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Review

Wnt signaling in physiological and pathological bone formation

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Summary. Wnt signaling, canonical or non-canonical, plays conserved roles in numerous physiological and pathological processes. However, it is well beyond the scope of this review to cover all functional aspects of Wnt signaling in different contexts at reasonable depth; therefore this review intends to cover only the roles of Wnt signaling in bone biology; more specifically, we intend to first update the roles of Wnt signaling in physiological bone process, including in osteogenesis and chondrogenesis, since recent years have witnessed tremendous progressions in this area, and then we seek to extend our understanding to the pathological bone process, especially to the heterotopic ossification (HO), even though the understanding of Wnt signaling in HO has been limited. We then further clarify the potential crosstalking between Wnt and other conserved signaling pathways, including FGF, GPCR and Hif1α pathways. Overall, our goal is to update the progressions, identify the general theme and the knowledge gaps and discuss the potential promising avenue for future applications in HO prevention and treatment.

Key words: Wnt signaling, Heterotopic ossification (HO), Fibrodysplasia Ossificans Progressiva (FOP), Osteogenesis, Chondrogenesis

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Introduction

Wnt signaling plays crucial conserved roles in numerous physiological processes, including embryonic development, axis patterning, cell fate specification, proliferation and migration, through at least three mechanisms, i.e., the canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway. Unsurprisingly, dysregulation of Wnt signaling has also been broadly implied in many disorders/pathological conditions (Khan and Bendall, 2006; Fujimaki et al., 2015). For example, in vertebrate development, loss of a single Wnt gene can produce dramatic phenotypes from embryonic lethal, CNS abnormalities to kidney and limb defects (Ikeya et al., 1997). Since Wnt signaling is essential for so many processes, it is almost impossible to cover all aspects of this conserved signaling at reasonable depth in one article.

Therefore, in this brief review, we intend to first focus on up-dating our understanding of the roles of Wnt pathway in physiological bone formation, since the field has made tremendous progress recently. Armed with this knowledge, we seek to further understand the pathological bone formation (i.e., heterotopic ossification, HO), since HO, acquired or hereditary (such as Fibrodysplasia Ossificans Progressiva, FOP), is a serious condition without effective treatment. More importantly, the specific roles of Wnt signaling in HO are still largely unknown, although much evidence has implied the involvement of Wnt signaling in this pathological process.

It is worth stressing that there might be fundamental

differences between physiological and pathological bone formation, even though Wnt signaling could play conserved roles in both physiological and pathological bone formation. For example, HO, unlike normal skeletal bone formation, is preceded by a series of stereotyped sequential disease specific events, such as injury induced abnormal inflammatory response (Foley et al., 2018).

With this in mind, we first review the features of three different Wnt pathways (Fig. 1): 1) In canonical Wnt pathway, Wnt proteins bind to the Frizzled (Fz)/low density lipoprotein (LDL) receptor-related protein (LRP) complex at the cell surface, and these receptors transduce signals through several intracellular proteins, including Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3), Axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin to regulate the target genes. 2) The non-canonical Wnt/PCP pathway uses Ror2, NRH1, Ryk or PTK7 as co-receptor instead of LRP5/6 to form a complex of DAAM1, which activates both small G-protein Rho and RAC1, which, in turn, modulate cytoskeletal structure and JNK activation, respectively (Komiya and Habas, 2008; Sugimura and Li, 2010; Lerner and Ohlsson, 2015). 3) The non-canonical Wnt/Ca^{2+} pathway regulates intracellular calcium levels by regulating endoplasmic reticulum calcium release; more specifically, Wnt-induced Frizzled activates PDZ and DEP domain of Dsh and trimeric G-protein to excite PLC and PDE, which repress canonical Wnt/β-catenin and regulate calcium-dependent NFAT to affect cell adhesion, migration and other functional aspects (Semenov et al., 2007; De, 2011). Recently, more and more Wnt proteins are identified to act through more than one Wnt signaling pathway, but according to their main model of actions and customary naming, we are still accustomed to classify them into canonical Wnts family (such as Wnt1, Wnt3a, Wnt9a, Wnt10b) and non-canonical Wnts family (such as Wnt4, Wnt5a/5b, Wnt7a/7b, Wnt11), while Wnt16 is thought to be a ligand that can work through both canonical and non-canonical pathways (Table 1).

