Chapter 2

Wnt signaling reaching gale force during multiple myeloma progression

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ABSTRACT

Aberrant activation of Wnt signaling plays a crucial role in the pathogenesis of various human cancers and is typically caused by mutations in the adenomatous polyposis gene (*APC*) or in the β-catenin gene (*CTNNB1*). As discussed in this review, multiple myelomas (MMs) often display evidence of Wnt pathway activation but do not contain these pathway intrinsic mutations. Instead, the Wnt pathway in MM is intact and activation results from auto- and/or paracrine stimulation by Wnt ligands. During tumor progression, this activation is promoted by epigenetic silencing of (soluble) negative feed-back regulators, like secreted frizzled-related proteins (sFRPs) and Dickkopf-1 (DKK1). Moreover, signaling may also be enhanced by genetic events affecting several recently identified positive- and negative Wnt-pathway regulators. Functional evidence indicates that deregulated Wnt signaling in MM plays two distinct pathogenic roles: i) aberrant activation of Wnt canonical and non-canonical Wnt pathways promotes tumor dissemination, proliferation, and drug resistance; ii) overexpression of soluble Wnt inhibitors like sFRPs and DKK1 by MMs contributes to osteolytic bone disease by inhibiting osteoblast differentiation. The ligand dependence of the aberrant Wnt signaling activity in MM implies that targeting Wnt secretion with small molecule inhibitors presents an interesting option for the treatment of MM.

INTRODUCTION

MULTIPLE MYELOMA

Multiple myeloma (MM) is a malignant plasma cell disorder that accounts for approximately 10% of all hematological cancers. It develops from a pre-malignant condition termed monoclonal gammopathy of undetermined significance (MGUS). In about 50% of MGUS and MM patients, the clonal plasma cells harbor translocations involving the immunoglobulin heavy chain (IgH) locus on chromosome 14q32 and one of the following partners: 11q13 (*CCND1*, cyclin D1 gene), 4p16.3 (*FGFR-3* and *MMSET* gene), 6p21 (*CCND3*, cyclin D3 gene), 16q23 (*MAFC* gene) and 20q11 (*MAFB* gene). Most of the remaining cases (IgH non-translocated MM) are associated with hyperdiploidy characterized by trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21.[1] Genetic abnormalities including Ras mutations, p16 (*CDKN2A*) promotor methylation, changes involving the MYC family of oncogenes, secondary chromosomal translocations and deletions, and p53 mutations have all been identified in clonal plasma cells in association with progression to the advanced stage MM.[2–5] In addition, the bone marrow (BM) microenvironment becomes heavily modified during disease progression and plays a crucial role in the biology of MM. This interaction between MM cells and the microenvironment is bi-directional: MM cells disrupt the homeostasis of the BM, resulting in anemia, aberrant angiogenesis, and osteolytic bone disease, while the BM microenvironment supports the growth and survival of the malignant plasma cells through signals mediated by adhesion molecules, cytokines, and growth factors.[6]

Major signaling routes deregulated in MM include the extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, Janus kinase (JNK), signal transducer and activator of transcription (JAK/STAT), and NF-κB pathways. In addition, as discussed in this review, over the last decade

evidence has accumulated indicating that deregulated Wnt signaling plays an important role in the pathogenesis of MM. This deregulation leads to aberrant expression of multiple Wnt pathway components, including agonists and antagonists, as well as illegitimate activation of canonical[7] and non-canonical Wnt signaling.[8–10] Although the full impact of this deregulation on the biology of MM is not yet defined, effects on tumor cell migration, proliferation and drug resistance have been reported.[7,8,10,11] In addition to these direct consequences for the tumor, aberrantly expressed Wnt pathway components also strongly affect the tumor microenvironment, inhibiting osteoblast differentiation and promoting angiogenesis.[6,12–14] Although loss of *APC* or gain of function mutations in *CTNNB1* have not been found in MM, recent data suggests that genetic and epigenetic alterations affecting Wnt pathway regulators play a critical role in the aberrant activation of the pathway.

