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EDITORIAL

Novel Signaling Pathways in Osteosarcoma

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ABSTRACT

Osteosarcomas are the most common primary malignant bone tumors. Major advances in the treatment of osteosarcoma (OS) have revolutionized their clinical and surgical care. Despite these improvements, no specific targeted therapy has yet been employed for OS treatment. The present review will outline significant cytokine signaling pathways important in OS tumor biology, including the Bone morphogenetic protein (BMP), Hedgehog, and Wnt signaling pathways. Also discussed are the inherent challenges in studying cytokine signaling in OS, including the diversity in tumor location, grade, and lines of differentiation. Multiple BMP ligands and receptors are expressed across most OS cell lines and OS subtypes. Available data suggest that BMP signaling has pro-migratory effects in OS cells, as in the case with other sarcomas. Activation of Hedgehog and Wnt signaling has been observed in OS cell lines and/or primary human OS specimens. Emerging data suggests that Wnt signaling inhibition may prevent osteosarcomagenesis and/or sensitize OS cells to traditional chemotherapeutic agents. Likewise, interference with Hedgehog signaling transduction may reduce OS cell proliferation and *in vivo* tumor growth. In summary, multiple signaling factors show preclinical promise for cell targeted therapy in osteosarcoma.

Key words: Osteosarcoma; BMP; Hedgehog; Wnt

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INTRODUCTION

Osteosarcoma is the most common, non-hemopoietic, primary malignancy of bone. When untreated, osteosarcoma (OS) is essentially a fatal disease characterized by early hematogenous dissemination. With a combination of surgical resection and chemotherapy, long term disease-free survival is achievable in the majority of patients. Despite these vast improvements in patient outcome, targeted therapies for OS have not yet been realized.

The characterization of cell signaling pathways in OS presents multiple practical challenges, principally derived from the significant variation from tumor to tumor. For example, OS tumors vary by line of differentiation, histologic grade, tumor location, and treatment history. While all OS show some element of osteogenic differentiation, this can be highly variable. By convention, OS can be classified into osteoblastic, chondroblastic, and fibroblastic differentiation. Accordingly, the extent of bone matrix formation has been observed to correlate with various osteoinductive cytokine expression. Meanwhile, tumor grading predicts biological behavior based on histological appearance, with higher grade tumors showing increased cellularity, nuclear atypia, and mitotic figures. Considering the signaling cascade activity of BMP alone, for example, there exists a spectrum of activity and expression between low-grade and high-grade tumors. Perhaps the simplest distinguishing feature of OS is the anatomic location. Intramedullary tumors tend to be more common (conventional OS), while superficial (periosteal) lesions are more infrequent. Finally, OS specimens vary in their treatment history and responsiveness to preoperative chemotherapy. Tumor necrosis with or without inflammation shows dramatic changes to OS cytokine signaling. With this variation of OS in mind, this review will focus

on cell signaling pathways important in OS tumor biology.

Focus of review

The development of new, targeted therapies for OS hinges on improved understanding of cell signaling pathways important in osteosarcomagenesis, tumor growth, invasion, and metastasis. Surprisingly, few studies have specifically examined key signaling cascades in the context of OS tumor biology. For example, BMP, Hedgehog, and Wnt signaling cascades are relatively infrequently studied in the context of OS. Further, the majority of studies treat OS as a single disease entity, rather than taking into account the variety inherent in OS tumors. Conversely, studies focusing on only a single OS subtype may be limited in scope and conclusions. With these limitations in mind, this review will specifically focus on the role of BMP, Hedgehog, and Wnt signaling in OS and other malignant skeletal tumors. This review will highlight the recent advances in understanding the role of each signaling pathway in OS biology, with an emphasis on the divergent findings by various researchers.

BMP SIGNALING IN OS

Introduction to BMP signaling

Members of the transforming growth factor-β (TGF-β) superfamily, Bone Morphogenetic Proteins (BMPs) are extracellular cytokines originally isolated from bone extracts[1]. While responsible for numerous cell regulatory processes, BMPs are most studied for their ectopic induction of bone and cartilage[2]. To date, 31 different types of BMP ligands have been identified[3], of which BMP-2, -4, -7, and -9 are most commonly studied in the context of osteogenesis[4,5]. As expected from a key regulator of osteogenesis, investigators have observed BMP expression in both benign and malignant skeletal tumors, including benign bone-forming lesions[6], as well as benign non-bone forming lesions[7]. BMP-like protein was first identified in murine OS in 1974[8], confirmed soon after in human OS[9]. Since then, the significance of BMP signaling in OS has been studied by multiple investigators. Despite these studies, however, conflicting conclusions have been drawn in regards to the role of BMPs in OS[10].

Overview of BMP signaling

The BMP signaling cascade initiates through extracellular ligand interaction with transmembrane type I and type II BMP receptors (BMPRs), both being serine-threonine kinases (Figure 1). Type II BMPRs handle initial BMP ligand interaction, binding via a high affinity mechanism which results in recruitment and activation of type I BMPRs, which are classified as low-affinity receptors[2,11,12]. While seven known variants of type I BMPRs exist, few actually contribute to osteogenic differentiation, predominantly BMPR-IA and BMPR-IB[13]. BMP signaling propagation then proceeds downstream to activate several pathways via phosphorylation, including the Smad1/5/8, MAP Kinase, and c-Jun N-terminal kinase (JNK) signaling pathways[13,14]. It is principally through Smad1/5/8 signaling that BMPs induce osteogenic programming (see[15] for a more detailed review of BMP signaling transduction).