WNT signaling modulates physiological bone development

Among the three Wnt pathways, the canonical Wnt

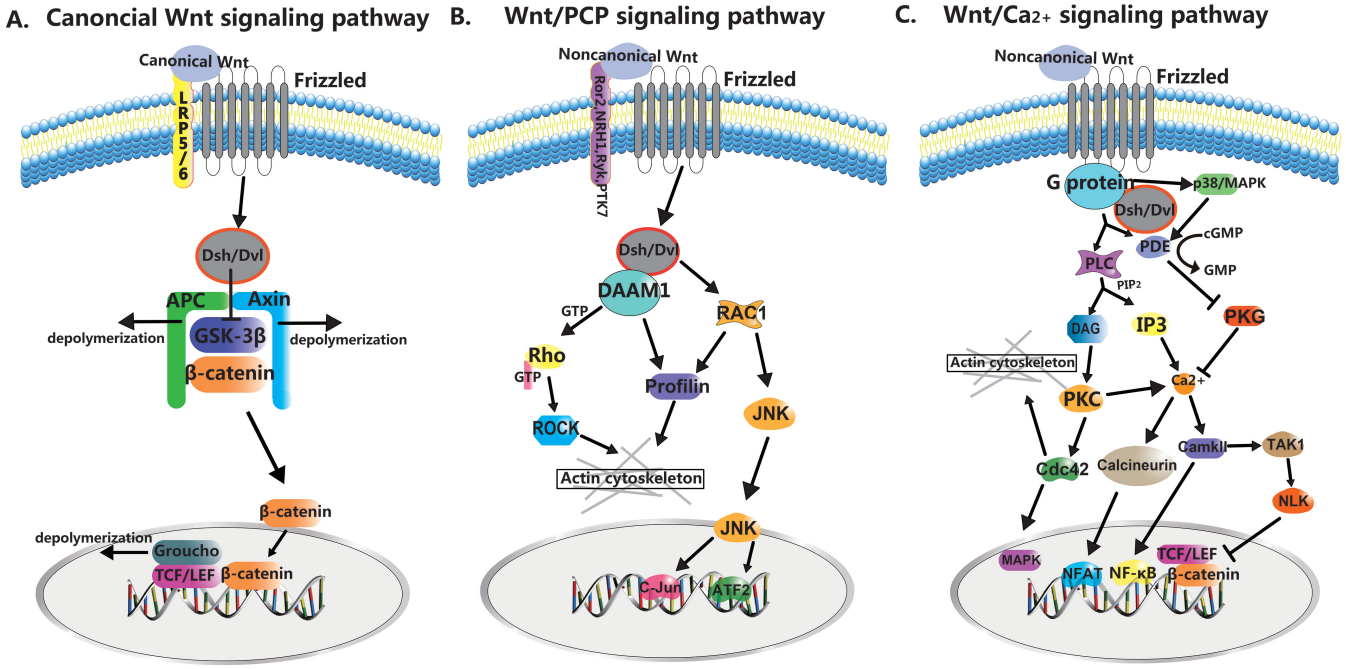

Fig. 1. A. The canonical Wnt pathway (or Wnt/β-catenin pathway). In the presence of Wnt, Wnt-induced Dsh/Dvl depolymerizes GSK-3β/APC/Axin/β-catenin tetramer, which causes the accumulation of dephosphorylated β-catenin, which then enters the nucleus, and binds TCF/LEF to remove negative regulatory factor, Groucho, and promote target genes expression. **B.** The non-canonical planar cell polarity pathway uses PDZ and DIX domain of Dsh to form a complex with Dishevelled-associated activator of morphogenesis 1 (DAAM1), which can induce Rho (the small G-protein) to activate Rho-associated protein kinase (ROCK). Dsh can also form a complex with RAC1 and stimulate JNK to activate target gene expression and remodel cytoskeletal actin. **C.** non-canonical Wnt/ calcium pathway is more complicated. Activated Frizzled can directly act on the PDZ and DEP domain of Dsh and trimeric G-protein to activate PLC and PDE pathway. PLC can then cleave PIP2 to generate DAG and IP3, which can induce the release of calcium, activate calcineurin-induced NFAT transcriptional activation and regulate CAMKII-TAK1-NLK to inhibit the canonical Wnt pathway, and DAG can induce Cdc42 expression to remodel cytoskeleton. Activated PDE, on the other hand, inhibits PKG, which subsequently causes the inhibition of calcium release from the ER. MAPK is also involved in the Wnt/ calcium pathway by stimulating p38/MAPK to inhibit calcium release by regulateing PDE.

pathway has been extensively studied in the context of physiological bone formation, including osteogenesis and chondrogenesis.

