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Wnt Pathway, an Essential Role in Bone Regeneration

Yan Chen[1] and Benjamin A. Alman[1,2*]

[1]*Program in Developmental & Stem Cell Biology, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada*

[2]*Division of Orthopaedic Surgery, Department of Surgery, University of Toronto, Toronto, Ontario, Canada*

ABSTRACT

Fracture repair is a complex regenerative process initiated in response to injury, resulting in optimal restoration of skeletal function. Although histology characteristics at various phases of fracture repair are clear and well established, much remains to be understood about the process of bone healing, particularly at the molecular signaling level. During the past decade, secreted signaling molecules of the Wnt family have been widely investigated and found to play a central role in controlling embryonic development processes. Wnt signaling pathway also plays a pivotal role in the regulation of bone mass. Recent published data reveal that Wnt signaling pathway is activated during postnatal bone regenerative events, such as ectopic endochondral bone formation and fracture repair. Dysregulation of this pathway greatly inhibits bone formation and healing process. Interestingly, activation of Wnt pathway has potential to improve bone healing, but only utilized after mesenchymal cells have become committed to the osteoblast lineage. These advances suggest an essential role of Wnt pathway in bone regeneration. J. Cell. Biochem. 106: 353–362, 2009.

KEY WORDS: Wnt; BETA-CATENIN; BONE REGENERATION

Wnt are 39–46 kDa cysteine-rich, secreted glycoproteins, that have been identified in organisms ranging from hydra to humans [Katoh, 2002]. The Wnt family is involved in embryonic development, tissue induction, and axial polarity [Gavin et al., 1990; Cadigan and Nusse, 1997]. Human and mouse genomes encode 19 Wnt and 18 Wnt genes, respectively [Akiyama, 2000]. Wnt ligands are unique, in that they can activate several different receptor-mediated signal transduction pathways. Wnt pathway is highly conserved across species, including *Caenorhabditis elegans*, *Drosophila melanogaster*, Zebrafish, *Xenopus laevis*, chicken, mouse, and human [Behrens, 2000]. Among the four Wnt pathways in literature, the canonical Wnt pathway is most extensively studied, which controls gene expression by stabilizing β-catenin (Wnt/β-catenin pathway) in regulating a diverse array of biological processes.

During the past decade, Wnt pathway has been found to play a central role in controlling embryonic bone development and bone mass [Westendorf et al., 2004]. In the developing skeletogenesis, Wnt signaling is required for limb bud initiation, early limb patterning, and, finally, late limb morphogenesis events. It has been reported that Wnt-3a and Wnt-7a are expressed in the limb bud and have roles in skeletal pattern determination [Kengaku et al., 1998], and that Wnt-14 is involved in joint formation [Hartmann and Tabin, 2001]. In addition, Wnt-3a, Wnt-4, Wnt-5a, and Wnt-7a all influence cartilage development [Hartmann and Tabin, 2000]. Abnormal Wnt signaling leads to developmental defects and human diseases affecting either tissue development or homeostasis [Yang, 2003]. Since the original description of the causal mutations in the low-density lipoprotein receptor related protein 5 (LRP-5) gene that were identified in patients with osteoporosis pseudoglioma syndrome (OPPG) [Gong et al., 2001] and in kindreds with autosomal dominant high bone mass (HBM) [Boyden et al., 2002; Little et al., 2002], considerable attention has been focused on the Wnt/β-catenin signaling pathway that it regulates for its role in the regulation of bone mass. It is reported that LRP-5 is expressed by osteoblasts of the endosteal and trabecular bone surface and

Abbreviations used: ALP, alkaline phosphatase; APC, adenomatosis polyposis coli; BMP, bone morphogenetic protein; CaMKII, calcium/calmodulin-dependent protein kinase II; Dkk, dickkopf; Dvl, Dishevelled; FGF, fibroblast growth factor; Fz, Frizzled; GSK-3β, glycogen synthase kinase 3β; HBM, high bone mass; Hh, Hedgehog; LEF, lymphoid enhancer factor; LRP, low-density lipoprotein receptor related protein; MSCs, mesenchymal stem cells; NF-AT, nuclear factor of activated T cells; NICD, notch intracellular domain; OPPG, osteoporosis pseudoglioma syndrome; PCP, planar cell polarity; PKC, protein kinase C; PTH, parathyroid hormone; Su(fu), suppressor of fused; TCF, T cell factor; TGF-β, transforming growth factor β; WISP-1, Wnt-1-induced secreted protein 1.

*Correspondence to: Benjamin A. Alman, Rm 13-601, TMDT Bldg, 101 College Street, Toronto, ON, Canada M5G1L7. E-mail: benjamin.alman@sickkids.ca

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regulates osteoblastic proliferation, survival and activity [Koay and Brown, 2005], targeted disruption of LRP-5 in mice reveals a significant reduction in the osteoblast surface density in both primary and secondary spongiosa in comparison to wild-type controls [Kato et al., 2002]. LRP-5 mutations have also been reported to cause severe forms of osteoporosis and recurrent fracture [Hartikka et al., 2005].

More recently, a striking finding that Wnt pathway also plays a crucial role in bone regenerative process (e.g., fracture repair) has been uncovered. Available published data strongly support the notion that modulation of this pathway may provide a promising therapeutic approach to enhance bone formation. In this review, we will mainly focus on the Wnt/β-catenin signaling pathway and discuss the involvement of this pathway in bone regeneration including ectopic bone formation and fracture healing, as well as the possibility to develop a new therapeutic approach targeting this pathway for enhancing bone formation.

