Noncanonical Wnt-4 Signaling Enhances Bone Regeneration of Mesenchymal Stem Cells in Craniofacial Defects through Activation of p38 MAPK

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Mesenchymal stem cells (MSCs) are multipotent cells that can be differentiated into osteoblasts and provide an excellent cell source for bone regeneration and repair. Recently, the canonical Wnt/β-catenin signaling pathway has been found to play a critical role in skeletal development and osteogenesis, indicating that Wnts can be utilized to improve de novo bone formation mediated by MSCs. However, it is unknown whether noncanonical Wnt signaling regulates osteogenic differentiation. Here, we find that Wnt-4 enhanced in vitro osteogenic differentiation of MSCs isolated from human adult craniofacial tissues and promoted bone formation in vivo. Whereas Wnt-4 did not stabilize β-catenin, it activated p38 MAPK in a novel noncanonical signaling pathway. The activation of p38 was dependent on Axin and was required for the enhancement of MSC differentiation by Wnt-4. Moreover, using two different models of craniofacial bone injury, we found that MSCs genetically engineered to express Wnt-4 enhanced osteogenesis and improved the repair of craniofacial defects in vivo. Taken together, our results reveal that noncanonical Wnt signaling could also play a role in osteogenic differentiation. Wnt-4 may have a potential use in improving bone regeneration and repair of craniofacial defects.

Human adult mesenchymal stem cells (MSCs)² are multipotent cells originally isolated from the bone marrow that are able to differentiate into several cell types, including osteoblasts, chondrocytes, adipocytes, and myocytes (1–5). Because of their osteogenic potential, MSCs could be potentially used for repairing critical size bone defects that normally cannot undergo spontaneous healing (2, 3). Growing evidence has suggested that MSCs also exist in a number of nonmarrow tissues. For example, MSCs isolated from adipose tissue and muscle express some common cell surface markers that are identified in marrow MSCs from bone marrow and can differentiate into a variety of cell types, including osteoblasts, muscle cells, and neural cells (6–8). Recently, based on the primary characteristics of MSCs from bone marrow, we have isolated MSCs from craniofacial tissues, such as the dental pulp and periodontal ligament (9, 10). Like MSCs isolated from bone marrow, these cells are self-renewing, multipotent, and clonal. They can be induced to differentiate into osteoblast-like cells and adipocytes in vitro. When implanted into immunodeficient mice, these cells formed mineralized tissues or related craniofacial structures, suggesting that they can be used in the replacement of tooth and craniofacial (9, 10).

Although bone regeneration mediated by MSCs shows potential utility, the regenerative capacity of these cells is relatively low when taking into account the number of cells utilized for transplantation in vivo. Moreover, critical size defects in patients often require a large number of MSCs. To obtain enough MSCs for cell transplantation, MSCs must be expanded ex vivo (2). However, the potential problem for ex vivo expansion is that MSCs have a limited life span and may gradually lose their osteogenic potential. To overcome these potential problems, several strategies have been explored. We and others have found that overexpression of telomerase significantly extended the life span of MSCs and enhanced bone formation potential of MSCs both in vitro and in vivo (11). A variety of growth factors that promote osteogenic differentiation have also been utilized to enhance bone regeneration mediated by MSCs. For example, studies by Peng et al. (7) have demonstrated that vascular endothelial growth factor and bone morphogenetic protein (BMP)-4 could significantly improve bone formation and healing by MSCs isolated from muscle.

