Wnt Signaling in Osteosarcoma

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Abstract

Wnt molecules are a class of cysteine-rich secreted glycoproteins that participate in various developmental events during embryogenesis and adult tissue homeostasis. Since its discovery in 1982, the roles of Wnt signaling have been established in various key regulatory systems in biology. Wnt signals exert pleiotropic effects, including mitogenic stimulation, cell fate specification, and differentiation. The Wnt signaling pathway in humans has been shown to be involved in a wide variety of disorders including colon cancer, sarcoma, coronary artery disease, tetra-amelia, Mullerian duct regression, eye vascular defects, and abnormal bone mass. The canonical Wnt pathway functions by regulating the function of the transcriptional coactivator β-catenin, whereas noncanonical pathways function independent of β-catenin. Although the role of Wnt signaling is well established in epithelial malignancies, its role in mesenchymal tumors is more controversial. Some studies have suggested that Wnt signaling plays a pro-oncogenic role in various sarcomas by driving cell proliferation and motility; however, others have reported that Wnt signaling acts as a tumor suppressor by committing tumor cells to differentiate into a mature lineage. Wnt signaling pathway also plays an important role in regulating cancer stem cell function. In this review, we will discuss Wnt signaling pathway and its role in osteosarcoma.

Keywords

Osteosarcoma · Wnt · β-Catenin · Dickkopf · Cancer stem cell · Wnt inhibitory protein · Frizzled- related protein

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Introduction

Wnt molecules are a class of cysteine-rich secreted glycoproteins that participate in various developmental events during embryogenesis and adult tissue homeostasis. Since its discovery in 1982, the roles of Wnt signaling have been established in various key regulatory systems in biology. The term Wnt combines the *Drosophila* segment polarity gene *Wingless* and the mouse proto-oncogene *Int-1*. Currently, 19 different Wnt proteins have been identified in the human genome [1, 2]. Wnt signals exert pleiotropic effects, including mitogenic stimulation, cell fate specification, and differentiation. It is a complex, tightly regulated pathway with many functions, whose involvement in cancer reinforces the notion that oncogenesis is a form of development gone awry. The Wnt signaling pathway in humans has been shown to be involved in a wide variety of disorders including colon cancer, coronary artery disease, tetra-amelia, Mullerian duct regression, eye vascular defects, and abnormal bone mass [2, 3].

The canonical Wnt pathway functions by regulating the function of the transcriptional coactivator β-catenin, whereas noncanonical pathways function independent of β-catenin. In the development of the bone, the canonical Wnt/β-catenin pathway is required for osteoblast differentiation, enhanced ossification, and suppression of chondrocyte formation. Recent reports of conditional inactivation of β-catenin in skeletal progenitors using Cre lines have revealed that β-catenin is essential for differentiation of mature osteoblasts and consequently for bone formation. Lack of β-catenin leads to a failure of perichondral and periosteal cells to express the osteoblast commitment factor Osterix, resulting in a chondrogenic fate [4, 5]. Loss-of-function and gain-of-function mutations in low-density lipoprotein receptor-related protein 5 (LRP5), a Wnt receptor, are associated with osteoporosis-pseudoglioma syndrome and high bone mass phenotypes, respectively [6, 7]. LRP5 expression has also been shown to be a marker for disease progression in high-grade osteosarcoma (OS), and its suppression may lead to reduction in local or systemic disease burden [8, 9].

The association between Wnt/β-catenin signaling and colon cancer is well recognized. Blocking β-catenin signaling has generated a significant interest in colon cancer treatment [10]. Although the role of Wnt signaling is well established in epithelial malignancies, its role in mesenchymal tumors is more controversial. Some studies have suggested that Wnt signaling plays a pro-oncogenic role in various sarcomas by driving cell proliferation and motility [11–14]; however, others have reported that Wnt signaling acts as a tumor suppressor by committing tumor cells to differentiate into a mature lineage [15–18]. In addition, the Wnt signaling pathway plays an important role in regulating cancer stem cell (CSC) function [19]. In osteosarcoma, stem cells have activated Wnt/β-catenin signaling, and Wnt inhibition can thus reduce drug resistance [20, 21].

Osteosarcoma (OS) is the most common primary bone malignancy in children and young adults. With the current multidisciplinary treatments, 60–70% of patients with localized disease survive [22]. Unfortunately, the long-term survival of patients with relapsed disease is only about 20% [23]. Despite aggressive efforts to strengthen and modify chemotherapy, the outcome of patients with OS has not significantly improved over the past few decades [24]. In this review, we will discuss Wnt signaling pathway and its role in osteosarcoma.

Overview of Wnt/β-Catenin Signaling Pathway

The Canonical Wnt Pathway

In the inactive state, there is an absence or inhibition of Wnt, which enables cytoplasmic β-catenin to form a complex with multiple proteins, including Axin, adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β) [2, 25–27] (see Fig. 8.1). Within this complex, CK1 and GSK3β