MECHANISM OF BONE HEALING

Fracture healing is a complex, however, well orchestrated, regenerative process initiated in response to injury, resulting in optimal skeletal repair and restoration of skeletal function [Einhorn, 1998; Dimitriou et al., 2005]. Unlike other adult tissues, which generate scar tissue at the site of an injury, the skeleton heals by forming new bone that is indistinguishable from adjacent, uninjured tissue.

In classical histological terms, fracture repair has been divided into direct (primary) and indirect (secondary) healing [Dimitriou et al., 2005]. Direct bone healing occurs only when there is anatomic reduction of fracture fragments by rigid internal fixation and decreased intrafragmentary strain [McKibbin, 1978]. This process involves a direct attempt by the cortex to reunion new havesian systems. Little or no periosteal response (no callus formation) is seen during this process [Einhorn, 1998].

The majority of fractures heal by indirect healing process, which consists of three main phases in the healing process: (1) the early inflammatory stage; (2) the repair stage; (3) the remodeling stage. Generally, the fracture repair starts with the activation of an inflammatory cell response (coagulation cascade). This early inflammatory phase is initiated after hemorrhage caused by vascular injury and the subsequent development of a hematoma. Recruitment and proliferation of undifferentiated mesenchymal stem cells (MSCs) from the tissue surrounding the fracture gap then occurs. The resulting granulation tissue is the foundation for the subsequent healing process. The repair stage is characterized by the formation of callus involving two mechanisms, intramembranous, and endochondral ossification [Olsen et al., 2000]. This stage begins with continued vascular ingrowth, secretion of osteoid, as well as the presence of fibrocollagenous fibers. During this phase, mesenchymal cells from the periosteum adjacent to the fracture site directly differentiate into osteoblasts and start to produce bone matrix (intramembranous ossification). Intramembranous bone formation results in hard callus formation and mainly occurs alone in the flat bones of the skull, the mandible, and the part of the clavicle [Einhorn, 1998; Ducy and Karsenty, 2000]. In the second phase of repair, the undifferentiated mesenchymal cells in the granulation tissue next to the subperiosteal bone proliferate and differentiate into chondrocytes, which synthesize and secret cartilage matrix, including collagen type II and proteoglycans. This type of fracture healing provides an early bridging callus which is histologically characterized as soft callus that stabilizes the fracture fragments [Reddi, 1998; Dimitriou et al., 2005]. Once firm mechanical stability is established, the cartilage undergo hypertrophy and mineralization in a spatially organized manner [Barnes et al., 1999], and the soft callus is eventually replaced by woven bone (endochondral ossification). Finally, the remodeling process substitutes the trabecular woven bone with compact bone. During this stage, the woven bone is first resorbed by osteoclasts, creating a shallow resorption pit known as a "Howship's lacuna." Then osteoblasts deposit compact bone within the resorption pit. Eventually, the fracture callus is remodeled into a new shape which closely duplicates the bone's original shape and strength.

In many aspects, fractures heal by recapitulating the stages of embryonic bone development [Bruder et al., 1994]. This idea is predicated on the histomorphological similarities between the cells that populate the wound site and those that are recruited during skeletogenesis. For example, both fetal and adult skeletal progenitor cells aggregate to form cell condensations that eventually differentiate into skeletal tissue [Thompson et al., 2002]. The same molecular markers of chondrogenesis and osteogenesis are expressed during development and repair, which suggests that the regulation of cell differentiation is also conserved [Ferguson et al., 1999; Karsenty and Wagner, 2002]. In both development and bone healing process, vascularization is a prerequisite for ossification, and disruptions in extracellular matrix remodeling can delay subsequent bone formation [Thompson et al., 2002]. Nevertheless, there are also inherent differences between fracture repair and fetal skeletogenesis. For example, fracture healing involves an inflammatory response that is absent in embryonic development. It is also not clear whether embryonic stem cells are equivalent to mesenchymal stem cells seen in an adult fracture. Furthermore, to some extent, there remains difference for some endogenous growth factors and cytokines in terms of their temporal and spatial expression manner during bone development or regeneration. Despite these obvious differences, the molecular signals involved in adult mesenchymal cell differentiation, chondrocyte maturation, angiogenesis, and ossification appear similar to those mediating parallel events during fetal skeletal development [Ferguson et al., 1998].

Wnt SIGNALING PATHWAY

Cell signaling cascades provoked by Wnt proteins have been well conserved through evolutional processes among a variety of species. Wnt proteins act on target cells by binding to Frizzleds (Fzs), seven-span transmembrane receptor proteins, and LRP-5/6, single-span transmembrane co-receptor proteins. Wnts activate at least four distinct intracellular signaling cascades: the Wnt/β-catenin pathway, the Wnt/Ca^{2+} pathway, the Wnt/planar cell polarity (Wnt/PCP)

pathway, or the Wnt/protein kinase A (Wnt/PKA) pathway [Moon et al., 2002; Nelson and Nusse, 2004].