The canonical Wnt/β-catenin signaling pathway has recently been found to play an essential role in bone development and postnatal maintenance of bone mass (12–20). Since Wnts are secreted growth factors, they may potentially be utilized as recombinant factors to improve bone regeneration.
There are 19 Wnt family proteins in total that are primarily divided into two main categories based on their role in cytosolic β-catenin stabilization: canonical and noncanonical (21–24). Canonical Wnts transduce their signals through intracellular β-catenin. In the absence of Wnt proteins, β-catenin is associated with a cytoplasmic complex containing adenomatous polyposis coli, glycogen synthase kinase-3β, Axin, and casein kinase 1. In this complex, glycogen synthase kinase-3β constitutively phosphorylates β-catenin, resulting in its ubiquitination and the degradation by the 26S proteasome. In the presence of Wnt stimulation, a frizzled receptor and the Wnt co-receptor LRP5 (low density lipoprotein receptor-related protein 5) or LRP6 transduce signals to inhibit Axin/adenomatous polyposis coli/glycogen synthase kinase-3β activity. This inhibition leads to the accumulation of free cytosolic β-catenin. The elevated cytosolic β-catenin can translocate to the nucleus, form a complex with members of the Tcf family of transcription factors, and activate the expression of Wnt target genes (21–24). In contrast, noncanonical Wnts transduce their signal independent of β-catenin. The noncanonical Wnt signaling pathway has been found to be associated with gastrulation movements, heart induction, dorsoventral patterning, tissue separation, and neuronal migration (21). Unlike the canonical Wnt signaling pathway, the noncanonical Wnt signaling pathway is quite diverse. It has been reported that noncanonical Wnts can activate calcium flux, G proteins, Rho GTPases, or c-Jun N-terminal kinase (JNK) (21, 25, 26).

Initial observations on the association between human LRP5 gene mutation and osteoporosis-pseudoglioma syndrome have provided a connection between Wnt signaling and bone formation (13, 14). Subsequently, studies from knock-out mice have demonstrated that the canonical Wnt/β-catenin signaling pathway plays a critical role in bone formation (15–20). This pathway might stimulate bone formation through several molecular mechanisms, including stem cell renewal, the stimulation of preosteoblast proliferation and/or differentiation, and the inhibition of apoptosis (15–20). These important discoveries provide a molecular basis to explore whether targeting Wnt/β-catenin signaling can improve the efficiency of bone regeneration by MSCs (27–30). Although some studies demonstrated that the activation of the Wnt/β-catenin signaling pathway promoted osteogenic differentiation, paradoxically, a number of works recently claimed that Wnt/β-catenin signaling inhibited osteogenic differentiation and mineralization of MSCs in vitro (31–37). For example, Boer et al. (31) and Boland et al. (32) have demonstrated that the canonical Wnt-3a and Wnt-1 inhibited osteogenic differentiation and promoted proliferation of MSCs in vitro. Cho et al. (33) found that Wnt/β-catenin signaling promoted proliferation and suppressed osteogenic differentiation of human adipose-derived MSCs. However, the underlying mechanism that is responsible for the inhibition of osteogenic differentiation of MSCs is not clear. Nevertheless, these findings suggest at least that, although canonical Wnt signaling plays a critical role in bone development, targeting Wnt/β-catenin may not be useful for improving osteogenic potential of MSCs for bone tissue engineering. During the course of studying the role of Wnts in craniofacial bone regeneration, consistent with the studies of Boer et al. (31), we also observed that several canonical Wnts strongly inhibited osteogenic differentiation of MSCs in vitro and in vivo (3). Unexpectedly, we identified that Wnt-4, a noncanonical Wnt member, played a novel role in promoting osteogenic differentiation of MSCs isolated from craniofacial tissues. Using two different models of craniofacial bone injury, we found that MSCs that were genetically engineered to express Wnt-4 exhibited robust bone formation capacities and improved the repair of craniofacial bone defects. We found that p38 MAPK was activated by Wnt-4 in a novel noncanonical signaling pathway in MSCs and played a critical role in enhancing osteogenic differentiation of MSCs. Our results suggest that Wnt-4 may be potentially utilized for enhancing the properties of MSCs in craniofacial bone regeneration and repair.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Retroviral Infection**—C2C12 cells were purchased from ATCC and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 15% fetal bovine serum. MSCs from human periodontal ligaments were grown as described previously. BMP4 was purchased from R & D Systems. To engineer C2C12 cells or MSCs stably expressing Wnt-4, the retrovirus-mediated infection was performed as described previously (38). Briefly, retroviruses were produced by transfecting the retroviral vector encoding Wnt-4 or control vector into 293T cells by the calcium phosphate method. Forty-eight h after transfection, retrovirus-containing supernatants were collected, filtered with 0.45-μm filters, and stored in −70 °C. C2C12 cells or MSCs were infected with retroviruses in the presence of 6 μg/ml Polybrene (Sigma). Forty-eight h after infection, cells were selected with neomycin (2 μg/ml) for 10 days. The surviving cells were pooled, and cells expressing Wnt-4 were confirmed by Western blot analysis.