Significance of BMP signaling in bone development

In regards to its role in bone development, the BMP signaling pathway plays a crucial role in the induction and regulation of osteogenic differentiation through both autocrine and paracrine pathways[16,17]. Through genetic modification of BMP receptors, inhibitors, and ligands in murine models, investigators have shown a role for BMP in signaling bone formation[18-21]. For instance,

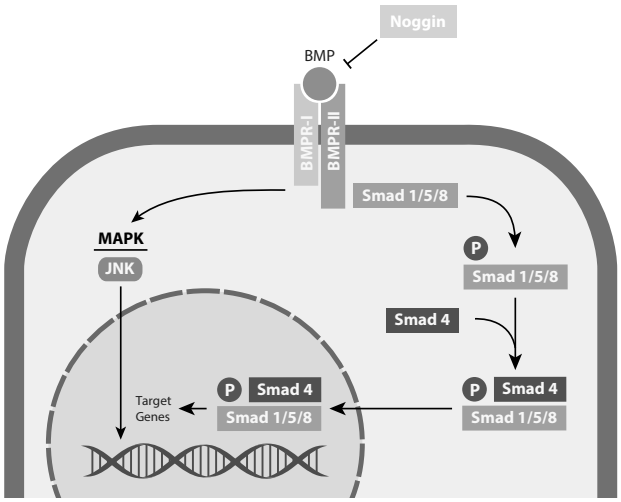

Figure 1 Signaling is initiated via BMP ligand binding to a heterodimeric formation comprised of two different surface receptors, BMPR I and II. Type II receptor phosphorylates the type I receptor, thereby inducing signal transduction through the Smad pathway and mitogen-activated protein kinase (MAPK) via phosphorylation. Under the Smad pathway, which is the principal one governing osteogenic regulation, Smad proteins 1/5/8 associate with Smad4 to enter the nucleus and regulate target gene expression.

modification of BMPR-IA receptors in transgenic mice results in reduced bone mass as well as irregular patterns of calcification[18]. Conversely, overexpression of BMP signaling inhibitors, such as Noggin and Gremlin, has led to impaired bone formation[22-24]. Of the 31 known BMP ligands, only several are known to induce osteogenic commitment and terminal differentiation in mesenchymal stem cells (MSC); these include BMP-2, -4, -6, -7, and -9[5,25]. Of these, BMP-2 is most well- studied, exhibiting induction of osteogenesis in MSC both in vitro and *in vivo*[26-33]. As BMP signaling exerts significant pro-osteogenic effects across multiple cell types and bone injury models, interest in the expression and manipulation of BMP signaling in OS tumor biology has been significant.

BMP Signaling in OS

BMP signaling elements and activity has been identified in several OS cell lines and primary cells, most notably in murine cell lines[8,34,35] and human OS cells lines[36-38]. Independent of their osteoinductive properties *in vivo*, expression of BMP-2, -4, and -6 have been observed in murine OS cell lines[39]. Via polymerase chain reaction, Gobbi *et al* observed BMP-2 through -8, along with BMP receptors, across five OS cell lines[40]. With regards to human OS specimens, BMP-2/4 expression has been observed in both immunohistochemical and in situ hybridization[41,42]. In fact, high-grade OS was shown to have the highest and most consistent expression of BMP-2/4[41]. In comparison, however, high-grade OS specimens exhibited reduced levels of BMP signaling activity in comparison to the benign osteoblastoma, as indicated by reduced phospho-Smad1 expression[43]. In a similar comparison, gene microarray compared osteoblastic to non-osteoblastic OS specimens[44]. BMP-7 expression was significantly increased in osteoblastic OS as compared to non-osteoblastic OS specimens. BMP-6, in contrast, was most highly expressed in chondroblastic OS[45]. Thus, the majority of OS specimens exhibit BMP signaling activity. Studies suggest that specific BMP ligand expression is influenced by the histological subtype and tumor grade, although this is incompletely defined.

Despite numerous studies examining BMPs in OS, relatively

few have addressed the potential role for BMP signaling in tumor formation or disease progression. BMP-2 has been reported to exhibit an inhibitory effect on in vivo sarcomagenesis as seen in OS 'cancer stem cells' (OS99-1 cell line)[46]. Conversely, BMP-2 and -9 were found to exert pro-mitogenic effects in OS with 'differentiation defects,' tumors where overexpression of either BMP ligand failed to induce terminal osteogenesis[47]. Instead, tumor growth was increased, which suggests that some OS cells gain resistance to BMP induced osteogenesis and develop a concomitant shift towards sarcoma growth[47]. Lastly, in another study, OS cells were exposed to various extracellular matrix components in either the presence or absence of BMP-2; results showed that BMP-2 induces OS cell migration through regulating fibronectin-Integrinβ1 signaling[48]. In summary, few studies have examined the mechanisms via which BMP signaling may affect sarcomagenesis, sarcoma growth, and invasion – with somewhat conflicting results.

