

Contributed Mini Review

Role of Wnt signaling in fracture healing

Huiyun Xu¹, Jing Duan¹, Dandan Ning¹, Jingbao Li¹, Ruofei Liu¹, Ruixin Yang¹, Jean X. Jiang² & Peng Shang^{1,*}¹Key Laboratory for Space Biosciences & Biotechnology, School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi Province 710072, People's Republic of China, ²Department of Biochemistry, University of Texas Health Science Center at San Antonio, Texas 78229, the United States

The Wnt signaling pathway is well known to play major roles in skeletal development and homeostasis. In certain aspects, fracture repair mimics the process of bone embryonic development. Thus, the importance of Wnt signaling in fracture healing has become more apparent in recent years. Here, we summarize recent research progress in the area, which may be conducive to the development of Wnt-based therapeutic strategies for bone repair. [BMB Reports 2014; 47(12): 666-672]

INTRODUCTION

'Wnt' was named after both the *Drosophila* Wg (wingless) gene (1) and the homolog of mouse mammary oncogene Int-1 (2). Wnts now comprise a family of secreted glycoproteins and play critical roles in embryonic development, carcinogenesis, and other important physical processes, such as bone metabolism. To date, 19 different Wnt proteins have been found in humans and mice, including WNT1-WNT11, and WNT16 (3). It is well known that the Wnt signaling pathway plays several major roles in skeletal development and homeostasis (4, 5). To a certain degree, the process of fracture repair is similar to that of embryonic bone development (6). Thus, in recent years, increasing attention has been placed on the role of Wnt signaling in fracture healing (4, 7, 8).

As a regenerative tissue, bone is able to repair itself after a fracture. However, ~3-10% of fractures fail to heal properly, with issues such as delayed union and non-union (9). In the United States, it is estimated that 100,000 fractures lead to non-union each year (10). Thus, it is important to find new anabolic agents that enhance bone regeneration and promote bone repair to improve the quality of treatment for fracture

patients. In this article, we summarize some of the findings on the role of Wnt signaling pathway in fracture healing.

WNT SIGNALING PATHWAY

In the canonical Wnt signal pathway, the Wnt protein binds to the membrane receptor 'Frizzled' (Fzd) (11), which is a seven-transmembrane protein. Then, together with other coreceptors, LRP5 and LRP6 (low-density lipoprotein receptor-related protein) (12), the protein activates 'disheveled' (Dsh), which inhibits the activation of glycogen synthase kinase-3 β (GSK-3 β). Inactive GSK-3 β is unable to phosphorylate β -catenin, so the unphosphorylated β -catenin escapes degradation by the proteasome complex, then translocates into the nucleus and associates with transcription factors 'T cell factor 7' (Tcf7) and 'lymphoid enhancing factor 1' (Lef1) to regulate the expression of relevant genes (13). In the β -catenin-independent non-canonical Wnt signal pathway, calcium signaling is thought to be the central mediator (14-16). The interaction of Wnts and Fzd leads to the formation of a tri-protein complex of Dsh-Axin-GSK, which mediates the phosphorylation of co-receptor tyrosine-protein kinase transmembrane receptor 1/2 (Ror1/2). The binding of Wnts to Fzd and Ror1/2 activates membrane-bound phospholipase C (PLC) and causes an increase in the concentration of inositol triphosphate (IP₃), 1,2 diacylglycerol (DAG), and intracellular calcium. This leads to alterations in downstream cellular function (17). Additionally, some secreted proteins, such as Dkk (dickkopf), Sost (sclerostin), and Sfrp (secreted frizzled-related proteins), may interact with LRP5/6 or Fzd receptor, and act as antagonists, inhibiting the Wnt signaling pathway (18-20).

FRACTURE HEALING

Fracture healing is a complex biological process that involves different types of bone cells and the interactions between cells, growth factors, and extracellular matrix. The repair consists of four overlapping stages: inflammatory response (also known as hematoma formation), soft callus formation, hard callus formation, and bone remodeling (21). During the process, bone cells are sequentially activated to form new bone. After hematoma formation, mesenchymal stem cells are recruited and proliferate and differentiate into osteogenic cells: chondrocytes

*Corresponding author. Tel: +86-29-88460391; Fax: +86-29-88461671; E-mail: celldon@126.com; shangpeng@nwpu.edu.cn

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and osteoblasts. The chondrocytes form a soft callus, which gives the fracture a stable structure. Later, the soft callus is mineralized and replaced with bone through endochondral ossification. At the same time, osteoblasts mineralize, generating a hard callus through intramembranous ossification. Finally, osteoclasts and osteoblasts are responsible for the bone remodeling process, which establishes new bone tissues (21-24).