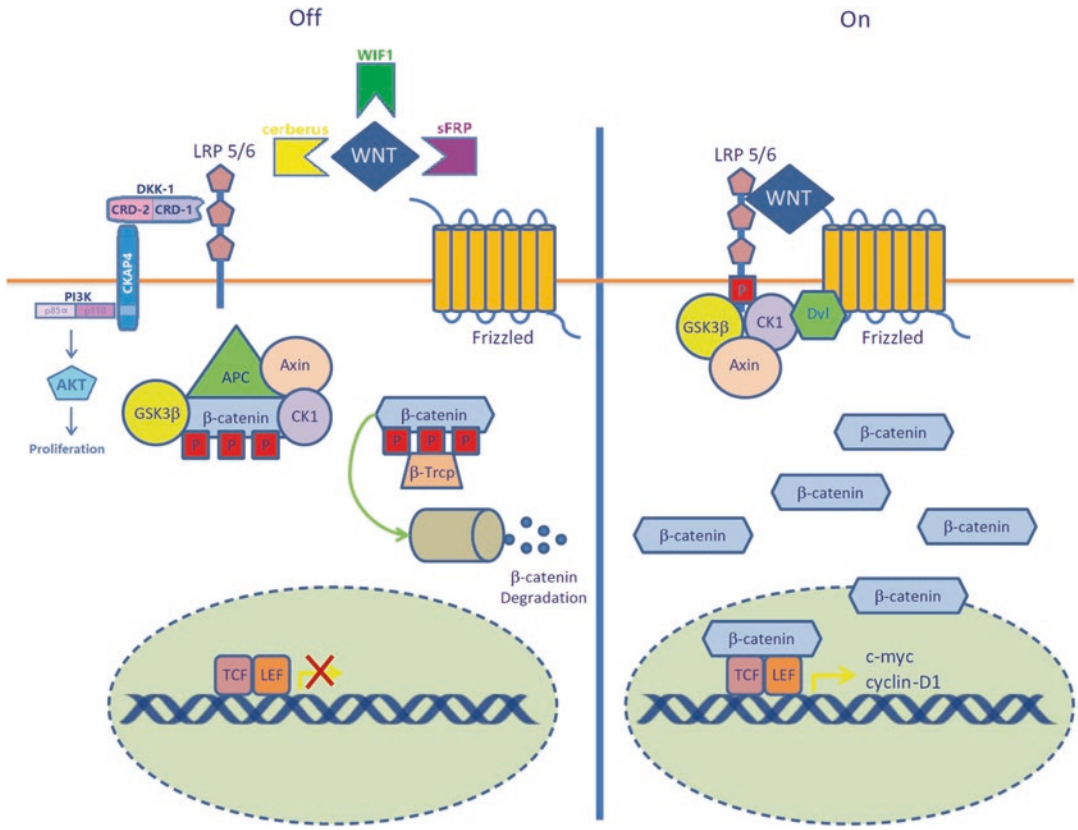

Fig. 8.1 Overview of Wnt/β-catenin signaling
In the absence or inhibition of Wnt, the cytoplasmic β-catenin forms a complex with Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β). CK1 and GSK3β phosphorylate β-catenin. β-Trcp (E3 ubiquitin ligase subunit) recognizes this complex and targets β-catenin for proteasomal degradation. The Wnt antagonists WIF1, sFRP, and cerberus bind directly to Wnt ligands. The DKK family proteins competitively bind to Wnt receptor LRP5/6. The DKK proteins have two cysteine-rich domains, cysteine-rich domain 1 (CRD1) and CRD2. DKK-1 binds to LRP5/6 through the CRD1 domain, and DKK-1 binds to cytoskeleton-associated protein (CKAP) 4 through CRD2 to induce proliferation in normal and tumor cells in a β-catenin-independent manner via the PI3K/AKT pathway
In the presence of Wnt binding to targeted receptors frizzleds, low-density lipoprotein receptor-related protein 5 and 6 (LPR 5/6), and disheveled (Dvl), the complex becomes phosphorylated, leading to the inhibition of GSK3β. Cytoplasmic non-phosphorylated β-catenin accumulates, inhibiting its degradation and promoting translocation to the nucleus. A complex with transcription factors, including T-cell transcription factor (TCF), lymphoid enhancer-binding factor (LEF), and transcriptional coactivators, leads to transcriptional activity of multiple downstream target oncogenes

act in concert to phosphorylate β-catenin, which is then targeted by the E3 ubiquitin ligase β-Trcp for proteasomal degradation. When Wnt ligands bind to their target membrane receptors frizzled and LRP5/6, cytoplasmic disheveled (Dvl) causes phosphorylation of the complex, leading to an inhibition of GSK3β. The resulting cytoplasmic accumulation of non-phosphorylated β-catenin promotes its translocation into the nucleus. Within the nucleus, β-catenin forms a complex with T-cell transcription factor (TCF), lymphoid enhancer-binding factor (LEF), and other transcriptional coactivators to induce the transcription of multiple downstream target genes (e.g., c-Myc, cyclin D1) that promote cellular proliferation [2, 28].

In addition to Wnt ligands and receptors, five families of Wnt antagonists have been identified:

Wnt inhibitory factor 1 (WIF1), secreted frizzled-related proteins (sFRP1–5), Dickkopf (DKK) proteins, cerberus, and the Wise/SOST family. Among these, WIF1, sFRP, and cerberus bind directly to Wnt ligands. The DKK family proteins and SOST competitively bind to Wnt receptor LRP5/6. The DKK proteins have two cysteine-rich domains, cysteine-rich domain 1 (CRD1) and CRD2. DKK-1 binds to LRP5/6 through the CRD1 domain, and more recently, it has been recognized that DKK-1 binds to cytoskeleton-associated protein (CKAP) 4 through CRD2 to induce proliferation in normal and tumor cells in a β-catenin-independent manner via the PI3K/AKT pathway [29].

