinical groups of leukemic patients for activation of components of WNT pathway and DNA microarray studies that will identify new target genes in various tissues. Such identification strategies may potentially open new targets for development of novel therapies targeting aberrant WNT activation.

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