Lastly, studies have shown that the BMP signaling pathway is upregulated in conditions predisposing to OS, such as Paget's disease and fibrous dysplasia. For example, Urist *et al* examined patients with Paget's disease, finding significantly increased BMP levels by radioimmunoassay in serum as compared to the normal control group[49]. Other investigators have examined BMP signaling in fibrous dysplasia. For example, Khurana *et al* described BMP-2 and -4 expression fibrous dysplasia by RT-PCR, Northern blot, and immunohistochemistry[7]. Likewise, in fibroblast-like cells of fibrous dysplasia, Sakamoto *et al* found more frequent immunoexpression of TGF-β and fibroblast growth factor-2 (FGF-2), in addition to BMP-2[50]. Lastly, in bone tissue of women with fibrous dysplasia, a gene analysis using RT-PCR found nine upregulated genes and 18 downregulated genes in comparison to non-fibrous dysplasia bone tissue[51]. These altered genes corresponded to collagen molecules, adhesion molecules, and growth factors, to name a few. Taken together, these studies regarding predisposing conditions of osteosarcoma further reinforce the central role that BMP plays in bone formation, and why regulation of this signaling pathway holds potential in context of OS tumor biology.

HEDGEHOG SIGNALING IN OS

Introduction to Hedgehog signaling

Hedgehog (HH) signaling pathway is essential for mesodermal tissue patterning and differentiation. Originally identified in Drosophila, Hedgehog was later identified across all vertebrate organisms. Three homologues exist for this protein: Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH)[52]. While DHH expression is limited and not of known importance in bone biology[53-56], both SHH and IHH are critical in embryonic development. Knockout of either SHH and IHH in murine models results in multiple congenital anomalies and neonatal lethality[57-59]. While IHH is more prominent in regulating chondrogenesis and endochondral formation[52], SHH has a role in vertebrate organogenesis, promoting adult stem cell proliferation in hematopoietic, mammary, and neural tissues[60]. Furthermore, SHH plays a role in skeletogenesis and development of facial, appendicular, and axial skeletal patterns[52]. With a significant role in cell differentiation and stimulation of osteogenesis, the HH pathway serves as an attractive potential target in OS tumor biology.

Overview of Hedgehog signaling

The Hedgehog signaling pathway mechanism remains conserved across the three different homologues. Following initial pathway activation via autocatalytic cleavage, the C-terminus is modified with

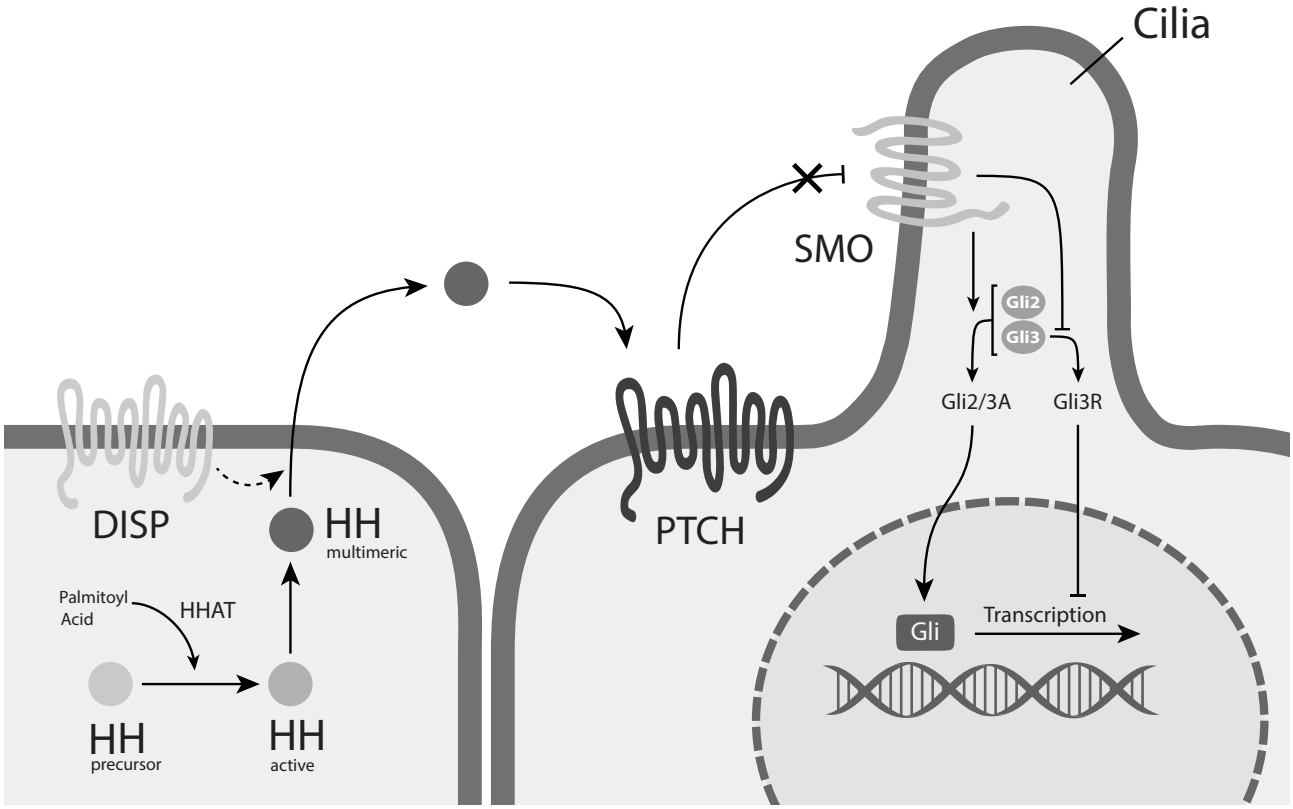

Figure 2 The precursor form of Hedgehog (HH) ligand is modified with cholesterol and palmitoyl acid to reach an active, multimeric form; HHAT is necessary for the palmitoylation step. Twelve-transmembrane Dispatched (DISP) then secretes the HH ligand, which binds to Patched (PTCH) on the receiving cell. This releases Smoothened (SMO) from constitutive inhibition, causing it to migrate to the cilia where it activates Gli2/3 complex (Gli2/3A) while also repressing Gli3 repressor (Gli3R). Collectively, HH activation of SMO upregulates target gene expression.