WNT SIGNALING AND FRACTURE HEALING

During the repair process, the expression of many Wnt ligands (WNT4, 5b, 10b, 11, and 13) and receptors Fz1, 2, 4, and 5 are upregulated during fracture healing (25). Also, some target proteins of the Wnt pathway, such as c-myc and connexin 43, are activated (26, 27). These results have shown the role of Wnt signaling in regulating bone formation during the repair process.

β -catenin

Several studies have shown the activation of β -catenin signaling at fracture sites (28-31). Chen *et al.* have shown that β -catenin protein is highly expressed during the entire period of fracture repair (25). They used loss-of-function and gain-of-function approaches and found that in the early stage of healing, β -catenin controls the differentiation of mesenchymal cells, into osteoblasts and chondrocytes. Either an increase or a decrease of β -catenin interferes with the early stage of bone healing. In the later stages, when cells are committed to be osteoblasts, β -catenin promotes the differentiation of osteoblasts into bone and stimulates fracture healing (25).

LRP

LRP5 and LRP6 are required for successful fracture repair. The common genetic variants of LRP5 and LRP6 lead to decreased bone mass and bone mineral density (BMD). Furthermore, these variants have been shown to increase fracture risk in large cohorts of elderly Australian women (32) and Caucasian men (33). LRP5 mutations in mice have been shown to delay the repair of mandibular bone (28). *Lrp5*^{-/-} mice show impaired bone repair, with reduced callus area, bone mineral content (BMC), BMD, and biomechanical properties (34). A gain-of-function mutation in LRP5 delayed mandibular (28) and tibial (31) skeletal repair due to early repair stage exuberant cell proliferation, which postponed osteoblast differentiation at the injury site.

GSK-3 β

The function of GSK-3 β during fracture healing has been investigated using inhibitors of GSK-3 β . Oral treatment with lithium chloride (LiCl), a known inhibitor of GSK-3 β (35), can activate Wnt signaling and accelerate fracture repair. However, this effect only occurred in the later phases of repair when mesenchymal cells have committed to become osteoblasts. Early lith-

ium treatment, before the fracture, causes the aggregation of undifferentiated mesenchymal cells and reduces bone at fracture sites (25). LiCl attenuates the damaging effects of alcohol exposure on healing by restoring cartilaginous callous formation and endochondral ossification at fracture sites (36). For human cases, LiCl treatment is associated with a decreased risk of fractures (37). The oral administration of AZD2858, a bioactive GSK3 inhibitor, heals fractures rapidly and increases the strength of healed bone versus vehicle-treated controls (38).

Sfrp1

Sfrp1 interacts directly with Fzd or Wnts to antagonize canonical Wnt signaling (39). The expression of sfrp increases in Wnt-dependent early bone formation, along with the enhancement of β -catenin expression. Sfrp1-deficient mice have increased BMD, bone volume, and mineral apposition in the trabecular region, but not in the cortical region (40). Microarray expression analysis shows a significant decrease in the expression of sfrps 4 days after fracture. However, sfrp1 and sfrp4 are upregulated at both day 8 and day 14 after fracture, indicating negative regulation of bone formation during the osteogenic phase of repair at the injury site (29). The loss of sfrp1 function *in vivo* improves fracture repair by directly shifting progenitor cells into osteoblast lineage to promote early bone union. The sfrp1^{-/-} mice showed a dramatic reduction in the cartilage callous, and increased intramembranous bone formation at day 14 after fracture. These mice also exhibited earlier bone remodeling during the 28 day fracture repair process than wild-type mice (41).

Sost

Sost is a secreted glycoprotein expressed primarily by osteocytes in bone tissue. Sost binds to the extracellular domain of LRP5 and LRP6 and disrupts the formation of Wnt-LRP complex (42). Sost knockout mice have increased BMD, bone volume, bone formation, and bone strength (43, 44). Also, these mice have more bone in the fracture healing defect, which is due to an enhancement of the thickness of trabecularized spicules and osteoblast numbers (44, 45). Kambiz Sarahrudi *et al.* showed the first evidence demonstrating that Sost increases significantly during human fracture repair (46). Several *in vivo* studies have shown that systemic administration of Sost antibodies increased bone formation significantly at the site of fractures in several animal models, including mice, rats, and cynomolgus monkeys (44, 47-53). Antibody treatment improved bone density and the strength of non-fractured bones (47, 52). Anti-Sost therapy represents a promising approach for osteoporosis and fracture healing.