THE Wnt PATHWAY

The term "Wnt" is derived from a combination of the names for the Drosophila melanogaster segment polarity gene *Wingless* and its vertebrate homolog-*Integrase-1* (*Int-1*), a mouse proto-oncogene that was discovered as an integration site for mouse mammary tumor virus.[15,16] There are 19 *Wnt* genes in the human genome, all encoding lipid-modified secreted glycoproteins, which act as ligands for cell surface receptor-mediated signal transduction pathways regulating a variety of cellular activities, including cell fate determination, proliferation, migration, and cell polarity. The lipid modification of Wnt proteins involves covalent attachment of palmitic acid on the first cysteine residue and palmitoleic acid on a highly conserved serine residue. Whereas the palmitoylation of Wnts is required for binding to their cognate frizzled receptors, initiating signaling, glycosylation of Wnts is required for their secretion. Studies in Drosophila and vertebrates have shown that Wnt signals can be transduced in distinct ways; by a well-defined "canonical" Wnt/β-catenin pathway, or by either of two "non-canonical" β-catenin independent pathways.[17–19]

CANONICAL Wnt SIGNALING

Wnt/β-catenin signaling is the best characterized Wnt signaling pathway. A major effector of the canonical Wnt signaling pathway is the transcription factor β-catenin. In the absence of Wnt proteins, β-catenin interacts with APC and Axin scaffold proteins in the cytoplasm and is a phosphorylation substrate for the kinases Casein Kinase 1 (CK1) and glycogen synthase kinase (GSK)3β. Phosphorylated β-catenin is ubiquitinated and subsequently destructed by the proteosome. Wnt ligand binding to Frizzled (Fz) family receptors, in complex with the coreceptor LRP5/6 and Dishevelled (DVL), promotes the phosphorylation of LRP5/6 by CK1 and GSK3β (the same kinases that are involved in β-catenin phosphorylation in the destruction complex). Phosphorylation of LRP5/6 creates a docking site for AXIN1, resulting in its sequestration from the destruction complex and β-catenin stabilization. Active, non–phosphorylated β-catenin translocates to the nucleus where it binds TCF/LEF transcription factors and mediates expression of Wnt responsive genes (Figure 1).[20–22]