cholesterol while the N-terminus is modified with palmitoyl acid, thus increasing membrane solubility in the process (Figure 2)[61]. Hedgehog acyltransferase (HHAT) has also been deemed necessary in the post-translational palmitoylation of HH; in the absence of HHAT, HH secretion is downregulated[62]. The resulting multimeric form of the activated ligand undergoes paracrine signaling; secretion via 12-pass transmembrane protein, Dispatched (DISP), allows for the activation of 12-pass transmembrane Patched (PTCH) receptor on the receiving cell[63]. In its unbound state, PTCH functions as an inhibitor of Smoothened (SMO). From interaction with the activated HH ligand, Smoothened is released from constitutive inhibition by PTCH. Once free, SMO shuttles through the cilia and activates downstream target genes through utilization of the transcription factor glioblastoma gene product family, Gli2/3 complex and Gli1[63]. At the same time, this action inhibits the Gli3 repressor form. HH-Gli activators have been shown to function in regulating lineage commitment during cell differentiation[64].

Hedgehog signaling in bone biology

The HH signaling pathway plays a critical role in differentiation, directing cells toward an osteogenic lineage. HH signaling (SHH, Gli1) regulates osteoprogenitor cell differentiation by enhancing osteogenic differentiation and inhibiting adipogenic differentiation in multiple mesenchymal cell types[65]. For example, SHH added to adipose derived stromal cells (ASC) simultaneously induced osteoblast differentiation while suppressing adipocyte formation, both *in vitro* and *in vivo*[66]. Likewise, the IHH ligand has also been shown to exhibit an inverse relationship between osteogenic and adipogenic mesenchymal stem cell differentiation[67]. Expectedly, ossification is greatly impaired in developing animals with improper SHH and IHH signaling, leading to a variety of mutations; SHH mutant mice develop a cleft palate associated with disrupted FGF signaling[68]. In mice with mutations for IHH, an endochondral ossification defect led to severely shortened limbs while irregular intramembranous ossification led to skull vault defects[69]. Furthermore, endogenous HH is involved in regulation of angiogenesis and bone vascularization. Similarly, exogenous HH induces vascular formation *in vitro*. Specifically, implantation of recombinant SHH catalyzes *in vitro* vascular structure formation and enhances in vivo perfusion of artificial tissue and formation of de novo mature bone tissue[70]. The critical role of HH in stimulation of calvarial and appendicular bone development and basic bone biology has led to interest in the manipulation of HH signaling in OS tumor biology.

Hedgehog signaling in the biology and treatment of OS

Dysregulation of the HH pathway has been associated with tumorigenesis, angiogenesis, and metastasis in a variety of cancers. For example, in the benign cartilage tumor osteochondroma, the lack of EXT expression and heparin sulfate production disrupts IHH signaling, resulting in excess proliferation[71]. To date, three key methods have been proposed to explain how HH signaling leads to tumor formation[72]. In the first, both increased expression of HH ligands and ectopic Gli or PTCH will lead to autocrine or juxtacrine HH activation. Second, activation of HH occurs independent of the ligand, where mutation of either transmembrane PTCH or SMO enables SMO to remain active on downstream Gli elements. By contrast, in the last approach, paracrine HH activation occurs in a ligand-dependent manner; ectopic expression or mutated elements can be passed from the tumor to the supporting stroma. This signaling has been shown to increase both tumor invasiveness and proliferation. Furthermore, activation of the HH pathway causes increase in Snail protein expression and decrease in E-cadherin and tight junctions[73], leading to a stimulation of angiogenesis and metastasis[74]. Lastly, it is worth noting that abnormal activation of HH has been shown to upregulate angiogenic factors angiopoietin-1 and angiopoietin-2[75], as well as antiapoptotic genes[76].

In comparison to regular osteoblasts, human OS cell lines show increased mRNA expression of HH ligands, receptors, and downstream transcriptional activators[77]. Accordingly, in keeping with the known roles of HH in tumorigenesis, pharmaceutical antagonists of HH signaling have been examined[78]. One recent example is the development of Vismodegib, the first FDA approved drug to manipulate the HH pathway to inhibit tumor growth in basal cell carcinoma[79]. Its mechanism involves SMO inhibition, which inactivates transcription factors Gli1, thus hindering expression of tumorigenic genes. Currently, Vismodegib is undergoing clinical trials by the National Cancer Institute to test its potency in treating extraskeletal osteosarcoma, metastatic osteosarcoma. Likewise, FDA-approved arsenic trioxide (ATO) used for the treatment of leukemias has shown preclinical success in OS both *in vitro* and *in vivo*. Specifically, ATO induces apoptosis of p53 deficient OS MG63 cells by inhibiting catalase[80]. Moreover, injection of ATO in vivo reduced tumor size in mice by 300% after an eight week period[81]. Finally, ATO also inhibits OS invasiveness and metastasis by reducing cell motility, migration, and adhesion[82].