The Noncanonical Wnt Pathways

Noncanonical Wnt pathways play important roles in embryonic and tissue development, homeostasis, and bone formation. Wnt5a is one of the major ligands for noncanonical Wnt signaling. Planar cell polarity (PCP) and Wnt/calcium are the major noncanonical Wnt pathways (Fig. 8.2). These pathways are initiated by Wnt/frizzled signaling rather than β-catenin transcriptional function. Disheveled (Dvl) is downstream of both canonical and noncanonical signaling pathways and has three different domains, i.e., DIX, PDZ, and DEP. DIX and PDZ domains function in canonical pathway to stabilize β-catenin. In noncanonical planar cell polarity (PCP) pathway, activation of small

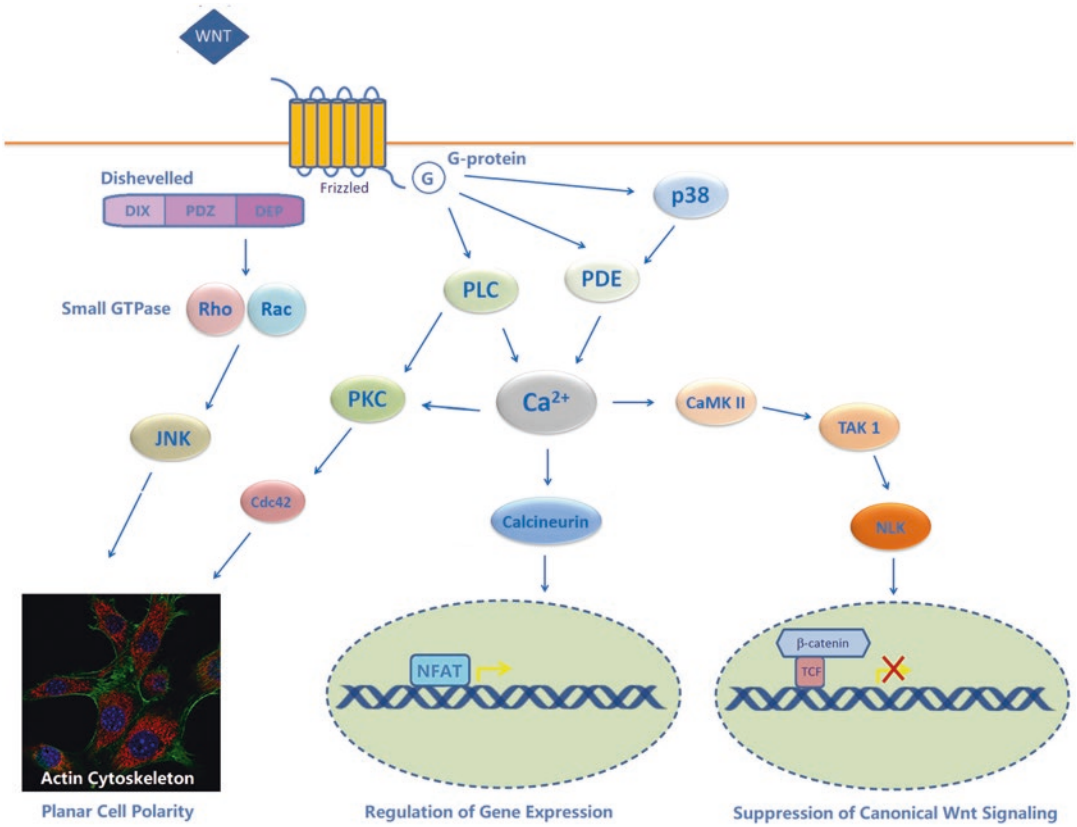

Fig. 8.2 Noncanonical Wnt signaling. The activation of frizzled by Wnt is mediated by disheveled or heterotrimeric G-proteins. The planar cell polarity pathway is mediated by small GTPase (Rho and Rac), JNK, and Cdc42, which is activated by PKC. Ca^{2+}-calcineurin pathway activates NFAT to regulate the gene expression. Ca^{2+}-induced CaMKII-TAK1-NLK pathway suppresses canonical Wnt signaling by inhibiting β-catenin-dependent transcription

GTPases, Rho and Rac, occurs downstream of DEP domain. The PCP pathway regulates the cytoskeletal architecture to affect cell migration via the activation of c-Jun NH2-terminal kinase (JNK).

In the Wnt/Ca^{2+} pathway, frizzled activation by Wnt leads to activation of heterotrimeric G-proteins. Activated G-protein regulates phospholipase C (PLC), phosphodiesterase (PDE), and p38, which on activation release intracellular Ca^{2+} to activate calcium-sensitive enzymes such as protein kinase C (PKC), calcineurin (CaCN), and Ca^{2+}/calmodulin-dependent kinase II (CaMKII). PKC activates the small GTPase Cdc42, which is a key regulator of PCP to remodel the actin cytoskeleton and control the polarity of cells. Nuclear factor of activated T-cells (NFAT) is the downstream target of CaCN. NFAT enters the nucleus to regulate target gene expression and regulates cell proliferation and differentiation. TGF-β-activated kinase-1 (TAK1)/Nemo-like kinase (NLK) are downstream targets of CaMKII that antagonize β-catenin-mediated canonical signaling [30, 31].