**Western Blot Analysis**—Cells were either untreated or treated with BMP4 (50 ng/ml) and then harvested and washed once with PBS. Cells were pelleted and lysed with cell lysis buffer containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, and 1:100 protease inhibitor mixtures (Sigma). The protein concentration was measured according to the manufacturer’s instruction (Bio-Rad). 30–50 aliquots of protein extracts were subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad) using a semidy gel transfer cell. The membranes were blocked with 5% nonfat milk overnight at 4 °C and then probed with the primary antibodies. The immunocomplexes were visualized with horseradish peroxidase–coupled goat anti-rabbit or anti-mouse IgG (Promega) using the SuperSignal reagents (Pierce), as described previously (38). The primary antibodies were from the following sources: anti-α-tubulin monoclonal antibodies from Sigma; anti-HA monoclonal antibodies were from Convance; anti-β-catenin monoclonal antibodies were from Clontech; anti-phospho-p38 polyclonal antibodies were from Cell Signaling; and anti-p38 and anti-Axin polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

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Northern Blot Analysis—Cells were lysed with TRIzol reagent (Invitrogen). Total RNAs were extracted according to the manufacturer’s protocol. 5–10 μg aliquots of RNA samples were separated on a 1.4% agarose-formaldehyde gel and transferred onto a nylon membrane for 16–24 h (Bio-Rad). RNAs were cross-linked with a UV cross-linker (Promega). Blots were prehybridized with PerfectHyb (Sigma) buffer containing salmon sperm DNA (100 μg/ml) for 20 min and then hybridized with PerfectHyb buffer containing 32P-labeled osterix (Ox), type I collagen, or glyceraldehyde-3-phosphate dehydrogenase cDNA probes. The probes were labeled with a random primed labeling kit (Amersham Biosciences) in the presence of [α-32P]dCTP (ICN). The probes were purified with a micro-G50 Sephadex column (Amersham Biosciences). After hybridization, the blots were washed twice in 2× SSC, 0.1% SDS for 10 min at room temperature and twice in 0.1× SSC, 0.1% SDS for 20 min at 42 °C, as described previously (38).

Alkaline Phosphatase (ALP), von Kossa, Alizarin Red Staining, and Transplantation in Nude Mice—Cells were grown in differentiation-inducing media containing 100 μg/ml ascorbic acid, 5 mM β-glycerophosphate, and 1 μM dexamethasone. After 1–7 days, cells were fixed with 70% ethanol and stained with an ALP staining kit according to the manufacturer’s protocol (Sigma). For detecting mineralization, cells were induced for 2–3 weeks, fixed with 70% ETOH, and stained with 2% alizarin red or 1% silver nitrate (Sigma).