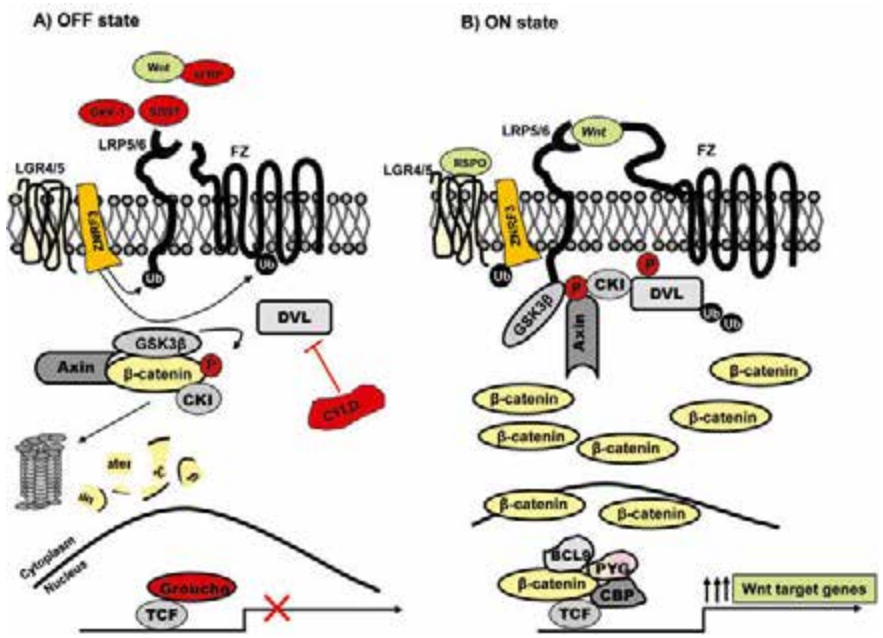

Figure 1. The canonical Wnt signaling pathway

Off state: In the absence of Wnt signaling free cytoplasmic β-catenin is kept at very low levels by proteosomal degradation. β-catenin degradation is accomplished through active phosphorylation at conserved regions by glycogen synthase kinase 3β (GSK-3β) and Casein Kinase 1 (CK1). In addition, without the presence of R-spondin, ZNRF3 promotes turnover of Fz and LRP6 by acting as an ubiquitin ligase for these proteins. DKK1, sFRP, WIF1, and sclerostin are soluble Wnt cascade inhibitors, preventing Wnt signaling, even in the presence of Wnt ligands. CYLD acts as negative Wnt pathway regulator by deubiquitinating Dishevelled (DVL). In nucleus, in the absence of β-catenin, TCF/LEF occupies and represses Wnt target genes, assisted by transcriptional co-repressors such as Groucho. On state: Upon binding of a Wnt to Fz-family receptors in complex with the co-receptor LRP5/6 and DVL, phosphorylation of LRP5/6 by CK1 and GSK3β is initiated. Phosphorylation of LRP5/6 creates a docking site for AXIN1, resulting in its sequestration from the destruction complex and β-catenin stabilization. Active, non-phosphorylated β-catenin translocates to the nucleus, replaces Groucho from TCF/LEF, and recruits transcriptional co-activators to drive target gene expression. Moreover, if present, R-spondin binds to both, ZNRF3 and LGR4, promoting ZNRF3 turnover, likely through autoubiquitination, thereby stabilizing Fz and LRP5/6 for a greater Wnt response.

As most other signaling cascades, the Wnt pathway contains multiple negative and positive regulatory elements, which either limit or enhance the strength and duration of the signal triggered by the initial stimulus. Thus, paracrine and/or autocrine stimulation of Wnt signaling induces expression of several negative intracellular and secreted feedback regulators, including AXIN2, DKK1, and sFRPs, resulting in signal attenuation once a certain threshold has been reached.[23,24] Recent studies have revealed a crucial role of protein ubiquitination in the regulation of Wnt signaling. It was shown that the deubiquitinase (DUB) CYLD negatively regulates Wnt signaling by removing K63-linked polyubiquitin chains from Dishevelled (Dvl).[25] In addition, ubiquitination and deubiquitination of Fz was found to modulate the cellular responsiveness to Wnts.[26] This ubiquitination is controlled by transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) or its homologue RNF43. ZNRF3/RNF43 ubiquitinates Fz and promotes the degradation of frizzled and LRP6, leading to attenuated canonical and non-canonical Wnt signaling. R-spondin a positive modulator of Wnt signaling was shown to bind to ZNRF3, in addition to transmembrane LGR4/5 receptors, resulting in membrane clearance of ZNRF3 thereby increasing Fz and LRP6 membrane expression levels and enhancing responses to Wnts.[27,28]

NON-CANONICAL Wnt SIGNALING

The non-canonical Wnt signaling pathways in Drosophila and vertebrates are less well characterized. They also involve Wnt-Fz binding, but are independent of LRPs and β-catenin. Based on the major intracellular mediators used, they are designated the Wnt/Jun N-terminal kinase (Wnt/JNK) or Wnt/Ca^{2+} pathway. The Wnt/JNK pathway largely overlaps with the planar cell polarity pathway, originally described in Drosophila. In this pathway, receptor triggering leads to Dvl mediated activation of the small GTPase RhoA and downstream protein kinases, including JNK and Rho kinase, which affects cytoskeletal dynamics.[29–32] In the Wnt/Ca^{2+} pathway, G-proteins, phospholipase C (PLC), and phosphodiesterase (PDE) are activated. Elevation of intracellular calcium levels activates enzymes such as Ca^{2+}/calmodulin dependent kinase II and protein kinase C, resulting in altered cell motility. Importantly, the Wnt/Ca^{2+} pathway has been linked to the activation of Nemo-Like Kinase (NLK), which is able to phosphorylate TCF transcription factors and thereby inhibits canonical Wnt signalling.[29,30]

SOLUBLE Wnt PATHWAY INHIBITORS

Wnt signaling is negatively regulated by a multitude of secreted proteins. These include Wnt inhibitory factor 1 (Wif1), secreted frizzled related proteins (sFRPs),

Dickkopfs (DKKs), and sclerostin. The sFRP family consists of five members, each containing a cysteine rich domain (CRD) with 30–50% sequence homology with the CRD of Fz receptors. Both, WIF1 and sFRPs bind to Wnt ligands thereby inhibiting Wnt pathway activity. In addition, sFRPs may also inhibit Wnt signaling by forming an inhibitory complex with Fz receptors. DKKs have a different mode of action: They act by forming a ternary complex with LRP5/6, resulting in endocytosis and thus depletion of LRP receptors from the cell surface. In this way they prevent Fz activation, even in the presence of Wnt ligands. Sclerostin, which was recently added to the list of extracellular Wnt antagonists, also binds to LRP5 and LRP6 co-receptor but with a lower affinity than DKK1.[33–35]