The HH pathway has also been observed to be altered in patients with predisposing conditions of OS. For instance, Cohen noted that abnormal bone in fibrous dysplasia were caused by mutations in GNAS1, downstream of the SHH pathway[83]. Likewise, Regard *et al* had previously demonstrated that GNAS gain-of-function contributed to fibrous dysplasia in osteoblast progenitor cells, but via upregulated WNT signaling[84]. In a subsequent study, Regard *et al* found that Gαs, which is encoded by GNAS, restricts bone formation via inhibition of HH[85]. This suggests a crosstalk phenomenon between the WNT and HH signaling pathways, in which Gαs serves as a key regulator of osteoblast differentiation. Thus, the consistent findings in predisposing conditions of OS further reaffirm the central role of HH signaling in OS development.

In summary, with mutation and dysregulation of the HH pathway correlated to tumor progression, targeted inhibition of HH signaling has enabled new strategies in the development of novel OS treatments. Inhibition of the cell surface receptors PTCH and SMO downregulates Gli1 and Gli2 transcriptional activity, thereby impeding OS progression through apoptosis. Collectively, these findings warrant additional research, as the possibility of using Hedgehog pathway inhibitors holds therapeutic promise for the treatment of osteosarcoma.

WNT SIGNALING IN OS

Introduction to Wnt signaling

The Wnt signaling pathway has been shown to play a variety of crucial roles in regards to cell fate, development, self-renewal, and tissue morphogenesis[86]. In bone development, Wnt signaling is required for limb bud initiation, early limb patterning, and late limb morphogenesis events[87]. Dysregulation of Wnt signaling can lead to osteoporosis[88], but also is associated with numerous and diverse diseases such as Parkinson's disease[89], retinopathy[90], colon cancer[91], and melanoma[92]. Over 19 known Wnt ligands have been identified to date, with 15 receptors and co-receptors across seven protein families[86,92]. Furthermore, Wnt ligands activate distinct intracellular

signaling cascades, including the Wnt/β-catenin pathway, the Wnt/Ca^{2+} pathway, and the Wnt/planar cell polarity (Wnt/PCP) pathway[93]. Due to its myriad of cellular functions, tremendous attention has been focused particularly on the key role of the Wnt/β-catenin signaling pathway in the regulation of bone mass[94,95] and on characterizing the ligands, receptors, and effectors of the Wnt pathway.

Overview of Wnt signaling

The Wnt signaling pathways can essentially be separated into two main categories: the β-catenin-dependent pathway (canonical Wnt pathway) and the β-catenin-independent pathway (non-canonical Wnt pathway). The canonical Wnt pathway has been studied more extensively, and functions by regulating the amount of the transcriptional coactivator β-catenin, which controls key developmental gene expression programs[96]. This β-catenin dependent mechanism initiates with Wnt ligand binding to the cysteine-rich portion of the seven-pass transmembrane receptors of the Frizzled family (Frz), as well as co-receptors such as lipoprotein receptor-related protein (LRP)-5/6 (Figure 3)[96-98]. The Wnt-Frz-LRP-5/6 complex then pairs with the intracellular phosphoprotein Disheveled (Dsh), thereby phosphorylating LRP-5/6 and recruiting Axin. As a result, the cytoplasmic Axin complex—consisting of the scaffolding protein, Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3)—is unable to phosphorylate cytoplasmic β-catenin. Thus, the E3 ubiquitin ligase β-Trcp fails to recognize the β-catenin for proteosomal degradation[96,99]. This stabilization and accumulation of cytoplasmic β-catenin allows β-catenin to enter the nucleus, combine with transcription factors of the lymphoid enhancer-binding factor/T cell factor (LEF/TCF) family, and regulate gene expression[100]. Moreover, the canonical Wnt pathway regulates mesenchymal lineage specification via this mechanism[101].

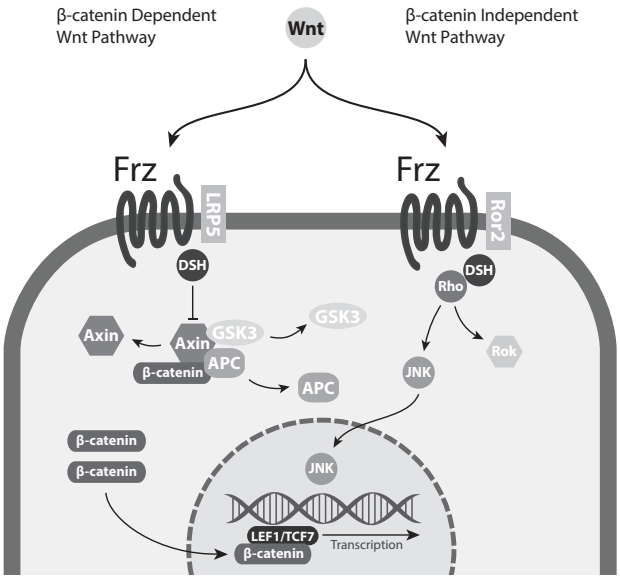

Figure 3 Under the canonical, β-catenin-dependent Wnt pathway, Wnt ligand binding to transmembrane protein Frizzled (Frz) and co-receptor LRP5 causes binding to Disheveled (Dsh) intracellularly. This activated complex recruits cytoplasmic Axin complex, consisting of APC and GSK3 among others. Cytoplasmic β-catenin degradation is then inhibited, as normally it is phosphorylated by free Axin; the net result is β-catenin accumulation and translocation across the nucleus to bind to LEF/TCF. By contrast, with the non-canonical β-catenin-independent Wnt pathway, Ror2 acts as one of the co-receptors for Frz. Activation again recruits Dsh, which then activates Rho, a G-protein. Rho then activates Rho-associated kinase (Rok) and JNK downstream.