THE CANONICAL Wnt PATHWAY

The Wnt/β-catenin pathway is commonly referred to as the canonical Wnt pathway, and is the most extensively studied pathway (Fig. 1). At least 7 of 19 Wnt proteins, including Wnt-1, Wnt-2, Wnt-3, Wnt-3b, Wnt-4, Wnt-8, and Wnt-10b, have been reported to activate this pathway. The canonical Wnt pathway affects cellular functions by regulating β-catenin levels and subcellular localization [Akiyama, 2000]. The canonical Wnt pathway is initiated by the binding of appropriate Wnt ligands to the Fzs and LRP-5/6 co-receptor. In the absence of appropriate Wnt ligands, β-catenin is phosphorylated in the its NH2-terminal degradation box, polyubiquitinated by βTRCP1 (a component of ubiquitin E3 ligase) or βTRCP2 complex for the following proteasome-mediated degradation by a multi-protein complex comprising glycogen synthase kinase 3β (GSK-3β), adenomatous polyposis coli (APC), and axin [Ikeda et al., 1998; Eastman and Grosschedl, 1999; Behrens, 2000]. In the presence of an appropriate Wnt ligand, binding of Wnt to receptor complex leads to the activation of the intracellular protein, Dishevelled (Dvl), an intracellular mediator that plays a central role in transducing the signal from the receptor complex. The activation of Dvl leads to the inhibition of GSK-3β, results in the collapse of the multi-protein complex [Seto and Bellen, 2004]. Hence, β-catenin cannot be targeted for degradation and it accumulates and translocates to the nucleus, where in concert with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family, activates the transcription of a wide range of genes, including c-myc and cyclin D1.

The complexity of Wnt intracellular signaling pathways parallels the complexity observed in the diversity of Wnt receptors. To date, there are 10 human Fz receptors [Adler and Lee, 2001]. It should be emphasized that, although the role of Fz in acting as a receptor for Wnts is long established, LRP-5 and its closely related homologue, LRP-6, are two important molecules in mediating the Wnt/β-catenin pathway [Gordon and Nusse, 2006], in that both of them act as co-receptors for Wnt proteins, and this cannical Wnt pathway can be antagonized by secreted proteins from the Dickkopf (Dkk) family that bind with high affinity to LRP-5 or LRP-6 and thereby directly prevent Wnt binding [Wu et al., 2000; Bafico et al., 2001].

THE NON-CANONICAL Wnt PATHWAYS

The Non-canonical Wnt pathway functions in a β-catenin-independent manner [Gordon and Nusse, 2006]. Non-canonical Wnt signals are transduced through Fz family receptors and co-receptors, such as ROR2 and RYK, but not LRP-5 or LRP-6 [Katoh, 2007]. There are three other non-canonical Wnt signaling pathways that have been described in the literature.

In the Wnt/Ca^{2+} pathway, Wnt protein (e.g., Wnt-5a) binds to a Fz receptor and co-receptor such as Knypek or ROR2. This binding stimulates heterotrimeric G proteins, increases intracellular calcium levels, decreases cyclin GMP (cGMP) levels, and activates protein kinase C (PKC) or calcium/calmodulin-dependent protein kinase II (CamKII) to induce nuclear factor of activated T cells (NF-AT) and other transcription factors [Wang and Malbon, 2003; Kohn and Moon, 2005]. Wnt/Ca^{2+} pathway can affect cell adhesion and cell movement during gastrulation [Torres et al., 1996]. Wnt/Ca^{2+} pathway also inhibits the canonical Wnt pathway by promoting GSK-3-independent β-catenin degradation [Topol et al., 2003].

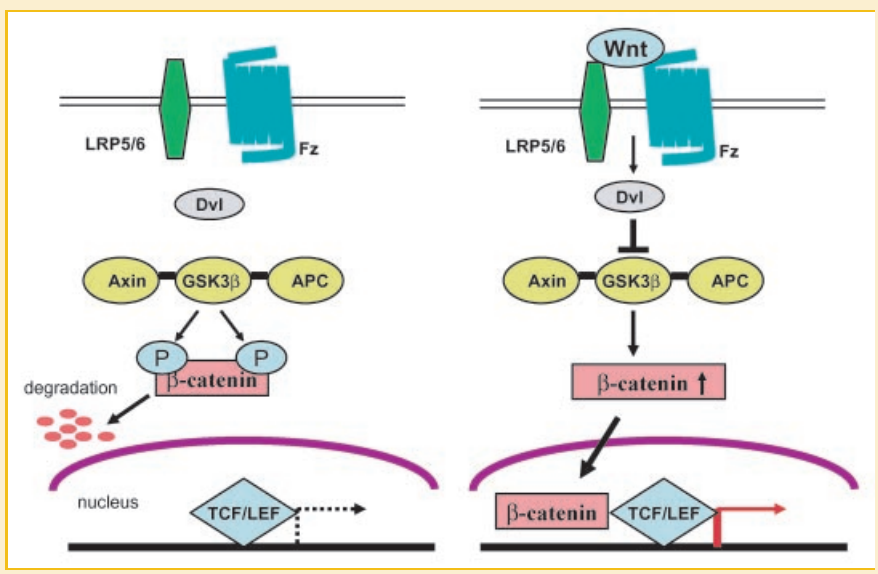

Fig. 1. An overview of the Wnt/β-catenin signaling pathway. In the absence of Wnt ligand, β-catenin is targeted for phosphorylation and degradation by a multi-protein complex comprising GSK-3β, APC, and axin. In the presence of an appropriate Wnt ligand, Wnt bind to the receptor Fz and co-receptor LRP-5/6, and this binding leads to the activation of the intracellular protein, Dvl, an intracellular mediator that plays a central role in transducing the signal from the receptor complex. The activation of Dvl leads to the inhibition of GSK-3β, results in the disassociation of the multi-protein complex and the intracellular accumulation of β-catenin. Hence, β-catenin cannot be targeted for degradation and it accumulates and translocates to the nucleus, where in concert with members of the TCF/LEF family, activates the transcription of a wide range of genes, including c-myc and cyclin D1.