THE ROLE OF Wnt SIGNALING IN MULTIPLE MYELOMA

THE ROLE OF NON-CANONICAL Wnt SIGNALING IN MULTIPLE MYELOMA

The first evidence for a role of (non-canonical) Wnt signaling in MM came from a study by Qiang *et al*.[9] These authors demonstrated that Wnt3a induces rearrangement of the actin cytoskeleton as well as striking morphological changes in myeloma cells. These responses were completely inhibited by sFRP-1, indicating the involvement of Fz receptors, but the DKK1 and DKK2 proteins had no effect, implying that LRP co-receptors were not involved. The morphological changes were associated with Rho activation and could be completely blocked by a Rho-associated kinase inhibitor.[10] Subsequent studies from the same laboratory showed that multiple members of the Wnt family (Wnt3a, Wnt1, Wnt4) promote myeloma cell migration and invasion. This Wnt-mediated migration was associated with activation of RhoA and members of the protein kinase C (PKC) family, including PKCα, PKCβ, and PKCμ and involved induction of macromolecular signaling complexes containing Dvl, RhoA, and PKCs.[10] Thus, by stimulating the migration of malignant plasma cells, non-canonical Wnt signaling may promote MM cell dissemination within the bone marrow.[10] An additional functional consequence of non-canonical Wnt signaling in MM involves its regulatory effect on integrin-mediated cell adhesion. As shown by Kobune *et al*.[8] Wnt stimulation promotes myeloma cell adhesion to extracellular matrix and stromal cells, through activation of the RhoA/ROCK kinase pathway. This integrin-mediated adhesion was shown to regulate cell-adhesion mediated drug resistance (CAM-DR) to doxorubicine.

ACTIVATION AND FUNCTION OF CANONICAL Wnt SIGNALING IN MULTIPLE MYELOMA

Initial evidence for a role of canonical Wnt pathway activation in the pathogenesis of MM came from a study by Derksen *et al.*[7], demonstrating that MM plasma cells, unlike normal BM plasma cells, express nuclear and non-phosphorylated β-catenin, suggesting active β-catenin/TCF mediated transcription. Stimulation of Wnt signaling with exogenous Wnt ligand (Wnt3a), LiCL, or a constitutively active mutant of β-catenin (S33Y), enhanced accumulation and nuclear localization of β-catenin and promoted proliferation. In contrast, disruption of β-catenin/TCF activity by dominant negative TCF led to inhibition of MM cells proliferation. Importantly, no mutations in *APC* or β-*catenin* (*CTNNB1*) were found in MMs. These data indicate that MM cells are dependent on active Wnt signaling, involving autocrine Wnts, which was further stimulated by exogenous (paracrine) Wnt ligands.[7] Consistent with these observations, analysis of gene-expression profiling data of primary MMs[36] revealed co-expression of various Fzs (*e.g.* Fz 1, 3, 6, 7 and 8) and the co-receptor LRP6, as well as various Wnts (including 4, 5A, 5B, 6, 10A and 16). Approximately half of the primary MMs co-expressed LRP6 with at least one Fz genes and one Wnt gene, indicating that these MMs are well equipped to evoke autocrine Wnt pathway activation. Furthermore, Wnt ligands are produced by bone marrow stromal cells,[7,37] providing a source of paracrine Wnt pathway activation. These findings were supported by Sukhdeo *et al.*,[38] who also observed massive upregulation of multiple Wnt signaling pathway genes in primary MM cells. In line with the study of Derksen,[7] active, non-phosphorylated β-catenin was found in the nucleus of the cells of the majority of MM cell lines. Blockage of β-catenin/TCF mediated transcription, by using the β-catenin/TCF complex inhibitor PKF115-584, resulted in downregulation of Wnt target genes as well as cell cycle arrest, apoptosis, and activation of apoptotic regulators.[38] These findings were supported by *in vivo* data, showing that MM growth in SCID mice was effectively inhibited by the compound.[38] Subsequent studies from the same laboratory demonstrated that Wnt/β-catenin pathway does not only affect the G1 phase of the cell cycle but also G2/M transition.[11] By targeting β-catenin with shRNAs, new potential Wnt target genes involved in cell cycle progression and checkpoint regulation were identified. Among these genes, AURKA/B was shown to act as a key regulator of β-catenin-mediated effects on cell cycle and MM growth, suggesting an important role for this protein in the Wnt-mediated pathogenic effects. Importantly, targeting β-catenin protein expression caused significant tumor reduction and increased survival in a xenograft mouse model of MM.[11] The finding that silencing of β-catenin by siRNAs inhibits of MM tumor growth *in vivo*