Five weeks postsurgery, rats were sacrificed by CO2 euthanasia, and the mandibulae were harvested, immediately fixed in 10% neutral buffered formalin for 48 h, and then scanned by microcomputed tomography (μCT) (model Pxs5–928EA; GE Healthcare, London, Canada) to evaluate osseous structures. The mandibulae were subsequently decalcified with a 10% EDTA solution for ~2–3 weeks, dehydrated with gradient alcohols, and embedded in paraffin. Coronal sections ~5 μm in thickness were cut and stained with hematoxylin and eosin. μCT scanning produced three-dimensional images, allowing for detailed visualization and analysis of hard tissue architecture and anisotropy. The samples were scanned at 18 μm resolution for mineralized tissue with accelerated potential of 80 kV and a beam current of 80 μA. The eXplore Microview version 2.0 software provides image analyzing tools with coronal, sagittal, and transversal views two- and three-dimensionally. Linear distances of roots were measured (mm) from the root furcation to root apex on the first molar (M1) distal root of the buccal side of mandibula by a single calibrated examiner. The μCT can create sections with 18-μm thickness from a three-dimensionally reconstructed image. Tooth fusions and root apices were determined through a coronal view, and the number of frames was counted. The exposed root length was measured between furcation and the root apex. The fractions of bone-covered root surface were calculated with the fraction exposed root length/total root length, as previously described (44–46). All results from the scans were statistically calculated with SPSS version 12 (SPSS Inc. Chicago, IL) with a statistical significance at p < 0.05.

Craniofacial Defect Model—Eighteen 5-week-old female SCID mice (N:NIH-bg-nu-xid; Charles River Laboratories, Raleigh, NC) were divided into three groups randomly. Animals were anesthetized with intraperitoneal injections of ketamine and xylazine. A linear scalp incision was made from the nasal bone to the occiput, and full-thickness flaps were elevated. The periosteum overlying the calvarial bone was completely resected. A trephine was used to create a 5-mm craniotomy defect centered on the sagittal sinus, and the wounds were copiously irrigated with Hanks’ balanced salt solution while drilling. The calvarial disk was removed carefully in order to avoid injury to the underlying dura or brain. After careful hemostasis, polymeric scaffolds previously loaded with 5 × 106 cells were placed into the defects. The scaffolds filled the entire defect and attached the bone edges around the entire periphery. The incisions were closed with 4-0 Chromic Gut suture (Ethicon/Johnston & Johnson, Sommerville, NJ), and the mice recovered from anesthesia on a heating pad. All mice were sacrificed 5 weeks after the implantation (47). Calvaria were harvested, immediately fixed in 10% neutral buffered formalin for 48 h, and then scanned for μCT analysis. Calvaria were subsequently decalcified with a 10% EDTA solution for ~2–3 weeks, dehydrated with gradient alcohols, and embedded in paraffin. Coronal sections ~5 μm in thickness were cut and stained with hematoxylin and eosin.

For μCT scanning, the specimens were fitted in a cylindrical sample holder, 15.4 mm in diameter, with the coronal aspect of the calvarial bone in a horizontal position. Specimens were scanned with the scanning direction parallel to the coronal
Osteogenic Differentiation of Mesenchymal Cells Enhanced by Wnt-4—C2C12 mouse mesenchymal cells are well-characterized cells that can be induced to differentiate into osteoblast-like cells in vitro following BMP stimulation (38). Since Wnt/β-catenin signaling has been found to play an essential role in osteoblast differentiation, we screened several Wnts to determine if they could enhance BMP-4-induced osteogenic differentiation of C2C12 cells. C2C12 cells were engineered to express Wnt-4 by retroviral transduction as described previously (39). As shown in Fig. 1A, Western blot analysis detected ectopic Wnt-4 from the cell lysates of C2C12 cells expressing Wnt-4 (C2C12/Wnt-4), but not from control cells expressing empty vector (C2C12/V). Also, Wnt-4 could be detected from the conditioned media from C2C12/Wnt-4 cells, but not C2C12/V cells, suggesting that Wnt-4 could be secreted (data not shown). To determine whether Wnt-4 promoted osteogenic differentiation, both C2C12/V and C2C12/Wnt-4 cells were stimulated with BMP-4. The induction of ALP activity by BMP-4, an early marker for osteogenic differentiation (40, 41), was significantly enhanced in C2C12/Wnt-4 cells compared with C2C12/V cells (Fig. 1B). Northern blot analysis revealed that Wnt-4 and BMP-4 also synergistically induced the expression of Osterix, a specific transcription factor for osteoblast differentiation (38) (Fig. 1C). Consistently, we found that Wnt-4 also enhanced the expression of type I collagen induced by BMP-4.