Wnt signaling in osteogenesis

Wnt signaling is crucial for osteogenesis. For example, *in vivo* studies of the frameshift (loss-of-function, LOF) mutations of Wnt1 found that LOF of Wnt1 caused the inherited early-onset osteoporosis and osteogenesis imperfecta (OI), which shows down-regulated osteogenesis and extreme bone fragility (Keupp et al., 2013; Makitie et al., 2016). Conversely, the gain-of-function (GOF) of Wnt signaling through suppressing the Wnt signaling inhibitors, such as through anti-sclerostin and anti-DKK1 antibodies, could effectively improve bone mass and fracture repair (Florio et al., 2016). Similarly, up-regulating Wnt signaling in osteocytes can also increase osteoblast numbers and osteogenic activity (Joeng et al., 2017). These data suggest that Wnt signaling is strictly regulated in physiological contexts, and dysregulated Wnt signaling often leads to changes in bone mass and affects physiological structure of bone. Consistently, many clinical trials suggested that Wnt plays beneficial roles in bone fragility (Keupp et al., 2013). Mechanistically, rapamycin, an inhibitor of mTOR signaling, can suppress osteogenesis induced by Wnt1, suggesting that Wnt participates in bone formation at least partially through regulating mTOR signaling (Joeng et al., 2017).

Furthermore, non-canonical Wnt signaling pathways are also involved in osteogenesis. For example, non-canonical Wnts, such as Wnt4, Wnt5a, Wnt7b and Wnt11, play significant roles in osteogenesis, but the underlying mechanisms for different non-canonical Wnt signals might not be exactly the same (Table 2). Furthermore, the non-canonical Wnt pathway normally crosstalks with other conserved signaling components, such as G protein-coupled receptor, PLC-MAPK, PI3K-AKT-mTORC1 and JNK-RANKL pathways, which makes dissecting the exact relationship among these components extremely hard (Chang et al., 2007; Tu et al., 2007; Maeda et al., 2012; Chen et al., 2014). Moreover, non-canonical Wnts can also interact with canonical Wnts, for example, classical Wnt3a and non-canonical Wnt7b can work synergistically with non-canonical G protein-linked PKC pathway to promote osteogenesis (Tu et al., 2007). Another non-canonical Wnt5a was found to activate Ror2-JNK pathway and PLC-PKC-CaMKII pathway to promote osteoblast differentiation, but the same Ror2-JNK can also activate

Table 1. Different Wnt family members participate in canonical and/or non-canonical Wnt signal pathway.

Ligand	Canonical Wnts	Non-canonical Wnts	Reference
Wnt1	Wnt/β-catenin	—	Keupp et al., 2013; Makitie et al., 2016; Joeng et al., 2017
Wnt3a	Wnt/β-catenin	Wnt/Ca^{2+}	Qu et al., 2013
Wnt4	Wnt/β-catenin	Wnt/Ca^{2+}	Chang et al., 2007; Lee and Behringer, 2007; Yu et al., 2014
Wnt5a	—	Wnt/PCP ,Wnt/Ca^{2+}	Maeda et al., 2012; Hosseini-Farahabadi et al., 2013; Martineau et al., 2017
Wnt5b	—	Wnt/PCP	Liu et al., 2015
Wnt7a	Wnt/β-catenin	Wnt/Ca^{2+}	Hwang et al., 2004; Gibson et al., 2017
Wnt7b	—	Wnt/Ca^{2+}	Tu et al., 2007; Chen, 2014
Wnt9a	Wnt/β-catenin	—	Day et al., 2005
Wnt10b	Wnt/β-catenin	—	Bennett et al., 2005
Wnt11	Wnt/β-catenin	Wnt/PCP, Wnt/Ca^{2+}	Friedman et al., 2009; Li et al., 2015
Wnt16	Wnt/β-catenin	Wnt/PCP	Moverare-Skrtic et al., 2014

Table 2. Wnt signal pathway in osteoblasts.