Dkk1

Dkk1 forms a complex with Lrp5/6 and disrupts the Wnt signaling pathway (54). Dkk1 allele deletion mice have increased bone mass without affecting the bone resorption process (55).

Table 1. Summary of the published *in vivo* studies of the role of Wnt signaling in fracture healing

Animal	Fracture Model	Treatment	Results	References
Rats	Femoral fracture		Activated Wnt signaling during fracture healing process.	Hadjiargyrou et al. [26]
6-month-old SD rats	Femoral fracture		Activated Wnt signaling pathway during bone regeneration.	Zhong et al. [27]
10-12-week-old male Lrp5 transgenic mice	1.0 mm hole in mandibular bone	Ad-Dkk1 injection	Activated Wnt signaling pathway during the early phase of bone regeneration. Ad-Dkk1 resulted in a delay in cranial skeletal repair. Lrp5 mutation caused delay in mandibular bone repair.	Leucht et al. [28]
6-week-old male SD rats	Drill-hole in tibia		Activated Wnt-dependent pathway in the early bone formation.	Macasai et al. [29]
8-week-old male C57BL/6 mice	Femoral fracture	30 µg/kg rhPTH injection	PTH promoted the levels of Dkk1 and Sost during the fracture healing.	Kakar et al. [30]
10-12-week-old male mice	1.0-mm hole drilled through a single cortex of the tibia	Adenovirus expressing Dkk1 (Ad-Dkk1) injection	Activated Wnt signaling at the site of injury. Ad-Dkk1 caused a delay in bone regeneration.	Kim et al. [31]
12-week-old male mice	0.5 mm hole on the tibia	0.02 M LiCl oral administration; Ad-Dkk1 injection	Up-regulated Wnt signaling during fracture healing. Either increase or decrease of β-catenin interfered with the early stages of bone healing. LiCl accelerated the fracture repair only in later phases of repair. Dkk1 treatment reduced chondrogenic differentiation.	Chen et al. [25]
17-week-old mice	Femoral fracture		Lrp5/ mice showed impaired bone repair.	Komatsu et al. [34]
6-7-week-old male C57BL/6 mice	Tibial fracture	100 mg/kg LiCl/day	LiCl attenuated the damaging effects of alcohol exposure on healing.	Lauing et al. [36]
9-week-old SD rats		30 µmol/kg AZD2858/day	GSK-3 inhibition promoted fracture healing and increased the strength of healed bone.	Sisask et al. [38]
8 weeks old male mice	Tibial fracture		Sfrp1/ mice promoted the fracture repair process	Gaur et al. [41]
13-week-old male mice	0.7 mm-diameter hole in femur		Sost/ mice had more bone in the fracture healing defect.	McGee-Lawrence et al. [44]
9-10-week-old male mice	Femoral fracture		Sost knockout mice showed increased bone formation and strength in the fracture callus.	Li et al. [45]
7-7.5-month-old male SD rats	Femoral fracture	25 mg/kg of Sost antibody (Scl-Ab) twice/week for 7 weeks	Sost antibody increased bone formation at the site of fracture.	Ominsky et al. [47]
14-week-old male Lewis rats	Femoral fracture	25 mg/kg of Scl-Ab for 12 weeks	Sost antibody increased bone formation at the site of fracture.	Virk et al. [48]
8-10-week-old female nude mice	0.8 mm femoral defect	25 mg/kg of Scl-Ab twice/week for 4 weeks	Scl-AbIII resulted in earlier healing and maturation of a non-critical-size bone defect.	Jawad et al. [49]
6-month-old male SD rats	Femoral fracture	25 mg/kg of Scl-Ab twice/week	Scl-Ab treatment enhanced bone healing.	Suen et al. [51]
10-week-old male SD rats	1.2 mm-diameter hole in tibia	25 mg/kg of Scl-Ab twice/week for 2 or 4 weeks	Scl-Ab treatment increased bone formation during metaphyseal repair but also in untraumatized bone.	Agholme et al. [52]
12-week-old male SD rats	3 mm defects in femur	25 mg/kg of Scl-Ab twice/week for 6, 10 and 12 weeks	Scl-Ab treatment enhanced bone repair in a bone defect and in the surrounding host bone, but lacked the osteoinductive activity to heal it	Alaee et al. [53]
6-6.5-month-old male SD rats	Femoral fracture	25 mg/kg of DKK1-Ab twice/week	Inhibition of Dkk1 enhanced the healing process	Li et al. [56]
10-12-week-old female mice	Femoral fracture	200 mg/kg/day LiCl	Inhibition of GSK-3β activity rescued the alterations in healing in Cx43-deficient mice	Loiselle et al. [57]
Mice	Tibial fracture	10 µg BMP2/7 injection	BMP treatment activated Wnt pathway in fracture calluses	Yu et al. [70]