In the Wnt/PCP pathway, Wnt protein signaling through Fz receptors mediates asymmetric cytoskeletal organization [Habas et al., 2003] and the polarization of cells by inducing modifications to the actin cytoskeleton. Two independent pathways, which are initiated by Dvl trigger the activation of the small GTPases Rho and Rac. Activation of Rho requires Daam-1 and leads in turn to the activation of the Rho-associated kinase ROCK. Rac activation is independent of Daam-1 and stimulates c-Jun N-terminal Kinase (JNK) and nemo-like kinase (NLK) signaling cascades [Huelsken and Behrens, 2002; Habas and Dawid, 2005]. In addition, the Wnt/PKA pathway activates cyclin AMP (cAMP) response element binding protein (CREB), and thus stimulates CREB-mediated transcription in a PKA-dependent manner. This non-canonical pathway modulates myogenic gene expression during development [Chen et al., 2005].

Taken together, although the specificity between Wnt molecules and Fz receptors remains unclear, it is known that different Wnt ligands will preferentially activate one of these four canonical or non-canonical Wnt pathways [Johnson and Kamel, 2007].

Wnt SIGNALING PATHWAY IN BONE REGENERATION

Studies on embryonic skeletogenesis provide clues that Wnt pathway is very likely involved during bone regeneration. As was stated above, members of the Wnt family participate and play a central role in a variety of fundamental processes during embryonic development, such as limb skeletogenesis [Yang, 2003]. Wnt pathway also represents a mechanism in mesenchymal cells proliferation and differentiation during chondrogenesis or osteogenesis. For example, Wnt-3a has been shown to promote proliferation and suppresses osteogenic differentiation of adult MSCs [Boland et al., 2004], suggesting that canonical Wnt signaling pathway functions in maintaining an undifferentiated, and proliferating progenitor MSC population. Wnt-3a also enhances BMP-2-mediated chondrogenesis in murine C3H10T1/2 mesenchymal cells [Fischer et al., 2002a]. Additionally, Wnt-10b has been shown to promote osteoblastogenesis via induction of the osteoblastic transcription factors, Cbfa-1, Dlx-5, and Osterix. These data strongly indicate that Wnt pathway is intimately associated with bone regenerative process.

Wnt/β-CATENIN PATHWAY IN ECTOPIC ENDOCHONDRAL OSSIFICATION

Enomoto-Iwamoto et al. reported that Wnt/β-catenin pathway stimulates maturation and hypertrophy of cultured chick chondrocytes. Experimental inactivation of this pathway blocks chondrocyte maturation in the chick limb in vivo and that nuclear translocation of β-catenin occurs in mineralizing hypertrophic chondrocytes both in vivo and in vitro [Enomoto-Iwamoto et al., 2002]. These findings suggest that β-catenin-mediated Wnt pathway may regulate endochondral ossification. To test this possibility, Kitagaki et al. [2003] established a chondrocyte transplantation model to induce ectopic endochondral ossification in nude mice. In this study, the authors implanted intramuscularly the collagen gel-containing mature chick chondrocytes in the athymic mice, and reported that transplantation of chondrocytes results in initial formation of cartilage followed by endochondral bone and marrow development with increasing time in vivo. By using a constitutive gain-of-function or loss-of-function approach, the authors found that activation of β-catenin-mediated TCF/LEF signaling in chondrocytes accelerates chondrocyte maturation and greatly enhances ectopic bone formation, while inactivation of Wnt/β-catenin pathway suppresses it [Kitagaki et al., 2003].

Recently, our research group discovered a crucial role of β-catenin signaling pathway in BMP-2-induced endochondral ossification [Chen et al., 2007b]. In this study, we implanted recombinant BMP-2 into the muscle of mice to induce endochondral bone formation, a model that has been widely used for endochondral ossification studies. We showed that β-catenin-mediated TCF-dependent transcription is activated during BMP-2 induction of endochondral bone formation, in particular, during early chondrogenesis (but not late stage of chondrogenesis) and throughout osteogenesis. We also found that Wnt/β-catenin pathway is required not only for osteoblast differentiation but also for cartilaginous differentiation during BMP-2-induced endochondral bone formation. When Wnt/β-catenin pathway was inactivated either by treatment of Dkk-1, a soluble antagonist of Wnt ligand-mediated signaling, or by activating conditional null alleles of β-catenin, not only did we observe an inhibition of chondrocytes on histological evaluation and bone formation, but we also found a significant downregulation of Sox-9 [Chen et al., 2007b], a marker of chondrocyte differentiation [Lefebvre et al., 1997; Bridgewater et al., 1998], and Runx-2 (also known as Cbfa-1), a marker of osteoblast differentiation [Ducy, 2000]. These findings clearly suggest that in BMP-2-sitmulated ectopic endochondral ossification, β-catenin is induced by the BMP-2 through a Wnt ligand-dependent manner, and that the Wnt/β-catenin pathway is the dominant mechanism regulating new bone formation. Interestingly, these data are in contrast to studies of embryonic skeletogenesis, which demonstrated β-catenin is required only for osteogenesis but is dispensable for chondrogenesis [Day et al., 2005; Hill et al., 2005]. Hence, although bone regenerates by recapitulating the stages of embryonic skeletogenesis, embryonic development pathways are not always recapitulated during postnatal regenerative processes, and the biochemical pathways utilized to regulate cell differentiation may be different.