Several similarities exist between the canonical and non-canonical pathway mechanisms, namely the binding of Wnt ligand to Frz receptors to activate Dsh. However, the downstream pathways are activated by binding of a non-canonical Wnt ligand which no longer require β-catenin; hence, the non-canonical Wnt pathway is also known as the β-catenin-independent pathway. The non-canonical Wnt pathway contains two main pathways: the planar cell polarity (PCP) pathway and the Ca^{2+} pathway. In the Ca^{2+} pathway, the binding of a non-canonical Wnt ligand to Frz and a co-receptor, increases intracellular calcium levels, decreases cyclin GMP (cGMP) levels, and activates protein kinase C (PKC)[102].

The binding of Frz receptors in the PCP pathway, on the other hand, activates Dsh, which then signals modulation of cytoskeletal elements including actin and microtubules via small GTPases[102,103]. By inducing modifications to the actin cytoskeleton, the PCP pathway mediates asymmetric cytoskeletal organization and the polarization of cells[103]. Both non-canonical pathways play a role in development, cell polarization, motility, and homeostasis[104]. Furthermore, non-canonical Wnt ligands can stimulate osteogenic differentiation through the activation of both PCP and Ca^{2+} pathways[105,106].

Significance of Wnt signaling in bone development

Wnt signaling plays vital roles in the development, growth, and homeostasis of various organs including the skeletal system[107-110]. Extensive investigation has adequately characterized the role of canonical Wnt signaling in maintaining bone homeostasis. Riddle et al. demonstrated that expression of both LRP-5 and LRP-6 are required within mature osteoblasts for normal postnatal bone development[111]. When LRP-5 was mutated upstream of β-catenin, loss-of-function LRP-5 mutations resulted in osteoporosis pseudoglioma (OPPG) syndrome and a low bone mass phenotype, whereas gain-of-function mutations in LRP-5 led to an osteosclerotic, high bone mass phenotype[112-115]. Furthermore, mice selectively lacking either LRP-5 or LRP-6 in mature osteoblasts demonstrated significant reductions in whole-body bone mineral density. LRP-6 mutant mice failed to accumulate normal amounts of trabecular bone, while LRP-5 mutant mice exhibited normal trabecular bone volume but later showed trabecular bone loss as they matured. Both mutants exhibited significant alterations in cortical bone structure[111]. Mice lacking both LRP-5 and LRP-6 developed severe osteopenia, and calvarial mesenchymal cells deficient for both LRP-5 and -6 fail to form osteoblasts when cultured in osteogenic media, instead attaining a chondrocyte-like phenotype[111]. Additionally, the various roles of β-catenin through canonical Wnt signaling have been verified as a regulator of both osteoblast and osteoclast function in mature cells as well as in the early stages of osteogenesis and postnatal development[116]. Reduced levels of β-catenin in mesenchymal progenitor cells arrests osteoblast development at an early stage, resulting in fetal skeletal defects[116-119]. Furthermore, genetically induced β-catenin deficiencies in terminally differentiated osteoblasts can result in impaired maturation and mineralization along with upregulation of an osteoclast differentiation factor, leading to significant bone resorption[120,121].

Inhibiting Wnt antagonists have also been studied as a method of stimulating canonical Wnt pathway activity to induce formation of new bone along with limiting bone resorption. In particular, Wnt antagonists Sclerostin and Dickkopf-1 (DKK-1) inhibit canonical Wnt signaling by bind to co-receptors LRP-5/6 in order to downregulate the accumulation of β-catenin, thus inhibiting β-catenin's influence on gene expression[122,123]. Morvan *et al* demonstrated that mice lacking a single allele of DKK-1 showed a

markedly increased trabecular bone volume and elevated trabecular bone formation rate[124]. Yaccoby *et al* used antibodies raised against DKK-1 in the treatment of a mouse model of multiple myeloma revealed increased numbers of osteoblasts, reduced number of osteoclasts, and reduced myeloma burden in the antibody-treated mice[125]. Inhibiting the production or action of sclerostin resulting in enhanced canonical Wnt signaling led to increased bone mass in preclinical studies, where monoclonal antibody against sclerostin promoted trabecular bone formation rapidly in monkeys and rats without increases in resorption parameters[126]. Moreover, introducing inhibitors of antagonists Sclerostin and DKK-1, anti-Sclerostin and anti-DKK-1 respectively, has demonstrated the ability to stimulate bone formation and increase bone mineral density, with successful Phase I and II clinical trials[127,128]. Overall, the Wnt signaling pathway has been shown to have numerous and diverse effects in bone biology, including skeletal development, regulation of osteoblast/osteoclast function, and maintenance of bone homeostasis. Thus, research regarding this multifaceted signaling pathway in context of OS tumor biology has been significant.