was confirmed by Ashihara *et al.*[39] In the 5TGM1 mouse myeloma model, Edwards *et al.*[40] found that Wnt pathway activation by LiCl induces accumulation of β-catenin and enhanced Wnt target gene expression, but does not alter the *in vitro* proliferation rate or the Wnt-pathway dependent tumor expansion in the BM microenvironment. However, LiCl enhanced the growth of subcutaneously inoculated 5TGM1 cells, which was prevented by overexpression of a dominant-negative *TCF4*, confirming the Wnt signaling dependency.[40] A recent study by Bjorklund *et al.*[41] indicates a role for Wnt/β-catenin signaling in drug resistance. Exposure of malignant plasma cells to lenalidomide enhanced β-catenin/TCF mediated transcription and expression of Wnt target genes. This lenalidomide-mediated Wnt pathway activation, or activation by Wnt3a or β-catenin, reduced the anti-proliferative effect of lenalidomide. These effects were reversed by shRNA-mediated down-regulation of β-catenin, suggesting that targeting Wnt/β-catenin signaling may help to overcome lenalidomide resistance in MM. In line with the results discussed above Qiang *et al.*[42] reported that Wnt3a, either alone or in combination with IL-6 and insulin-like growth factor (IGF)-1 induces β-catenin stabilization and Wnt reporter activity. However, these authors did not find an enhancing effect on MM proliferation but reported that Wnt3a overexpression attenuates bone disease and tumor growth of a human MM cell line transplanted in human bone implants in SCID mice.[42] In another study,[43] GSK3β inhibition by 6-bromoindirubin-3-oxime (BIO) led to improved bone quality at the bone-tumor interface as well as to increased tumor necrosis in a murine model for MM-bone disease, while a pro-apoptotic effect of BIO was found in human MM cell lines *in vitro*.

Taken together, although a few discrepant reports exist, which might be explained by the difference in experimental models or distinct levels of Wnt pathway activation,[44] most of the above studies support a scenario in which aberrant canonical and non-canonical Wnt pathway activation acts as an important factor enhancing MM aggressiveness by promoting cell motility, cell cycle progression, and drug-resistance. This Wnt pathway activation depends on autocrine and/or paracrine Wnts and not on mutations in the *APC* or *CTNNB1*, causing ligand-independent activation. However, as will be discussed below, loss of negative regulation of Wnt signaling is common in MMs and appears to be an important factor in Wnt pathway activation during disease progression.

EPIGENETIC AND GENETIC EVENTS AFFECTING Wnt SIGNALING IN MULTIPLE MYELOMA

During MM progression, negative regulators of Wnt signaling are common target of epigenetic silencing. Thus, Chim *et al.*[45] reported silencing by promoter