Wnt/β-CATENIN PATHWAY IN FRACTURE REPAIR

Two microarray gene expression studies in a rat closed fracture model revealed that several genes for Wnt signaling components and their target genes are upregulated, including Wnt-4, Wnt-5a, Wnt-5b, Fz, β-catenin, Dvl, TCF-1, LRP-5, fibronectin, phosphatases 2A, connexin 43, as well as c-myc [Hadjiargyrou et al., 2002; Zhong et al., 2006]. These data indicate that Wnt signaling pathway is activated during fracture healing. Noticeably, the transcription factor, LEF-1 is inhibited during the early stage of fracture repair, coinciding with the peak of osteoblast differentiation and subsequent intramembranous ossification [Zhong et al., 2006]. However, given that LEF-1 is known to repress Runx-2-dependent

activation of osteocalcin promoter in osteoblasts [Kahler and Westendorf, 2003], it is not surprising that LEF-1 is downregulated during healing process.

French et al. [2004] investigated the role of Wnt-1-induced secreted protein 1 (WISP-1), a member of the CCN (connective tissue growth factor, Cyr61, NOV) family of growth factors, during both embryonic development and fracture healing in mice. The authors found that during limb skeletogenesis, WISP-1 expression is restricted to osteoblasts and to osteoblastic progenitor cells of the perichondral mesenchyme. WISP-1 also interacts with perichondral mesenchyme and undifferentiated chondrocytes. During fracture healing, the WISP-1 expression recapitulates the pattern observed during skeletal development. WISP-1 also increases proliferation and saturation density but repressed chondrocytic differentiation [French et al., 2004]. These data demonstrate that WISP-1 is an osteogenic potentiating factor promoting mesenchymal cell proliferation and osteoblastic differentiation while repressing chondrocytic differentiation, suggesting an important regulatory role of Wnt pathway during bone development and fracture repair. Kakar et al. found that daily systemic treatment of parathyroid hormone (PTH) (1-34) following fracture not only induces a larger callus cross-sectional area, length, and total volume compared with controls, but also upregulates the expression of several Wnt molecules (i.e., Wnt-4, Wnt-5a, Wnt-5b, and Wnt-10b) and increased levels of unphosphorylated, nuclear localized β-catenin protein [Kakar et al., 2007], suggesting that the PTH-mediated enhancement of fracture repair is at least in part through the activation of Wnt/β-catenin signaling pathway.

Recently, our research group uncovered a crucial role of Wnt/β-catenin pathway in bone healing in a stabilized tibia fracture mouse model [Chen et al., 2007a]. Firstly, we found that β-catenin-mediated, TCF-dependent transcription is activated in both bone and cartilage formation during fracture repair. We also observed that several Wnt ligands and receptors (e.g., Wnt-4, Wnt-5b, Wnt-10b, Wnt-11, Wnt-13, Fz-1, Fz-3, Fz-4, Fz-5, and LRP-6) are upregulated during bone healing. Since Wnt-4 and Wnt-10b have been demonstrated to mediate the canonical Wnt pathway, it seems likely that these Wnt ligands are responsible for activating β-catenin signaling during fracture repair. Furthermore, treatment with Dkk-1 inhibits β-catenin signaling and the healing process, suggesting that canonical Wnt pathway is indeed the dominant mechanism regulating β-catenin during the healing process. Interestingly, bone healing is significantly repressed in mice conditionally expressing either null or stabilized β-catenin alleles. Fracture repair is also inhibited in mice expressing osteoblast-specific β-catenin null alleles. However, in stark contrast, there is dramatically enhanced bone healing in mice expressing an activated form of β-catenin, whose expression is restricted to osteoblasts only [Chen et al., 2007a]. Based on these data, it seems very likely that in early stages of fracture healing, precise regulation of β-catenin is required for pluripotent mesenchymal cells to differentiate to either osteoblasts or chondrocytes. Once these undifferentiated cells have become committed to the osteoblast lineage, β-catenin positively regulates osteoblasts. Conclusion can be drawn that Wnt/β-catenin pathway plays an essential and disparate role at different stages of fracture repair.

Using a simple transcortical defect, created by drilling a 1.0-mm hole through a single cortex in the tibia, Kim et al. [2007] found that several Wnt ligands and receptors (e.g., Wnt-2b, Wnt-3a, Wnt-5a, Wnt-5b, Wnt-11, and Fz-4) were expressed at the injury site. Surprisingly, Wnt inhibitory factor (WIF) was also induced following injury, suggesting a negative feedback mechanism regulating Wnt activity. Similar to our data, the author demonstrated that inactivation of Wnt pathway by Dkk-1 also greatly inhibited monocortical bone healing [Kim et al., 2007], providing further evidence for a crucial role of Wnt pathway in fracture repair.

Wnt/β-CATENIN PATHWAY IN CRANIOFACIAL BONE REGENERATION

Osteoblasts are derived from two distinct embryonic lineages: cranial neural crest, and mesoderm. Appendicular skeleton is derived entirely from mesoderm and forms bone through endochondral ossification, whereas the cranial skeleton is derived from paraxial mesoderm as well as the cranial neural crest and that form bone through both endochondral and intramembranous ossification [Noden and Trainor, 2005]. Although both populations of cells are capable of forming bone and cartilage during fetal development and during adult bone repair, it remains unknown if they use equivalent molecular pathways (e.g., Wnt pathway) to achieve osteoblast differentiation.