Wnt signaling in OS

Dysregulation of the Wnt/β-catenin pathway is closely associated with a variety of human cancers, including OS. β-catenin is required for differentiation and proliferation of cells in a wide variety of tissues, including the skeletal, neuromuscular, and cardiovascular system among many others. The critical involvement of aberrant activation of the Wnt/β-catenin pathway in oncogenesis is relatively well documented[129,130]; studies have demonstrated that overexpression of numerous Wnt components, including Wnt ligands and Frizzled and LRP receptors, highlighted the implications of aberrant Wnt/β-catenin signaled in the development and progression of OS. For example, Chen *et al* identified expression of multiple Wnt ligands (Wnt-2b, Wnt-3, Wnt-5a, Wnt-5b, and Wnt-14) and receptors (Frz-1, Frz-2, Frz-3, Frz-6, Frz-7, LRP-5, and LRP-6) across two human OS cell lines: MG63 and HOS[131]. Furthermore, two murine OS cell lines (K7M2 and K12) also demonstrated Wnt ligand and receptor expression[131]. Similarly, Hoang *et al* examined Wnts, Frzs, and LRP-5 expression using reverse transcription polymerase chain reaction (RT-PCR) in four OS cell lines: U2OS, HOS, 143B, and Saos-2[132]; all cell lines were found to express Frz-1, Frz-2, Frz-4, Frz-5, Frz-9, and LRP-5. Furthermore, out of 44 high-grade OS patient biopsies surveyed for mRNA, Hoang *et al* detected LRP-5 expression in 50% of samples, and found a significant correlation between LRP-5 expression and nuclear β-catenin accumulation with tumor metastasis[132]. Additional studies suggest a role for LRP-5 in OS; Guo *et al* demonstrated that Saos-2 cells transfected with dominant-negative, soluble LRP-5 resulted in an increase in E-cadherin and decrease in N-cadherin, leading to the reversal of the epithelial-to-mesenchymal transition (EMT), a hallmark of OS[133]. The increase in E-cadherin also caused a corresponding decrease in Wnt-responsive transcriptional repressors SLUG and TWIST, thereby suggesting that LRP-5 promotes EMT and OS invasiveness[133]. Using real-time quantitative PCR analysis, Ma *et al* found that all major elements in the Wnt/β-catenin pathway—including Wnt-3a, β-catenin, and Lef-1—were consistently upregulated in human OS cell lines Saos-2 cells compared to human fetal osteoblasts (hFOB)[134]. Ma *et al* also demonstrated that expression level of β-catenin consistently correlated with the invasiveness of OS. Western blotting analysis further confirmed that the protein levels of both total and active β-catenin were increased in Saos-2 cells compared to hFOBs[134]. Moreover, knocking down β-catenin in Saos-2 cells, Ma *et al* observed increased Saos-2 sensitivity to methotrexate (MTX) induced cell death, sensitizing the cells to chemotherapy[134].

In addition to the overexpression of numerous Wnt components in OS, the epigenetic silencing of genes encoding endogenous Wnt pathway inhibitors, such as Wnt inhibitor factor 1 (WIF-1), underscore the implications of aberrant Wnt signaling in the development and progression of OS. WIF-1 mRNA expression was significantly decreased in numerous OS cell lines in comparison to normal human osteoblasts, attributed to WIF-1 promoter hypermethylation; it was found that WIF-1 expression downregulates the expression of MMP-9 and 14, thereby preventing the invasion and mobility of OS cells[135]. Kansara *et al* further confirmed that WIF-1 is epigenetically silenced in human OS, and target disruption of WIF-1 accelerates OS formation in mice[136]. Expression of other Wnt/β-catenin inhibitors, such as FrzB/sFRP3, has been found to be consistently suppressed in OS[137]. Likewise, LEF/TCF transcription factors have been shown to be negative regulators of the Wnt pathway[138]. Another suspected negative regulator of the canonical Wnt pathway is TWIST, a basic helix-loop-helix transcription factor that is typically expressed in low levels in OS[139]. Inducing overexpression of TWIST *in vitro* in Saos-2 and MG63 lines led to a decrease in β-catenin, while knockout of TWIST with RNA interference (RNAi) led to increased levels of β-catenin[139]. Further studies have demonstrated that increased TWIST expression in Saos-2 OS cells causes an increase in sensitivity to cisplatin-induced apoptosis, whereas TWIST RNAi will actually increase resistance to apoptosis[139]. Taken together, the majority of the current literature supports the notion that aberrant activation of the Wnt pathway contributes to OS development.

Following the notion that aberrant activation of the Wnt pathway contributes to OS, investigated treatment methods commonly target the Wnt pathway and regulators to inhibit and downregulate Wnt signaling activity[140]. Cadigan *et al* conducted a review showing that in the absence of Wnt binding to Frz and LRP-5/6 cell-surface receptors, a cytoplasmic complex consisting of axin, adenomatosis polyposis coli (APC), and glycogen synthase kinase 3 (GSK3) phosphorylates β-catenin, thereby promoting degradation of β-catenin, thus contributing to the downregulation of Wnt signaling activity[141]. To reduce β-catenin-dependent Wnt pathway activity, Brun *et al* silenced FHL2 (four and a half LIM domains protein 2) using shRNA in OS cells, which reduced the expression of Wnt-responsive genes as well as Wnt-5a and Wnt-10a, inhibiting OS cell proliferation, invasion, and migration in vitro. Moreover, the study demonstrated that FHL2 silencing markedly reduced tumor growth and lung metastasis occurrence in mice, associated with the decreased Wnt signaling in the tumors[142].

Several studies have suggested that Wnt pathway inhibition has anti-proliferative, pro-apoptotic effects in OS cells[143,144]. Activating the Wnt pathway can inhibit apoptosis, but when combined with chemotherapeutic drugs, doing so can be counterproductive[142,145]. Whether or not Wnt inhibition can directly induce apoptosis is not well documented, and the precise mechanisms remain unknown.