Ligand	Mode of action	Phenotypes
Wnt1	Activates canonical Wnt/β-catenin pathway	Increased osteoblasts and improved bone mass. Holmen et al., 2005; Florio et al., 2016; Joeng et al., 2017
Wnt4	Induces Axin mediated PLC-p38/MAPK pathway, Inhibits RANKL to reduce NK-κB expression	Increased osteoblasts, osteoclasts and bone volume, decreased inflammation. Chang et al., 2007; Yu et al., 2014
Wnt5a	Induces Ror2-JNK, PLC-PKC/CAMKII pathway in osteoblasts, stimulates Ror2-JNK-RANKL pathway in osteoclasts	Increased osteoblasts and osteoclasts. Inhibited net osteogenesis. Maeda et al., 2012; Keller et al., 2016; Martineau et al., 2017
Wnt7b	Through G protein-linked PKC pathway, or PI3K-AKT-mTORC1 pathway	Increased osteogenesis. Tu et al., 2007; Chen et al., 2014
Wnt11	Through classic Wnt/β-catenin pathway, or Wnt/PCP pathway	Increased osteoblast differentiation and osteogenesis. Friedman et al., 2009; Li et al., 2015; Wang et al., 2016
Wnt16	Activates Dsh-JNK-c-Jun pathway, Wnt/β-catenin-OPG pathway, or Wnt/β-catenin-OPG suppress RANKL pathway	Increased osteoblasts, osteogenesis and cortical thickness, decreased osteoclasts. Moverare-Skrtic et al., 2014; Shen et al., 2018

RANKL in the osteoclasts to increase osteoclast activity, therefore it seems that Wnt5a might play key roles in both bone formation and bone resorption, which therefore could play key roles in maintaining the homeostasis of normal bone (Maeda et al., 2012; Keller et al., 2016; Martineau et al., 2017).

Wnt signaling in chondrogenesis

Reports indicated that stable Wnt/β-catenin expression (GOF of canonical Wnt signaling) inhibited the phenotype of chondrocytes or chondrogenesis. For example, Day et al. (2005) found that overexpression of Wnt14/β-catenin (also known as Wnt9a/β-catenin) in Col2a1-positive cells severely blocked chondrocyte proliferation, through promoting chondrocyte maturation and then endochondral bone formation *in vivo* (Day et al., 2005; Hill et al., 2005). Hill et al. (2005) used β-cat$^{\Delta Prx/-}$ mice to study the GOF of canonical Wnt signaling *in vivo*, and also found that chondrogenesis

Table 3. Wnt signal pathway in Chondrogenesis.

Ligand	Mode of action	Phenotypes
Wnt1	Activate canonical Wnt/β-catenin pathway	Inhibits early chondrogenesis and promotes chondrocyte maturation, hypertrophy. Day et al., 2005; Hill et al., 2005
Wnt4	Possibly through Wnt/β-catenin pathway	Inhibits chondrogenesis, due to prematured cartilage differentiation. Lee and Behringer, 2007
Wnt5a	Induces JNK/PCP pathway, but inhibits Wnt/β-catenin expression	Inhibited early chondrogenesis, accelerated hypertrophy and the final differentiation. Excessive cartilage matrix degradation. Church et al., 2002; Gao et al., 2011; Hosseini-Farahabadi et al., 2013; Huang et al., 2017
Wnt7a	Through Wnt/β-catenin, or PI3K and AKT expression	Increased chondrocyte de-differentiation and inhibited apoptosis. Hwang et al., 2004; Gibson et al., 2017
Wnt9a	Through Wnt/β-catenin signaling pathway	Promotes chondrocyte hypertrophy and osteoblast differentiation. Day et al., 2005
Wnt11	PKC pathway	Reduced cartilage damage. Church et al., 2002; Ryu and Chun, 2006; Liu et al., 2014
Wnt16	Inhibit wnt/β-catenin	Inhibited chondrocytes apoptosis. Nalesso et al., 2017