Kim *et al.* showed that adenoviral expression of Dkk1 effectively prevented the differentiation of osteoprogenitor cells and blocked bone formation at the injury site (31). Also, Dkk1 treatment caused a large amount of undifferentiated mesenchymal-like tissues and reduced chondrogenic differentiation at fracture sites (25). Dkk1 antibodies significantly promoted fracture repair only when treated on the first day, not 4 days after the operation (34). Inhibition of Dkk1 enhanced the healing process, resulting in mechanically stronger bone at the fracture site (56).

INTERACTION BETWEEN WNTS AND OTHER BONE REGULATORY MOLECULES DURING FRACTURE HEALING

There are also other molecules that play roles in fracture healing process also via interactions with Wnt signaling.

Cx43

As the most abundant gap junction protein in bone, connexin43 (Cx43) is essential for bone homeostasis. Also, recent studies by Loisel *et al.* have shown the role of Cx43 in fracture repair. Targeted deletion of Cx43 in osteoblasts/osteocytes delayed bone formation and impaired mechanical properties during fracture healing. In Cx43-deficient fractures, β -catenin expression was attenuated, while Sost expression was increased. The changes in fracture healing in Cx43-deficient mice can be rescued by restoring β -catenin expression through inhibition of GSK-3 β activity with LiCl treatment (57, 58). Cx43 may be a potential therapeutic target to enhance fracture healing via regulating β -catenin expression.

PTH

As a clinically approved anabolic drug used to treat osteoporosis, parathyroid hormone (PTH) is thought to also be effective for fracture repair (59), which is at least partially Wnt dependent (60). PTH enhances the expression of several Wnts and nuclear localized β catenin protein (30). It also induces Lrp5/6 expression in the fracture callus, and promotes levels of Dkk1 and Sost during fracture healing.

BMP

Another growth factor used to accelerate bone healing is bone morphogenetic protein (BMP) (61-64), which can also cooperate with the Wnt signaling to promote osteoblast differentiation and new bone formation (65, 66). Wnt signaling is involved in chondrogenesis process induced by BMP-2. LiCl treatment decreased the upregulation of LEF-1 and β catenin induced by BMP-2 during later chondrogenesis (67). BMP-induced bone formation could be inhibited by Sost, both *in vitro* and *in vivo* (68, 69). Yan Yiu Yu *et al.* have shown that in BMP-treated calluses, the Wnt pathway is activated (70).

CONCLUSIONS AND FUTURE PERSPECTIVES

In this review, we summarize the current state of knowledge on Wnt signaling during the fracture repair process, which involves a well-organized interaction of various bone cells and activated regulatory factors (Table 1). Generally, activation of Wnt signaling is helpful to accelerate bone repair, and mutations in β -catenin or LRP5/6 reduce bone healing. Moreover, inhibition of negative regulators in the Wnt signaling pathway, such as GSK-3 β and Sost, can improve bone formation at fracture sites. Thus, inhibitors of GSK-3 β and Sost neutralizing antibodies may be promising and feasible targets for bone repair. Unfortunately, efforts to develop Sost antibodies for fracture healing have been abandoned by Amgen and their partner UCB, mainly due to the high investment requirement and the smaller market for fracture healing than osteoporosis. Thus, more hope should be placed on inhibitors of GSK-3 β for developing drugs to promote bone repair. Nonetheless, there is a long way to go. More work remains to be done in clinical and basic research to optimize treatment strategies. For example, the disparate roles of Wnts in different phases of fractures should be considered in the future development of therapeutic strategies.

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