To address this issue, Leucht et al. [2008] established a mouse mandibular repair model and focused on the role of Wnt signaling pathway in the healing process. Using in vivo hybridization, the researchers found that at day 7 time point, three Wnt ligands including Wnt-2b, Wnt-3a, and Wnt-7a were broadly expressed throughout the early callus. Treatment of adenovirus expressing the Fc portion of the immunoglobulin (Ad-Fc) exhibited high reporter activity throughout the buccinator muscle in TOPgal mice 7 days after injury, as detected by *LacZ* staining, which indicated that injury triggers upregulation of the endogenous Wnt pathway. Interestingly, treatment with Ad-Dkk-1 to inactivate Wnt pathway resulted in greatly reduced Wnt signaling activity, and a lack of new bone matrix due to inhibition of osteoblast differentiation. However, constitutive activation of Wnt signaling in transgenic mice harboring a gain-of-function mutation in the Wnt co-receptor LRP-5 not only resulted in increased bone mass, as has been previously reported [Boyden et al., 2002], but also decelerated bone repair, apparently due to exuberant cell proliferation in the early repair process that delayed osteoblast differentiation [Leucht et al., 2008]. These findings indicate that Wnt/β-catenin signaling pathway is also required for craniofacial bone repair, suggesting a similar signaling pathway despite their different embryologic origin.

NON-CANONICAL Wnt PATHWAY IN BONE REGENERATION

Although recent advances demonstrated a crucial role of canonical Wnt pathway during ectopic bone formation and fracture healing, this does not rule out the possibility that non-canonical Wnt pathways are also possibly involved during these bone regenerative process. Indeed, we found that Wnt-5b, Wnt-11, as well as Wnt-13 are upregulated at their mRNA level during fracture healing. We also

observed an upregulation of Wnt-5a and PKC-α at their protein level in both BMP-2-induced ectopic endochondral ossification and fracture healing process [Chen et al., 2007a,b]. All these Wnts are non-canonical Wnt proteins. For example, Wnt-5a and Wnt-11 can signal through the Wnt/Ca^{2+} pathway, which regulates Ca^{2+} flux and Ca^{2+}-sensitive protein kinases and transcription factors, such as PKC-α and calcium/calmodulin-dependent protein kinase II (CaMKII) [Sheldahl et al., 1999; Veeman et al., 2003]. Wnt-11 can also activate the JNK pathway [Heisenberg et al., 2000; Carron et al., 2005].

Interestingly, we found that during ectopic bone formation and fracture healing, both Wnt-5a and its signal mediator, PKC-α, are highly expressed during early stage of bone regeneration, that is, at the stage of inflammatory and chondrogenesis stage. However, the expression level greatly reduced when bone become more mature [Chen et al., 2007a,b]. Similarly, a microarray study in a rat model noticed that the expression pattern of Wnt-5a increases at day 3 following fracture, declines at 5–7 days after the injury, rises again at 10–14 days, and decline back down to intact bone levels by 21 days after fracture [Hadjiargyrou et al., 2002]. These findings suggested that Wnt-5a mediated non-canonical Wnt pathway may be activated during inflammation and chondrogenesis, but not during osteogenesis or remodeling stage. These results imply that Wnt signaling in fracture repair is complex, potentially involving activation of both canonical and non-canonical Wnt pathways.

So far, the mechanism whereby non-canonical Wnt pathway regulates bone regeneration is still unknown. Studies on embryonic skeletogenesis demonstrate that Wnt-5a promotes chontrocyte differentiation in the distal limb bud by inhibiting the canonical Wnt activity, [Topol et al., 2003] and that its misexpression delayed the maturation of chondrocytes and the onset of bone collar formation [Hartmann and Tabin, 2000], as well as the truncation of long bones due to retarded chondrogenic differentiation [Kawakami et al., 1999]. These data provide a supporting evidence of the role of Wnt-5a in the early stages (e.g., chondrogenesis) of fracture repair and ectopic endochondral ossification. Furthermore, corroborating with Van Den Berg who showed that Wnt-5a is expressed by highly enriched populations of hematopoietic progenitor cells ($CD34^+$ Lin^-) [Van Den Berg et al., 1998], Baksh and Tuan [2007] found that both non-hematopoietic ($CD45^-$) and hematopoietic cells ($CD45^+$) expressed Wnt-5a protein, while neither of these populations expressed any detectable levels of Wnt-3a. Since it has been demonstrated that, in murine transplantation studies, cells of hematopoietic origin contribute to the early inflammatory and late morrow-repopulating stages of BMP-4-induced heterotopic ossification [Kaplan et al., 2007], these data may provide another evidence of the role of Wnt-5a in the early stages (e.g., inflammation) during bone regeneration.

INTERACTION BETWEEN Wnt AND OTHER PATHWAYS

Bone regeneration is a complex regenerative process, which remains to a great extent an unknown cascade of complex biological events, particularly at the interactions between various intracellular and extracellular molecular signaling pathways. Many signaling pathways are involved in bone regeneration, such as the Wnt/β-catenin pathway, BMP/Smad pathway, Hedgehog pathway, and Notch pathway. Due to BMP's utmost importance in stimulating bone formation, the crosstalk between Wnt and BMP pathway during chondrogenic or osteogenic differentiation has received increasing attentions.