Wnt Signaling in Osteosarcoma

The role of Wnt signaling in osteosarcoma is controversial. The majority of studies report that Wnt signaling is pro-tumorigenic (Table 8.1); however, other studies also suggest that Wnt is tumor suppressive (Table 8.2). Tissue samples from osteosarcoma patients have been used to correlate various components of the Wnt pathway with clinical outcomes. In our study, RNA isolated from fresh-frozen osteosarcoma tissue was used to examine the expression of the Wnt receptor LRP5 by RT-PCR. LRP5 RNA expression statistically correlated with worse event-free survival in patients [8], and a dominant-negative LRP5 decreased tumorigenicity and metastasis of OS in vivo in nude mouse experiments [9]. Furthermore, it appears that blocking Wnt/LRP5 signaling can reduce tumor invasiveness by reversing the epithelial-to-mesenchymal transition [32]. Some recent studies also report Wnt/β-catenin signaling activation in osteosarcoma [13, 33]; however, others have shown that the Wnt/β-catenin signaling activation occurs only in the cancer stem cell (CSC) subpopulation of osteosarcoma cells and not in parental cells. It is the CSCs that are thought to be responsible for relapse, metastasis, and resistance to chemotherapy, so if targeting Wnt signaling can eliminate these cells, this may offer a new therapeutic approach that may improve patient outcomes [20, 21, 34]. There are some reports of DKK-1, a Wnt inhibitor, possibly playing a pro-tumorigenic role [15–17, 35, 36].

By using siRNA to suppress Wnt5a, Enomoto et al. demonstrated reduced invasiveness and

Table 8.1 Recent studies suggesting Wnt is pro-tumorigenic

Author/year	Protein of interest	Clinical relevance
Fang et al. 2018 [11]	Small molecular inhibitor of Wnt, PRI-724	Treatment with it leads to decreased cell proliferation, migration, invasion, colony formation
Neves et al. 2018 [21]	IWR-1 tankyrase inhibitor	IWR-1 inhibits translocation of *β*-catenin to the nucleus. It impairs self-renewal capacity of stem cells and hampers the activity and expression of stemness-related markers
Zhao et al. 2015 [113]	Naked cuticle homolog 2 (NKD2) gene, a down regulator of Wnt signaling	Downregulation of NKD2 expression found in metastatic and recurrent OS. Overexpression of NKD2 decreases cell proliferation and metastasis ability in vivo and in vitro by inhibiting Wnt signaling
Neves et al. 2015 [34]	Nuclear *β*-catenin	Exposure to conventional chemotherapy induces transition to stem-like phenotype associated with activation of Wnt/*β*-catenin signaling
Neves et al. 2015 [20]	Nuclear *β*-catenin	Cancer stem cells show activation of Wnt/*β*-catenin signaling as evident by increased nuclear *β*-catenin, TCF/LEF activity, and Axin2 expression

Table 8.2 Recent studies suggesting Wnt is anti-tumorigenic in osteosarcoma

Author/year	Protein of interest	Clinical relevance
Goldstein et al. 2016 [16]	Wnt signaling inhibitor DKK-1	Anti-DKK-1 antibody slowed the growth of tumor and inhibits metastasis. This effect was correlated with increased nuclear beta-catenin and increased expression of bone differentiation marker osteopontin
Cai et al. 2010 [18]	GIN (GSK3β inhibitor)	Absence of nuclear staining of β-catenin is found in 90% of OS cell lines, whereas all osteoblastomas demonstrated strong nuclear β-catenin staining. GIN activates Wnt/β--catenin pathway as shown by translocation of β-catenin into the nucleus. GIN significantly reduces cell proliferation and enhances differentiation in OS cell lines
Lee et al. 2007 [35]	DKK-1	DKK-1 is highly expressed by OS tumors, and the level in blood is proportional to number of surviving OS cells in the tumor. DKK-1 is maximally expressed by OS in tumor periphery suggesting that it may have a role to prevent the repair of surrounding osteoid as the tumor expands
Krause et al. 2014 [36]	DKK-1	DKK-1 decreases cell differentiation potential, increases proliferation, and enhances osteolytic capacity. DKK-1 acts by shifting the balance of Wnt signaling in favor of Jun-mediated noncanonical Wnt pathways. This results in activation of RhoA and JNK and transcriptional activation of ALDH1 through Jun-responsive promoter elements
Gregory et al. 2003 [17]	Antibody to DKK-1	Anti-DKK-1 antibody increases the lag phase of OS cells

invadopodia formation in OS cells. These results suggest the role of noncanonical Wnt in conferring the invasive properties of osteosarcoma [37].

Wnt Antagonists in Osteosarcoma

WIF1

The antagonist Wnt inhibitor factor 1 (WIF1) is frequently downregulated in cancer cells, including prostate, breast, lung, and bladder cancer and in osteosarcoma [38, 39]. In these cancers, silencing of the WIF1 promoter by hypermethylation is associated with Wnt signaling activation [40–43]. In human osteosarcoma, silencing of WIF1 by promotor hypermethylation was shown to be associated with loss of differentiation, increased β-catenin levels, and increased proliferation, and in mice experiments, disruption of WIF1 accelerated osteosarcomagenesis [44]. Recently, we demonstrated that re-expressing WIF1 in OS cell lines inhibits anchorage-independent growth and cellular motility and decreases proteolytic enzyme matrix metalloproteinases (MMP-9 and MMP-14). In vivo, injecting WIF1-transfected OS cells into nude mice showed reduced tumorigenesis and pulmonary metastasis [39].

sFRP

Frzb, a member of the secreted frizzled-related protein (sFRP) family, is another Wnt antagonist that has been associated with cancer. It has an amino-terminal cysteine-rich domain (CRD) that is homologous to the ligand-binding domain of frizzled [45]. Frzb prevents receptor signaling primarily by binding to extracellular Wnt ligands, preventing the ligand-receptor interaction [46]. Frzb re-expression has been shown to inhibit tumorigenesis and invasiveness in both prostate and fibrosarcoma cancer cells. Recently, systemic and local levels of sFRP3 were found to be decreased in osteosarcoma [47].