hypermethylation of the Wnt antagonists *WIF1*, *DKK3*, *APC* as well as *sFRP1*, *-2*, *-4* and *-5* in MM cell lines[45] These cell lines displayed active Wnt signaling, which was suppressed by treatment with the demethylating agent by 5-azadC, re-expression of these antagonists. Of primary MM samples, 40% showed methylation of one or more of these seven genes, indicating that methylation of soluble Wnt antagonists is common in MM.[45] These data suggest that Wnt inhibitors may act as tumor suppressor genes that need to be inactivated in order to reach optimal levels of Wnt pathway activation during MM progression. Indeed, a study by Jost *et al.*[46] revealed that whereas hypermethylation of *sFRP1* and *-2* genes is already present in MGUS and remained present at all subsequent MM stages, *sFRP5* methylation was restricted to advanced stages of MM and plasma cell leukaemia (PCL).[46] Intriguingly, these studies did not explore the methylation status of *DKK1*, a major Wnt antagonist secreted by MM cells, which contributes to MM bone disease by inhibiting osteoblast differentiation (discussed below). We recently demonstrated[36] that *DKK1* is also a target of methylation and that *DKK1* methylation is largely limited to advanced stage MM. Hence, during the initial stages of MM evolution overexpression and secretion of DKK1 by malignant plasma cells modifies the bone marrow niche, inhibiting Wnt-signaling dependent osteoblast differentiation and creating an optimal environment for MM growth and progression. In advanced stage MM, however, silencing of DKK1 unleashes the Wnt pathway in MM cells, promoting proliferation of MM and drug resistance. Taken together, these data suggest that aberrant Wnt pathway activation in MM is the consequence of "releasing the brake" rather than of "hitting the gas". This hypothesis is corroborated by recent studies from our laboratory, identifying CYLD as an important negative regulator of Wnt signaling in MM. (manuscript in preparation).

CYLD was originally identified as a tumor suppressor gene mutated in familial cylindromatosis (Brooke-Spiegler syndrome), an autosomal dominant disorder predisposing to benign tumors of skin appendages.[47] Subsequent studies have linked loss of the tumor suppressor function of CYLD to the pathogenesis of several other tumors including melanoma, T-cell acute lymphoblastic lymphoma (T-ALL), and colon and hepatocellular carcinoma.[48–51] In MM, loss of CYLD, resulting from biallelic deletion or inactivating mutations, is among the most common genomic aberrations.[52,53] The CYLD protein is a member of the USP family of deubiquitinating enzymes (DUBs), which act by specifically removing lysine(K)-63-linked polyubiquitin chains from substrate proteins.[54] In contrast to lysine-48-linked polyubiquitination marking proteins for proteasomal degradation, K63-linked ubiquitination enhances protein stability and facilitates

protein-protein interaction. A number of studies have shown that CYLD acts as a negative regulator of nuclear factor-κB (NF-κB) signaling removing K63-linked polyubiquitin chains from TRAF2, TRAF6, and NEMO.[55,56] Other CYLD substrates important for NF-κB signaling include RIPK1, BCL3 and TAK1.[57–59] In addition, it was recently reported that CYLD may also acts as a negative regulator of proximal events in Wnt/β-catenin signaling. Loss of CYLD causes hyperubiquitination of the DIX domain of the adapter protein Dishevelled (Dvl), leading to enhanced Dvl polymerization and Wnt signaling.[25] Indeed, human cylindroma skin tumors that arise from mutations in *CYLD* were found to display hyperactive Wnt signaling, suggesting that the tumor growth instigated by loss of CYLD involves enhancement of Wnt responses.[25] We recently observed that CYLD expression in human myeloma cell lines as well as primary MMs is highly variable and that the protein is functionally involved in the regulation of MM cell growth and survival. In MM patients, low CYLD expression is associated with poor progression free survival and overall survival. Functional assays employing inducible CYLD silencing or overexpression revealed that CYLD represses autocrine as well as ligand-induced Wnt/β-catenin signaling, and that low CYLD expression in primary MMs is strongly associated with the presence of a Wnt signaling gene-expression signature. These findings identify CYLD as a regulator of Wnt/ β-catenin signaling in MM and suggest that loss of CYLD enhances MM aggressiveness through a mechanism involving Wnt pathway activation resulting in enhanced proliferation. (manuscript in preparation)

In conclusion, loss of negative regulators of Wnt signaling by epigenetic and genetic events causes hyperactivation of the Wnt pathway in advanced MM, contributing to enhanced cell proliferation and drug resistance.

THE ROLE OF Wnt PATHWAY IN THE PATHOGENESIS OF MULTIPLE MYELOMA BONE DISEASE

Wnt INHIBITORS AND MULTIPLE MYELOMA BONE DISEASE

During the last decade, it has become clear that osteoblast differentiation is critically dependent on Wnt signaling.[60] Interestingly, Tian *et al.*[61] showed that most primary MMs overexpress and secrete the Wnt inhibitor DKK1 and that this overexpression was strongly correlated to the presence of osteolytic bone disease. Similar data were reported by Oshima *et al.*[62] for the secreted Wnt signaling inhibitor sFRP2: MM cells from patients with advanced bone lesions secreted