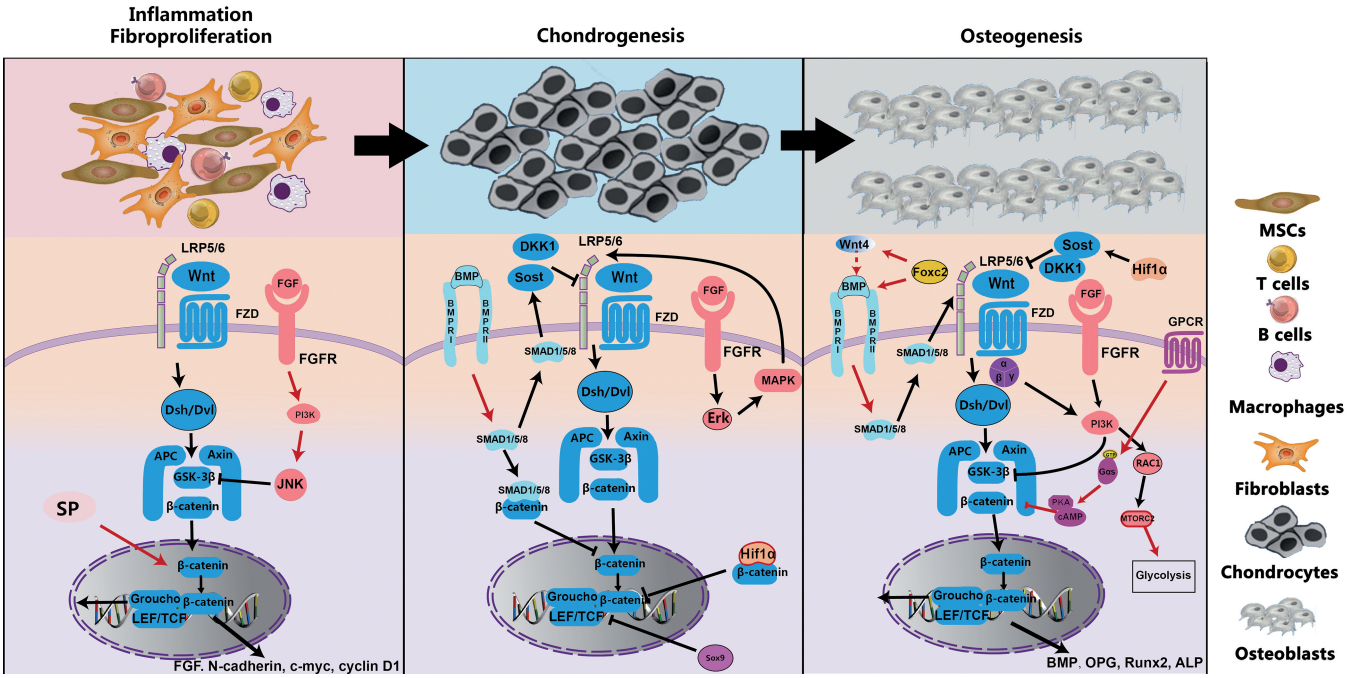

Fig 2. The interplaying of Wnts with several signaling pathways, such as BMPs, Gαs and FGFs, in the context of injury induced HO. These signal molecules form a huge network during the entire HO process, which constitute a complex microenvironment that regulates the HO process directly or indirectly. Left panel depicts the major signaling pathways and their potential cross-talking (i.e., Wnt and FGF pathways) involved in the early inflammation and the fibroproliferation stages; middle panel illustrates the major signaling pathways and their potential cross-talking (i.e., Wnt, FGF and Hif1α pathways) involved in the stage of chondrogenesis; right panel illustrates the major signaling pathways and their potential cross-talking (i.e., Wnt, FGF, GPCR and Hif1α) involved in the stage of osteogenesis. Red arrows indicate the known interactions found in HO, while the black arrows indicate the interactions mainly found in other contexts (could also occur in HO).

was inhibited, which caused malformed long bone without bone collar or trabecular structure. In contrast, LOF of canonical Wnt promoted chondrogenesis at the expense of osteogenesis, which caused patches of cartilage in the frontal bone at 17.5 dpc, where osteoblasts should occur (Hill et al., 2005; Qu et al., 2013).

The roles of non-canonical Wnts in chondrogenesis are quite complex. The general theme is that non-canonical Wnts on chondrocytes not only work through cells autonomously (Table 3), but also control the secretion of NCAM, N-cadherin, β-integrin, type II collagen and MMP13, through non-cell autonomous manner to complicatedly promote or inhibit chondrogenesis(Church et al., 2002; Hwang et al., 2004; Ryu and Chun, 2006; Gibson et al., 2017; Huang et al., 2017). More interestingly, the effect of some non-canonical Wnts on cartilage is significantly different from canonical Wnts, some non-canonical Wnts, such as Wnt16 and Wnt5a, might protect chondrocytes and simultaneously increase osteoblasts (Hwang et al., 2004; Hosseini-Farahabadi et al., 2013; Joeng and Long, 2014; Gibson et al., 2017; Nalesso et al., 2017), while other non-canonical Wnts like Wnt7a and Wnt16 apparently play key roles only in enhancing osteogenesis.