DKK-1: Tumor Suppressor or Pro-tumorigenic Factor?

The role of DKK-1 in Wnt signaling pathways is complex (Fig. 8.3). Human DKK-1 inhibits the canonical Wnt signaling pathway by binding to the transmembrane receptor LRP5/6, preventing interaction with Wnt ligands [48]. More recently, it has been recognized that DKK-1 also binds to cytoskeleton-associated protein (CKAP) 4 to induce cell proliferation in normal cells and in tumor cells in a β-catenin-independent manner by activating the PI3K/AKT pathway [29]. DKK-1 has immunomodulatory role by attenuating the canonical Wnt signaling pathway, thereby facilitating cell-mediated immune evasion by natural killer cells [49].

Inhibition of Wnt by recombinant DKK-1 decreases both nuclear β-catenin and cytoplasmic β-catenin. Cytoplasmic β-catenin helps in formation of adherens junctions. DKK-1 decreases cell-cell adherence which is required for differentiation of the cells. Inhibiting Wnt signaling by DKK-1 in human mesenchymal stem cells can transform them to form high-grade undifferentiated sarcoma-like tumors in mice, and conversely, re-establishing Wnt signaling in these tumors can differentiate them along mature connective tissue lineage [15]. DKK-1 has contrasting effects on tumors and surrounding stroma. They may not only slow down tumor cell proliferation but also exert potent osteo-inhibitory effects on the stroma and maintain the tumor niche. DKK-1 was shown to inhibit osteogenesis in osteosarcoma cells and the surrounding tissue when implanted in vivo. DKK-1 also had the unexpected effect of increasing proliferation and resistance to metabolic stress in vitro. This effect was attributed to the upregulation of the stress response enzyme and cancer stem cell marker aldehyde dehydrogenase-1 (ALDH-1) via noncanonical planar cell polarity Wnt signaling [36].

Inhibiting DKK-1 leads to positive signaling through canonical Wnt/β-catenin/LEF pathway which drove cells out of cell cycle toward differentiation and postmitotic state. The link between DKK-1 and noncanonical Wnt pathway was also suggested as DKK-1 downregulates Ap-1/JNK pathway and thereby decreases the expression of cell adhesion protein VCAM-1 [17].

Whether DKK-1 is pro- or anti-tumorigenic may depend on cellular context also. A tumor-suppressive role has been demonstrated in renal cell carcinoma and in colon cancer [50–53], but there are reports suggesting that DKK-1 is pro-tumorigenic in esophageal, pancreatic, hepatocellular, gastric, and prostate cancers and in multiple myeloma [54–63].

In prostate cancer, DKK-1 is expressed at high levels in early-stage disease and decreases once the primary tumor progresses to metastasize, which can unmask the Wnt-mediated osteoblastic activity and promote the development of osteoblastic osseous metastases [56]. DKK-1 has a complex role in organotropic metastasis in breast cancer, suppressing lung metastasis by suppressing noncanonical Wnt-JNK and Wnt/Ca^{2+} signaling and promoting bone metastasis through canonical Wnt signaling [64].

In osteosarcoma, DKK-1 has been shown to have an unexpected role in cancer survival and resistance to stress via tipping the balance of Wnt signaling in favor of the noncanonical planar cell polarity pathway [36]. Another recent report has shown increased nuclear β-catenin and expression of the bone differentiation marker osteopontin on

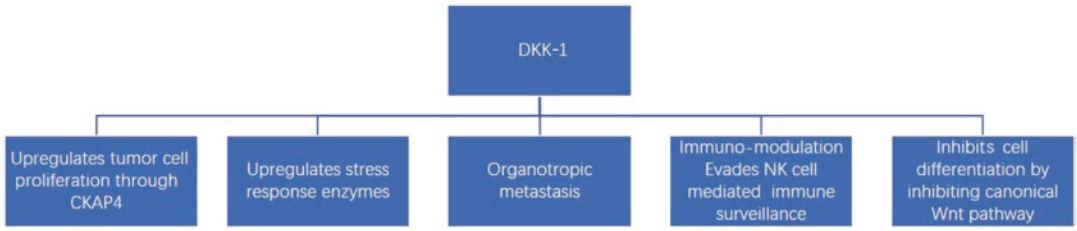

Fig. 8.3 Functions of DKK-1

treating the osteosarcoma with a neutralizing antibody against -DKK-1, resulting in decreased tumor growth and metastasis, suggesting that DKK-1 also regulates the canonical pathway [16].

Taken together, these studies have called into question the role of DKK-1 as a tumor suppressor and suggest that DKK-1 may be pro-tumorigenic in certain contexts [15–17, 35, 36].

DKK-3

Dickkopf-3 (DKK-3), also known as reduced expression in immortalized cells (REIC), is downregulated in multiple cancer cell lines, although its exact oncogenic suppressive mechanism is still unknown [65].