Finally, aberrant Wnt signaling is also observed in predisposing conditions of OS, such as fibrous dysplasia and Paget's disease. For example, Regard *et al* showed that activation of the Wnt/β-catenin pathway in osteoblast progenitors led to the fibrous dysplasia phenotype. Specifically, they analyzed the interaction between the Gα protein families and the Wnt/β-catenin pathway, and identified abnormally increased signaling levels as a cause of fibrous dysplasia[146]. Furthermore, the removal of the Gα protein mutations

decreased Wnt/β-catenin signaling and correspondingly decreased bone formation. In patients with Paget's disease of bone, an inhibitor of Wnt signaling, Dickkoph-1 (Dkk-1), was found to have elevated levels in the serum[147]. Overexpression of Dkk-1 is implicated in osteolytic disease as it is expected to inhibit bone formation, as well as induce bone breakdown. Previous studies have also demonstrated increased Dkk-1 expression and protein in osteoblastic and stromal cell cultures from Paget's lesions[148]. Thus, studies in predisposing conditions of OS reaffirm the importance of the Wnt signaling mechanism in OS development.

OTHER SIGNALING PATHWAYS IN OS

Numerous other signaling pathways play a role in osteogenic differentiation, and likewise are suspected to play a role in OS tumor biology. This review will briefly discuss Notch, Insulin-like Growth Factor (IGF), Transforming growth factor beta (TGF- β), and Fibroblast Growth Factor (FGF) in relation to OS.

The Notch signaling pathway plays a central role in the commitment of mesenchymal cells to osteoblastic lineage. For example, Ramasamy *et al* demonstrated that disruption of Notch signaling in mice led to impaired growth, reduced osteogenesis, shortening of long bones, chondrocyte defects, loss of trabeculae, and decreased bone mass[149]. Hughes *et al* showed that the Notch pathway is a significant contributor to the metastatic ability of osteosarcoma cells. In comparison to normal human osteoblasts and non-metastatic osteosarcoma cell lines, metastatic osteosarcoma cells exhibited higher levels of Notch1, Notch2, the Notch ligand DLL1, Hes1, a Notch-induced gene[150]. Thus, Notch is believed to hold an essential role in osteosarcoma metastasis, and regulation of this pathway can be crucial to the development of new therapies (please see[151] for a more detailed review).

IGF plays a critical role in skeletal maturation during fetal growth and development. Specifically, IGF is heavily involved in limb morphogenesis. Agrogiannis *et al* observed growth retardation and delayed skeletal development in mice lacking the Igf gene[152]. In regards to osteosarcoma, the IGF-1 receptor has been an area of focus due to its role in pathogenesis; compared to normal osteoblasts, osteosarcoma cells are characterized by overexpression of the IGF-1R signaling pathways. Thus, IGF-1R has served as a target of regulation in development of new therapeutic drugs. In one study, R1507, a monoclonal antibody to IGF-1R, is currently undergoing phase II clinical trials in patients diagnosed with OS[153]. Kuijjer *et al* also showed an inhibitor of IGF-1R, OSI-906, decreased osteosarcoma cell line proliferation via inhibition of phosphorylation of IRS-1, a downstream target of IGF-1R signaling[154]. Thus, regulation of the IGF signaling pathway holds potential in the development of novel OS therapies (please see[155] for a more detailed review).

TGF-β is another signaling pathway involved in numerous aspects of bone biology, including osteoprogenitor proliferation, differentiation, and commitment to the osteoblastic lineage. Lee *et al* demonstrated that TGF-β-2 can lead to more rapid calvarial bone expansion through activation of Erk-MAPK, which stimulates differentiation of osteoprogenitor cells into osteoblasts[156]. In the context of OS, Franchi *et al* showed that 17 of 23 different osteosarcoma cell lines demonstrated cytoplasmic reactivity for TGF-β1, -2, and -3. Furthermore, high-grade osteosarcomas showed higher expression of TGF-β1 in comparison to low-grade osteosarcomas[157]. Overall, due to its implication in cell differentiation and commitment to the osteoblastic lineage, TGF-β is a target pathway that warrants further research in the development of new OS therapies (please see[158] for a more detailed review).

Lastly, the FGF cascade is an important signaling pathway involved in skeletal development and bone regeneration. Through activation of FGF receptors (FGFR), this signaling pathway controls osteoprogenitor cell replication and differentiation into osteoblasts[159], aberrations in which can lead to various congenital skeletal diseases[160]. In context of OS tumor biology, Shimizu *et al* showed that Fgf2 enhanced proliferation, migration, and motility of osteosarcoma AX cells in mice[161]. With a significant role in osteosarcoma pathogenic progression, inhibition of the FGF pathway is a potential target area of research that can lead to new OS therapies (please see[162] for a more detailed review).

DISCUSSION

In summary, much remains unknown regarding those cytokine signaling pathways that drive osteosarcomagenesis, as well as sarcoma proliferation, invasion, and metastasis. Various signaling cascades with importance in basic bone biology have been studied in OS tumor biology, including BMP, Hedgehog, and Wnt signaling. Other signaling cascades not discussed here include Notch[163], Insulin-like Growth Factor (IGF)[153,164], Transforming growth factor beta (TGF-β)[165,166], and Fibroblast Growth Factor (FGF)[167,168], to name a few. Although not directly discussed, crosstalk between signaling cascades is a rich area of investigation. Therefore, it is vital to continue investigating inhibition of these signaling pathways as a strategy to combat diseases such as OS. Development of targeted therapies for osteosarcoma hinges on improved understanding of those signaling pathways important in OS tumor biology.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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