Mechanistically, there was a report indicating that Sox9 (a proposed chondrocyte biomarker) competes with β-catenin for the Lef/Tcf binding site and stimulates degradation of β-catenin by the ubiquitination pathway, which eventually promotes chondrogenesis (Akiyama et al., 2004). It was also reported that Wnts can also regulate osteoclasts by inhibiting glucocorticoid receptor-dependent RANKL and expressing OPG (Wang et al., 2014; Wang et al., 2017a). Of course, other mechanisms might also mediate the effort of Wnt signaling on chondrogenesis. For example, a study by Ryu (2002) indicated β-catenin was up-regulated during MSCs condensation but down-regulated in chondrocytes by N-cadherin-mediated cell-to-cell adhesion (Ryu, 2002), suggesting that dynamic expression pattern of Wnts could be partially responsible for the early chondrogenesis.

WNT signaling pathway in HO

With the insights gained from physiological bone formation, we seek to further understand the roles of Wnts in HO. We reason that HO is essentially osteogenesis in soft tissues, the insights gained from physiological bone formation might also be applicable to pathological bone formation, i.e., HO. However, it is worth stressing that HO, unlike normal skeletal bone formation, is preceded by a series of stereotyped sequential disease specific events, i.e., early injury induced inflammation, fibroproliferation, condensation and chondrogenic stages (Foley et al., 2018). Therefore, it is possible that the underlying mechanism of HO is fundamentally different from physiological bone formation.

Having said that, strong evidence still indicates that canonical Wnt signaling plays key roles in osteogenic differentiation of different stem cell populations *in vitro*, such as urine-induced stem cells, ESCs (embryonic stem cells) and iSCAP (immortalized stem cells of dental apical papilla) (Guan et al., 2015; Zhang et al., 2015; Keller et al., 2016). Importantly, in these contexts, unlike in the physiological bone formation, Wnt signaling is apparently not sufficient, because other modifiers such as VD3 or BMP were also needed to induce HO (Zhang et al., 2015; Keller et al., 2016). Consistently, De Boer et al. (2004) also found that different concentrations of Wnt/β-catenin can stimulate MSC proliferation or differentiation (De Boer et al., 2004). Similarly, it is reported that Wnt11 overexpression in MC3T3E1 pre-osteoblasts can activate β-catenin and augment BMP-induced osteoblast maturation and mineralization (Friedman et al., 2009). Therefore both canonical and non-canonical Wnt signalings are crucial for osteogenic differentiation of MSC into osteoblasts, at least *in vitro*.

Both *in vivo* and *in vitro* contexts show apparent evidence that Wnt signaling plays key roles in the whole process of inflammation, MSC proliferation and ossification. For example, at early stage, Wnt signaling can recruit or respond to cytokines to promote abnormal inflammation and tissue repair response, a pre-condition for HO process (Mei et al., 2014; Ahmad et al., 2017). In addition, further studies found that Wnt/β-catenin regulates MSC proliferation, and then in the presence of BMPs and/or other factors, Wnt/β-catenin co-regulates the lineage specific differentiation of MSCs into chondrocytes and/or osteoblasts, suggesting that canonical Wnt signaling pathway may regulate the initial HO process of HO *in vivo* (Ryu, 2002; Guerrero et al., 2014). In addition, some non-canonical Wnts, such as Wnt11, Wnt5a, can also promote chondrogenic differentiation of MSCs ex vivo (Liu et al., 2014). However, it was also reported that excessive non-canonical Wnt5a could stimulate degradation of mature cartilage matrix via activating canonical Wnt signaling, which might explain the self-limited characteristic of acquired HO (Hosseini-Farahabadi et al., 2013).

Moreover, Li et al. (2015) used mechanical tension to promote the osteogenesis of adipose-derived mesenchymal stem cells (ADSCs) *in vitro*, and in this context, they found that non-canonical Wnt11 activated osteogenesis of ADSCs through Wnt-PCP (RohA-ROCK) signaling pathway, while MIR-154-5P suppresses this process by directly inhibiting Wnt11 (Li et al., 2015). In another case, Gozo et al. (2013) found the patients with myositis ossificans had upregulated FOXC2 which directly activated Wnt4 and BMP4, and this led to HO (Gozo et al., 2013). In addition, Wnt signaling was also implied in nerve injury-induced HO, since Wnts is broadly expressed in the neural crest and its derivatives (Lewis et al., 2013). Consistently, it was reported that, in a neurogenic HO model ($Wnt1^{CreErt}$:Ai9Tm mice injected with BMP2-activated cells), all $SP7^{+}$ cells expressed Wnt1 (Olmsted-Davis et

al., 2017).

Together, a general theme emerges in HO, i.e., unlike normal bone formation, dysregulated Wnt signaling alone might not be enough to cause HO, unless other osteogenic inducers (i.e. HH or BMP) are present. Consistent with this idea, in the Progressive Osseous Heteroplasia (POH) mouse model, inhibiting Wnt signaling was not sufficient to inhibit ectopic bone formation (Regard et al., 2011, 2013).