In osteosarcoma cells, DKK-3 has been shown to block β-catenin nuclear translocation, leading to inhibition of downstream LEF/TCF activation. The ectopic expression of DKK-3 and dominant-negative LRP5 mutant in osteosarcoma cell lines substantially decreases cell invasion and motility [66]. Furthermore, DKK-3 suppresses tumorigenesis and pulmonary metastasis in nude mice when transfected into osteosarcoma cells [67].

SOST

Sclerostin is another glycoprotein known to antagonize Wnt/β-catenin signaling in osteoblasts by binding to LRP5/6 and inhibiting osteoblast differentiation, activity, and survival [68, 69]. The SOST gene encodes for sclerostin, and its inhibition has been an area of interest for the treatment of osteoporosis [70, 71]. The FDA recently approved romosozumab, a monoclonal antibody against sclerostin, in postmenopausal women with osteoporosis at high risk of fracture. This agent has shown promising results in recent clinical trials for fracture prevention in osteoporosis [72, 73]. A recent study has shown that after SOST gene silencing, mRNA and protein expression of Wnt-1, β-catenin, c-Myc, cyclin D1, MMP-7 and more, which promotes proliferation, invasion, and migration, inhibits apoptosis of osteosarcoma cells [74].

Naturally Occurring Small Molecules

The small-molecule compound curcumin is a natural ingredient in turmeric, which shows an inhibitory effect against β-catenin/TCF signaling among several cancer cell lines [75]. Hallett et al. found that PKIF118-310, a natural compound of microbial origin and a small-molecule inhibitor of Wnt signaling (β-catenin/TCF inhibitor II), given to breast-tumor-bearing syngeneic mice arrested tumor growth in vivo [76]. In osteosarcoma, Leow et al. demonstrated that both curcumin and PKIF118-310 suppressed both intrinsic and activated β-catenin/TCF transcriptional activities using luciferase reporter assays. These compounds also reduce nuclear β-catenin and inhibit osteosarcoma cell migration and invasion in a dose-dependent manner. These anticancer effects are associated with decreased expression of several oncogenes, such as cyclin D1, c-Myc, and survivin [77]. Resveratrol, a natural grape product, has shown to inhibit proliferation of osteosarcoma cells by downregulating the expression of β-catenin [78].

Other Small Molecules

Besides naturally occurring antagonists, two new classes of small molecules that perturb the Wnt pathway have been reported. The first class of compound inhibits the membrane-bound acyltransferase Porcupine, which is involved in Wnt protein posttranslational modification. The second class nullifies the destruction of Axin, which is known to suppress the Wnt/β-catenin signaling [79]. Huang et al. [80] have described tankyrase inhibition by stabilizing Axin. Tankyrase interacts with Axin and stimulates its degradation through the ubiquitin-proteasome pathway. Tankyrase inhibitors, by attenuating Wnt/β-catenin signaling, have shown potential therapeutic effects in hepatocellular and colorectal cancers [81, 82]. Stratford et al. [83] demonstrated the efficacy of tankyrase inhibitor in three osteosarcoma cell lines. They reported stabilization of Axin2 and reduced cell growth due to delay in cell cycle progression and induction of caspase-3-mediated apoptosis. They

also noticed that the miRNA of let-7 family was upregulated following treatment. Small-molecule inhibition of Wnt signaling by inhibiting tankyrase 1/2 enzymes was found to be cytotoxic to multiple osteosarcoma cell lines [84]. IWR-1, another tankyrase inhibitor, was shown to be specifically cytotoxic to osteosarcoma cancer stem cells [21] (Fig. 8.4).

A study by Grandy et al. identified another small-molecule inhibitor of Wnt which interacts with the PDZ domain of dishevelled [85]. Dishevelled (Dvl) is an essential component of the Wnt signaling pathway, which transduces Wnt signals from the frizzled receptor to downstream targeted components. Through structure-based ligand screening and NMR spectroscopy, these investigators were able to discover a small-molecule inhibitor (3289-8625) with an affinity to the PDZ domain of Dvl. It was shown to suppress the tumorigenesis of prostate cancer PC-3 cells and decrease Wnt signaling in the hyaloid vessel system and may prove to have similar affects in osteosarcoma cells.