We recently demonstrated that in cultured osteoblasts that had been treated with BMP-2, several Wnt ligands (e.g., Wnt-7a, Wnt-10b) and their receptors (e.g., Fz-1, LRP-10) were upregulated at their mRNA level, together with an increased β-catenin-mediated TCF-dependent transcription [Chen et al., 2007b]. Intramuscular implantation of BMP-2 in mice caused a highly expressed β-catenin and resulted in ectopic endochondral ossification. Blocking Wnt/β-catenin pathway using Dkk-1, or expressing β-catenin null alleles greatly inhibited BMP-2 induction of new bone. These data implied a functional crosstalk between BMP and Wnt signaling pathway [Chen et al., 2007b]. In another study, Fischer et al. [2002b] demonstrated that BMP-2 treatment upregulates expression of Wnt-3a and β-catenin in C3H10T1/2 cells, whereas overexpression of Wnt-3a in this cell lines not only increases total and nuclear levels of both β-catenin and LEF-1, but also leads to enhanced BMP-2-mediated chondrogenesis, an early stage during endochondral ossification and fracture healing. Interestingly, although total Smad-4, a known downstream component in the BMP-2 signaling pathway, do not increase in response to BMP-2, both nuclear levels of Smad-4 as well as the interaction between Smad-4 and β-catenin are enhanced upon BMP-2 treatment, thus providing a direct mechanism for a crosstalk between Wnt and BMP-2 pathway [Fischer et al., 2002a]. Moreover, TCF/LEF-1 as well as β-catenin has been shown to form a complex with Smad-4 [Nishita et al., 2000]. BMP-dependent activation of Msx-2 is also mediated by the cooperative binding of Smad-4 and LEF-1 to the Msx-2 promoter [Hussein et al., 2003]. Thus Wnt signaling pathway may interact with BMP/Smad pathway through various mechanisms.

Hedgehog (Hh) signal is another pathway that is crucial to embryonic skeletogenesis [Day and Yang, 2008]. For example, Indian Hedgehog (Ihh) regulates chondrocyte proliferation and differentiation and is essential for prenatal endochondral bone formation [St-Jacques et al., 1999], and that this pathway is directly required for osteoblast lineage in the endochondral skeleton [Long et al., 2004]. It has been shown that Wnt signaling controls Hh signal transduction through its regulation of Gli2 and Gli3, the key mediators to transduce Hh signals [Ogden et al., 2004], and provides a synergistic effect on surface ectoderm/neural tube and notochord signaling in somite cell specification [Borycki et al., 2000]. Additionally, Suppressor of fused (Su(fu), a negative regulator of the Hh pathway [Ogden et al., 2004]) is present in a complex with the oncogenic transcriptional activator β-catenin and functions as a negative regulator of TCF-dependent transcription [Meng et al., 2001].

Notch pathway is also involved in embryonic skeletogenesis. Mice lacking the gene encoding Notch ligand, Delta-like-3, or Notch signaling mediator, presenilin-1, show defects in the axial skeleton [Shen et al., 1997; Wong et al., 1997; Dunwoodie et al., 2002]. More recently, it is showed that Notch signaling pathway in bone marrow normally acts to maintain a pool of mesenchymal progenitors by

suppressing osteoblast differentiation, in that disruption of this pathway in the limb skeletogenic mesenchyme leads to undetectable mesenchymal progenitors and high bone mass [Hilton et al., 2008]. Among Notch ligands genes, JAG-1 gene is predicted as an evolutionarily conserved target of the canonical Wnt signaling pathway based on the conservation of double TCF/LEF-binding sites within the 5′ promoter region of mammalian JAG-1 orthologues [Katoh, 2006]. Additionally, when expressed in osteoblast cell line (MC3T3), the notch intracellular domain (NICD) impaired differentiation and blocked expression of TCF/LEF-1 target promoter, thus resulting in inhibition of the canonical Wnt pathway [Sciaudone et al., 2003]. NICD also interacts with the highly conserved high-mobility group (HMG) domain of LEF-1 [Ross and Kadesch, 2001] and thereby may have a direct effect on Wnt induced gene expression.

TARGETING Wnt/β-CATENIN PATHWAY AS A THERAPEUTIC APPROACH TO ENHANCE BONE REGENERATION

Bone regeneration for fracture repair and defect healing may be the first major attempted procedure in orthopedic surgery. Although internal fixation devices have already been developed that can successfully achieve short-term stabilization at virtually all orthotopic sites, long-term stability still requires bone fusion or bone augmentation. Autogenous bone graft is a commonly used approach for promoting bone repair, especially for a large-sized defect. However, it can only be performed on a limited scale, and its harvesting can involve substantial donor site morbidity. Allograft bone has potential for antigenicity and disease transmission. Biomaterials have increased infection rate and poor biomechanical properties [Altman et al., 1999]. Currently, BMP-2 and BMP-7 (also known as osteogenic protein 1, OP-1) are being increasingly employed in multiple clinical trials, in that they have been shown to be extremely effective in enhancing bone formation [Chen et al., 2004; De Biase and Capanna, 2005]. However, the requirement of large doses, the short half-life and thus short-term bioavailability of BMPs, and the lack of a practical and suitable method for sustained delivery of these exogenous proteins, have greatly limited the application of BMPs in humans.

The most ideal treatment for bone regeneration would be a pharmacologic agent that is cost-effective and does not require the addition of invasive procedures. Lithium is a suitable pharmacologic agent that can activate β-catenin signaling by inhibiting GSK-3β [Phiel and Klein, 2001; Zhang et al., 2003; Noble et al., 2005]. Previous report showed that lithium enhances bone formation and improves bone mass in mice [Clement-Lacroix et al., 2005]. We recently have demonstrated that oral lithium treatment increases the level of β-catenin in the fracture tissue. Interestingly, mice in which the lithium treatment was started 2 weeks before the fracture have reduced bone in the fracture site, whereas mice in which the lithium treatment was started 4 days after fracture enhances bone healing. Histological examination of the fracture sites showed undifferentiated mesenchymal cells at the fracture site with early lithium treatment, while there is increased bone volume at the fracture site with late treatment. These findings, in combination with our data that fracture repair is inhibited in mice expressing either β-catenin stabilized alleles or osteoblast-specific β-catenin null alleles, but dramatically enhanced in mice expressing osteoblast-specific β-catenin stabilized alleles [Chen et al., 2007a], strongly indicates that lithium therapy to activate Wnt/β-catenin pathway can be used to promote fracture repair. However, since activation of Wnt signaling inhibits differentiation of mesenchymal cells not yet committed to the osteoblastic phenotype, such treatment should be utilized only after cells are committed to the osteoblast lineage.