In addition to typical HO, reports suggested that Wnt/β-catenin signaling pathway is also essential for atypical HO, including tendonitis ossification, ankylosing spondylitis ossification and ossification of the posterior longitudinal ligament (OPLL). Similar to typical HO, atypical HO, also known as non-traumatic HO, is also preceded by inflammation and fibrous proliferation (Kashii et al., 2016). Studies also found that Wnt/β-catenin signaling is dysregulated in these disorders, for example, DKK1 and sclerostin (inhibitors of Wnt signaling) are down-regulated in ankylosing spondylitis ossification patients, indicating that up-regulated Wnt signaling likely plays key roles in these disorders, therefore, enhancing DKK1 or SOST could be a promising treatment to inhibit the pathological osteogenesis in these disorders (Haynes et al., 2012; Wendling and Claudepierre, 2013; Yu-cong Zou, 2016). However, contrary data also exist. For example, it has been reported that, in patients with OPLL, the disease phenotype is negatively correlated with Wnt/β-catenin signaling (Shi et al., 2016), and the underlying reason for this discrepancy is still unclear.

Crosstalking between WNT and other signaling pathways

As mentioned above, one of the key challenges to understand the precise roles of Wnt signaling in HO is the complex and probably context-dependent crosstalking with other conserved signaling pathways. Therefore, further clarification of the interaction between Wnts' other conserved signal components, including BMPs, FGFs and mTOR, in different contexts is especially crucial.

With BMPs

BMPs have been thought to be the most important signaling pathway in HO. In fact, FOP, a genetic HO, is caused by gain-of-function mutation in BMP type I receptor ACVR1/ALK2 (Shore et al., 2006). Our own study also proved that overexpression of BMP4 in mice (Nse-BMP4) could reproduce the hallmarks of FOP as well as acquired HO (Kan et al., 2004), however, the exact mechanism as to how Wnt cross-talks with BMP signaling is still unknown. Nevertheless, the evidence supports that the early expression of BMPs likely activates sclerostin and DKK to inhibit β-catenin concentration (Stewart et al., 2014; He et al., 2017), and then Smad1/5/8 could competitively recruit β-catenin to Smad-containing complexes to reduce canonical TCF/LEF transcriptional activity. Therefore, the early interaction of BMPs and Wnts in MSC leads to inhibition of Wnt signaling and the reduced stemness of early MSCs, which essentially promotes the lineage specific differentiation of MSCs (Salazar et al., 2013). However, Papathanasiou et al. (2012) also reported that in prehypertrophic chondrocytes, BMP2-Smad1/5/8 could also upregulate Wnt/β-catenin signaling through LRP5 which contributes to chondrocyte hypertrophy and cartilage degradation (Papathanasiou et al., 2012), suggesting the context dependent regulation of Wnt pathway by BMP signaling. Furthermore, when hypertrophic chondrocytes differentiate into osteoblasts, β-catenin/TCF/LEF complexes can bind to BMP promoter to stimulate BMP signaling in osteoblasts, suggesting interaction of Wnt and BMP could be bidirectional, i.e., Wnt signaling could also regulate BMP signaling (Zhang et al., 2013). However, future detailed studies are still needed to fully delineate the exact co-regulation relationship between Wnts and BMPs in a variety of different contexts.

With FGFs

Fibroproliferation is an important turning point in the process of HO, and Freeman et al. proposed FGF is essential for fibroproliferation in HO (Freeman et al., 2010). More interestingly, Wang et al. (2017a,b) found there is a positive feedback loop between FGF and Wnt in skin fibroblasts (Wang et al., 2017b). Further evidence suggested that FGF and Wnts can also synergistically promote osteogenesis (Felber et al., 2015). Mechanistically , some reports suggested that FGF acts downstream of Wnt via FGF-PI3K-GSK3β (Quarto et al., 2010) and FGF-MAPK-LRP6 (Buchtova et al., 2015) to promote osteogenesis (Fei et al., 2011; Xu et al., 2017), but inhibits chondrocyte differentiation (Buchtova et al., 2015). Together, these data suggested that FGF and Wnt can also interact with each other and co-regulate the osteogegesis and/or chondrogenesis in HO; however, the exact underlying mechanisms are still unknown.