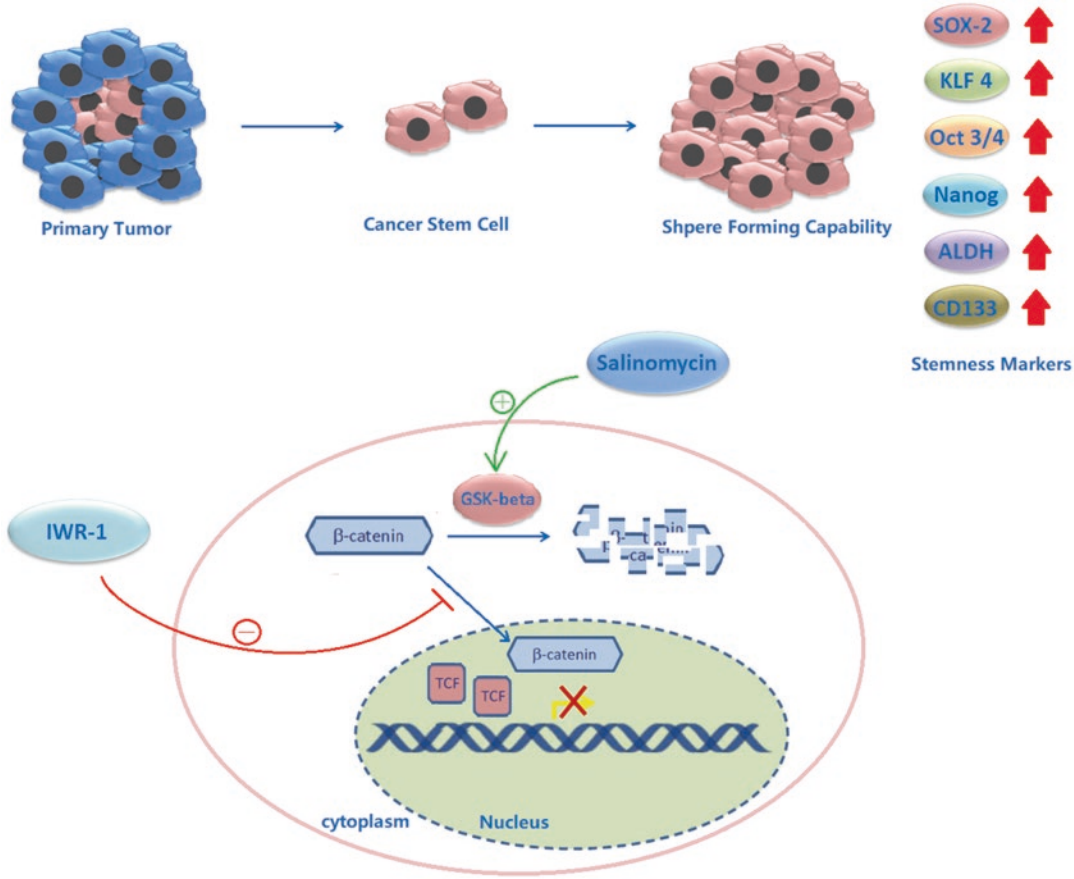

Fig. 8.4 Primary tumor contains few cancer stem cells which have the ability to self-renew. These stem cells have sphere-forming capability and have upregulated stem cell markers such as SOX-2, KLF4, Oct 3/4, Nanog, ALDH, and CD133

IWR-1, tankyrase inhibitor, inhibits the translocation of β-catenin from cytoplasm to nucleus, and salinomycin is a small-molecule inhibitor of LRP6 and it activates GSK3β to degrade the β-catenin in cytoplasm

Other Drugs Recently Shown to Inhibit OS

Tegavivint, a novel β-catenin/transducing β-like protein 1 (TBL1) inhibitor, has been shown to have antitumor activity in acute myeloid leukemia and multiple myeloma in preclinical trials and in a clinical trial for desmoid tumor. In osteosarcoma, this agent exhibits antiproliferative activity in vitro and reduces micro- and macrometastatic disease development in vivo. Metastatic osteosarcoma cell lines exhibited increased ALDH1 and β-catenin expression which was suppressed by tegavivint [86].

Niclosamide is a drug that inhibits Wnt/β--catenin signaling by suppressing LRP6 expression. This compound has been shown to inhibit the proliferation of human osteosarcoma cell lines by targeting multiple pathways and inducing apoptosis [87, 88]. Its role is also being studied in triple-negative breast cancer [89].

Therapy Against Wnt Target Genes in Osteosarcoma

Given the abundance of data suggesting Wnt/β--catenin involvement in tumorigenesis, there is a need to discover ways to mitigate Wnt transcriptional activation [27, 90]. Several strategies have been proposed to exploit the Wnt pathway for cancer therapy by targeting it at the extracellular, cytoplasmic, or nuclear level [91, 92]. The challenge to some of these strategies is that the Wnt pathway is a vast network that also regulates normal cell functions, tissue regeneration, and stem cell differentiation. Depending on how this pathway is targeted (extracellular, cytoplasmic, nuclear), detrimental side effects may be incurred. Targeting Wnt/β-catenin signaling at the extracellular level is a strategy that focuses on secreted Wnt antagonists, including WIF1, DKK-1, and sFRPs. Restoring the expression of these antagonists in antagonist-deficient tumors may prove to be helpful in reducing the proliferation of OS cells. Another strategy that simulates the mechanisms of Wnt antagonists is to create anti-Wnt monoclonal antibodies that can induce apoptosis of OS cells by blocking the Wnt-frizzled interaction. Therapeutic monoclonal antibodies against Wnt-1 and Wnt-2 have demonstrated inhibition of Wnt signaling and suppression of tumor growth in hepatocellular carcinoma and melanoma, respectively [93, 94]. This model can also be explored and potentially replicated for OS. Besides the extracellular level, we can aim to target the cytoplasmic components, such as β-catenin-binding domain of APC, for tumor suppression. Shih et al. showed that in colon cancer cells, re-expression of a recombinant adenovirus with APC (with known β-catenin-binding repeats) can inhibit nuclear translocation of β-catenin as well as β-catenin/TCF-mediated transactivation [95]. At the nuclear level, targeting the β-catenin/TCF transcriptional activity is widely regarded as impossible because the interacting surface between the transcription factor and DNA is huge and subject to significant changes during DNA binding. Targeting the downstream mediators, such as c-Myc, cyclin D1, and survivin, is being explored. In OS, the hepatocyte growth factor receptor c-Met is another Wnt target gene that is frequently overexpressed. Recent evidence suggests that c-Met can transform normal human osteoblasts into OS cells [96]. Therefore, c-Met is a candidate Wnt-related gene that can be explored for OS therapeutics. Nonsteroidal anti-inflammatory drugs (NSAIDS) are thought to impact the Wnt pathway by inhibiting the accumulation of prostaglandin E2, which ultimately decreases degradation of β-catenin. NSAIDs have mainly shown chemo-preventative effects against colon cancer [97]. Xia et al. demonstrated the effects of celecoxib (cyclo-oxygenase-2 inhibitor) on inhibiting β-catenin-dependent survival of a human OS cell line (MG-63). Not only did β-catenin protein decrease in the cytosol and nucleus following celecoxib treatment, but also there was a significant reduction of the Wnt target gene c-Myc and CCND1 [98]. As mentioned previously, using small-molecule inhibitors identified by high-throughput screens can be helpful to make an impact on OS therapy. These inhibitors are known to target β-catenin/TCF