Oral lithium has been used safely and effectively to treat humans with bipolar disease for over a half-century [Schou, 2001]. Additionally, a separate study that addressed the effects of lithium on fracture risk using a case–control study design revealed that lithium treatment is associated with a decreased risk of fractures, thus potentially pointing at bone-anabolic properties in humans [Vestergaard et al., 2005]. Based on these data, lithium treatment, via its activation of Wnt/β-catenin pathway, can be readily applied in clinical trials for patients with bone healing disorders.

Other GSK-3β inhibitors have been tested in animal models providing evidence for an increased bone mass and improved mechanical properties of bones. For example, oral administration of GSK-3β inhibitor, chemical compound LY6038-31-8, increases expression of several bone-specific genes including collagen-α1 (I) and -α1(V), biglycan, osteonectin, and Runx-2. This GSK-3β inhibitor also increases bone mineral content and bone mineral density in cancellous and cortical bone of ovariectomized rats, and this is associated with improved mechanical properties of lumbar vertebrae [Kulkarni et al., 2006], consistent with a role for the canonical Wnt pathway in osteogenesis.

Besides GSK-3β inhibitors, another alternative approach to activate Wnt/β-catenin pathway for bone regeneration is to antagnoize Dkk, especially Dkk-1. We have demonstrated that Dkk-1 treatment inhibits both chondrogenesis and osteogenesis during bone regenerative process [Chen et al., 2007a,b]. Others reported that Dkk-1 inhibits osteogenesis in cultured MSCs [Gregory et al., 2005]. In addition, anti-Dkk-1 strategies are also closely relevant to clinical oncology since high serum levels of Dkk-1 are thought to contribute to osteolytic lesion formation in multiple myeloma and possibly some forms of osteosarsoma [Tian et al., 2003].

CONCLUSIONS AND FUTURE DIRECTIONS

During the past decade, Wnt signaling pathway has been found to play a central role in controlling embryonic development from hydra to human. The most extensively studied Wnt pathway is the canonical Wnt pathway, which controls gene expression by stabilizing β-catenin in regulating a diverse array of biological processes. Extensive studies on embryogenesis have demonstrated that Wnt pathway, especially the canonical Wnt pathway, is essential for embryonic bone development, including limb bud initiation, early limb patterning, and late limb morphogenesis events. Wnt pathway has also been shown to play a major in bone mass regulation. Recently, we and others provide evidences that Wnt pathway is crucial to bone regenerative process. For example, Wnt/β-catenin signaling is required for both BMP-2-induced ectopic endochondral ossification and fracture repair. This canonical

pathway also plays an essential and disparate role in different phases of fracture repair. More importantly, pharmacological agents (e.g., lithium) targeting Wnt/β-catenin pathway may provide a new therapeutic approach for clinical bone augmentation. However, since activation of Wnt/β-catenin pathway inhibits differentiation of mesenchymal cells not yet committed to the osteoblastic phenotype, such treatment should be utilized only after these cells are committed to the osteoblast lineage. In addition, although not well understood, non-canonical Wnt pathway is also involved in bone regeneration, suggesting that Wnt signaling in bone regeneration is complex, potentially involving activation of both canonical and non-canonical Wnt pathways.

So far, our knowledge concerning how Wnt pathway regulates bone regeneration is still at its infancy, and there are many issues remain to be addressed. For example, what is the role of non-canonical Wnt pathway in bone regeneration? Which of the Wnts are the critical ligands to mediate canonical or non-canonical Wnt pathway in bone regeneration? What is the interaction between canonical and non-canonical Wnt pathway in bone formation? What about the specificity of interaction between Wnt ligands and Fzs receptors? What is the functional distinction between LRP-5 and LRP-6? What is the effect of Wnt target genes on osteoblast differentiation? These questions are topics of investigation that are being actively studied to better understand bone formation. Moreover, bone homeostasis requires balanced interplay between osteoblasts and osteoclasts. So far, the regulation of Wnt on osteoblasts has been extensively investigated, but the role of Wnt on osteoclasts is poorly understood, and this will be another important hot area in future research. In addition, bone regeneration is a complex process involving multiple molecular signaling pathways, thus another attention should focus on how these pathways interact with Wnt signal. In this review, we briefly reviewed the crosstalk between Wnt and BMP pathway, Hh pathway, and Notch pathway. There is an abundance of data from developmental and cellular models suggest that Wnt pathway interacts with several other pathways such as fibroblast growth factor (FGF) pathway. Obtaining a further understanding of how these pathways affect Wnt signaling during bone regeneration may provide knowledge for combined therapeutic interventions.

In general, based on recent discoveries, despite the fact that not all underlying mechanisms are well understood yet, Wnt signaling pathway indeed plays a crucial role in bone regenerative process, such as ectopic bone formation and fracture repair. Modulation of Wnt pathway using pharmacological agents (e.g., lithium) may provide a promising therapeutic approach to improve bone regeneration.

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