With mTOR

The physiological role of mTOR mainly promotes protein synthesis, energy metabolism, cell survival, inhibition of autophagy and lysosome formation. Interestingly, available data also support the idea that dysregulated mTOR might also play roles in both inherited and acquired HO by enhancing chondrogenesis and osteogenesis (Hino et al., 2017; Qureshi et al., 2017). More interestingly, Esen et al. (2013) found that Wnt-LRP5 works upstream of mTOR and regulates energy metabolism, and more specifically, the PI3K-mTOR pathway responds to Wnt-LRP5 to enhance glycolysis level by increasing the levels of key glycolytic enzymes during osteoblast differentiation

(Esen et al., 2013). However, it was also reported that insulin activates AKT/mTOR/p-GSK3 signaling to block BMP2-induced myoblast ossification (Zhang et al., 2014), which suggested negative correlation between Wnt/mTOR and HO. Detailed future studies will be needed to clarify the discrepancy.

With Hif1α

Hif1α is a subunit of a heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1). It is considered as the master transcriptional regulator of cellular and developmental response to hypoxia. The dysregulation of Hif1α has been strongly implicated in a number of pathological processes, including in injury that triggers HO (Wang et al., 2016). Hif1α is also an essential factor to maintain chondrocyte differentiation and homeostasis. More interestingly, evidence suggested that stable Hif1α could down-regulate Wnt signaling and provide suitable conditions for chondrogenesis. For example, Agarwal et al. (2016) found that PX-478, the Hif1α inhibitor, can prevent both trauma-induced and genetic HO (Agarwal et al., 2016). A further study found that, since β-catenin could bind preferentially to Hif1α, thus Hif1α could functionally sequestrate β-catenin and reduce the TCF-4-β-catenin interaction, which, in turn, could block the downstream Wnt/β-catenin signaling (Bouaziz et al., 2016). However, Chen et al. (2012) also reported that inhibition of Hif1α in osteoblasts *in vitro* caused decreased sclerostin, an inhibitor of Wnt signaling, and led to an increase of osteoblast growth and proliferation (Chen et al., 2012, 2013). The reason for the discrepancy is currently unknown.

With other signaling components

In addition to the aforementioned cross-talking, Shi et al. (2012) found that the non-canonical Wnt5a-RhoA pathway could crosstalk with uniaxial mechanical tension and promote osteogenic differentiation of rat tendon-derived stem cells (rTDSCs) (Shi et al., 2012). Others also reported that Wnt5a/Wnt5b could enhance HO through the JNK pathway (Liu et al., 2015). The inflammatory cytokine tumor necrosis factor α (TNFα) has been thought to induce DKK1 (a Wnt inhibitor) in bone erosion area (Diarra et al., 2007). However, local administration of TNFα could stimulate Wnt5a-Ror2 in chondrocytes while inhibiting Wnt5a in osteoblasts, which accelerates the syndesmophyte process (Briolay et al., 2013; Bougault et al., 2015). Notably, the inhibition of TNFα does not inhibit the ossification process, indicating that TNFα may only be involved in the early stage of ossification in this context. Unfortunately, the other potential cross-talking in HO remains largely unknown.

Conclusion and perspectives

In vivo and *in vitro* studies in the past decades have greatly enhanced our understanding of molecular mechanisms of Wnt signaling pathway in physiological and pathological bone processes. Current data suggest that Wnt signaling alone can work as a regulator of osteogenesis and chondrogenesis, but it can also crosstalk with other conserved signaling components, including BMPs, FGFs, mTOR and others to regulate the physiological and pathological bone processes. Based on these understandings, many translational investigations and animal experiments are underway to test the potential clinical applications. For example, hydroxyapatite containing Wnts can be used to treat bone regeneration after bone injury. Further studies, especially in the area of cross-talking between Wnts and other conserved signaling components in different contexts might provide more options for the treatment of different bone disorders. In addition, both canonical and non-canonical Wnts can also contribute to the atypical HO, such as OPLL and tendonitis ossification; therefore further understanding of the pathological mechanism will have positive impact on these disorders also.

In the future, it is important to 1) clarify the temporal and spatial expression pattern of Wnt/β-catenin singling components in the whole HO process, 2) understand the functional roles of Wnt/β-catenin in early inflammation and angiogenesis, 3) find disease specific changes of canonical and non-canonical Wnts, so that we can identify potential therapeutic targets. 4) Finally, it is interesting to understand why some Wnts, such as Wnt5a and Wnt16, play opposite roles in HO. By clarifying these issues, we hope we will be a step closer to finding the effective treatment for HO.

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Conflict of interest. The authors declare that there is no conflict of interest.

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