antagonists and transcriptional coactivator modulators along with the PDZ domain of Dvl [85]. Juan et al. have shown antitumor effect of blockade of Porcupine, an acyl-transferase essential for Wnt ligand secretion and activity, that diminished WNT → β-catenin → c-MYC signaling [99].

Wnt/B-catenin Signaling and Stem Cells

The Wnt/β-catenin pathway not only has a role in tumorigenesis but also has been suggested to exert diverse regulatory effects on cancer stem cells (CSC) [100]. Stem cells in general are defined as having the ability to self-renew along with creating specialized cells. Several groups of investigators have examined the Wnt pathway and its effects on specific stem cell functions [101]. As early as the 1990s, Korinek et al. demonstrated the association between mutated TCF4 and subsequent depletion of intestinal stem cells. Studies on the role of stem cells in hair follicle formation have suggested that Wnt inhibitors play a prominent role in regulating the stem cell microenvironments [102]. Gibbs et al. were first to identify a subpopulation of cells in osteosarcoma which were able to grow in spheres in serum-free conditions [103, 104]. In OS cell lines, Tirino et al. identified a subpopulation of CD133+ cells with self-renewal properties, higher proliferation, spherical formation, and expression of the stem cell-associated gene OCT3/4 [105]. In addition, elevated aldehyde dehydrogenase (ALDH) activity in normal stem cells and solid tumor CSC has led to the use of ALDH as a means of identifying CSC in sarcomas. Wang et al. found that OS cell lines containing a subpopulation of cells with high ALDH activity possess increased tumorigenic capacity, proliferative capacities, self-renewal, and expression of stem cell markers [106]. Neves et al. isolated the cancer stem cells from MNNG/HOS cell lines using the sphere formation assay. These cells possessed self-renewal and multipotential differentiation capabilities [107]. They expressed several markers of pluripotent embryonic stem cells such as Oct4, Nanog, ABC transporter P-glycoprotein, and BRCP. Compared to parental cells, CSC exhibits low metabolic activity and is more resistant to chemotherapy and irradiation. In subsequent studies, these investigators found that CSC has active Wnt/β-catenin signaling and overexpress SOX2 and KLF4, which are stemness-related genes. In osteosarcoma, chemotherapeutic drugs promote a stem-like phenotype through Wnt/β-catenin pathway, and hence targeting this pathway might be effective in overcoming the stemness that non-stem cells might acquire after treatment [20, 21, 34].

IWR-1, tankyrase inhibitor, was shown to be specifically cytotoxic to osteosarcoma cancer stem cells by inhibiting the translocation of β-catenin from cytoplasm to nucleus [21]. Salinomycin is a novel small-molecule inhibitor of LRP6, and it activates GSK3β in cancer cells [108]. Salinomycin can also block β-catenin/TCF4 complex formation and has demonstrated to selectively inhibit stem cells in breast cancer, colorectal cancer, and leukemia [109]. Tang et al. found that salinomycin selectively targets osteosarcoma stem cells both in vitro and in vivo, potentially through Wnt/β-catenin signaling pathway. They demonstrated that tumor samples treated with salinomycin have decreased expression of both β-catenin and cyclin D1 by immunohistochemistry confirmed with western blotting [110]. To overcome the poor solubility of salinomycin, Ni et al. developed salinomycin-entrapped nanoparticles labeled with CD133 aptamers which could target and kill CD133+ osteosarcoma CSCs [111]. Chen et al. recently constructed salinomycin-entrapped nanoparticles labeled with EGFR and CD133+ aptamers to simultaneously target both osteosarcoma cells and CSCs [112].

Summary

Wnt signaling plays an important role in osteosarcoma proliferation, metastasis, and cancer stem cells. The Wnt ligands can play their role through canonical and noncanonical signaling pathways which are tightly regulated and demon-

strate cross talk with each other. Wnt signaling also plays a role in the tumor microenvironment and immunomodulation. Most of the studies which suggest that Wnt signaling is tumor suppressive studied the Wnt antagonist DKK-1, which affects both canonical and noncanonical Wnt signaling. There is probably a fine balance between these different pathways, and it is tipping the balance in one way or the other that can affect the response. This is a promising area for the development of targeted therapies, though with concern for toxicities given the key role Wnt signaling plays in normal stem cell function. Future studies are needed to study this balance more closely and create therapeutic interventions to help patients with osteosarcoma and other cancers related to this important signaling pathway.

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