Wnt-4 Enhances Osteogenic Differentiation of MSCs Isolated from Human Craniofacial Tissues—Recently, we isolated several MSCs from human adult oral and craniofacial tissues, such as periodontal ligament and dental pulp. These cells are capable of forming bone-like tissues following in vitro transdifferentiation, suggesting that they may be utilized to repair craniofacial bone defects (9, 10). Therefore, we explored whether Wnt-4 could enhance osteogenic differentiation of MSCs from periodontal ligament. MSCs were also engineered to express Wnt-4 using retrovirus-mediated transduction. As shown in Fig. 2, Western blot analysis confirmed that MSCs stably expressed Wnt-4. To induce osteogenic differentiation of MSCs, both MSCs expressing Wnt-4 (MSC/Wnt-4) and control cells (MSC/V) were treated with differentiation-inducing medium containing ascorbic acid, β-glycerophosphate, and dexamethasone. As shown in Fig. 2B, ALP activities were more significantly induced in MSC/Wnt-4 cells than in MSC/V cells upon stimulation with differentiation-inducing medium. Northern blot analysis and Western blot analysis also found that Wnt-4 enhanced the expression of specific bone matrix proteins, type I collagen and OCN (Fig. 2, C and D). von Kossa staining revealed that Wnt-4 potentiated mineralization of MSCs in vitro (Fig. 2E). Of note, Wnt-4 expression did not significantly affect cell proliferation during the induction of cell differentiation or cell death (supplemental Fig. 1).

Wnt-4 Enhances Osteogenic Formation of MSCs in Vivo—We have previously found that transplants of MSCs mixed with a hydroxyapatite/tricalcium phosphate carrier could generate mineralized tissues in immunodeficient mice, although their osteogenic potentials were relatively weaker compared with MSCs from human bone marrow (9, 10). Thus, we examined whether Wnt-4 could enhance the formation of mineralized tissues in vivo. Both MSC/Wnt-4 and MSC/V cells were implanted into nude mice for 6 weeks. In these experiments, MSC/Wnt-4 cells formed more mineralized tissues than MSC/V cells in vivo (Fig. 3, A, D, and F). Immunostaining revealed that the osteoblast-like cells lining the surface of mineralized tissues reacted with human specific mitochondrial antibodies, suggesting that the mineralized tissues were formed by the transplanted MSCs (Fig. 3, B and E).
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**Activation of the Noncanonical Wnt Signaling Pathway by Wnt-4**—Since the canonical Wnt signaling pathway regulates skeletal development through β-catenin, we first examined whether or not Wnt-4 could enhance osteogenic differentiation by increasing the cytosolic level of β-catenin. Subcellular fractionation was performed to determine the cytosolic level of β-catenin, as described previously. As shown in Fig. 4A, the cytosolic level of β-catenin was identical in both C2C12/Wnt-4 and C2C12/V cells. Similarly, we found that Wnt-4 did not increase the cytosolic level of β-catenin in MSCs under uninduced and induced conditions (Fig. 4B). As a positive control, overexpression of Wnt-1 in MSCs significantly increased the cytosolic level of β-catenin (Fig. 4B). Additionally, Wnt-4 did also not induce the transcription activity of β-catenin/Tcf as determined by Topflash luciferase reporter assays (supplemental Fig. 2). These results suggest that Wnt-4 might promote osteogenic differentiation of MSCs independent of β-catenin.

We further screened other signaling pathways associated with osteogenic differentiation that were activated by Wnt-4. Since JNK signaling can be activated by the noncanonical Wnts (26), we first examined whether Wnt-4 could potentiate JNK activation induced by BMP-4 in C2C12 cells. We found that BMP-4 and Wnt-4 negligibly induced JNK activation in C2C12 cells (data not shown). In contrast, as shown in Fig. 4C, the basal level of p38 phosphorylation was significantly higher in C2C12/Wnt-4 cells than in C2C12/V cells, as determined by Western blot analysis (Fig. 4C; compare lane 1 with lane 5). Moreover, BMP-4 induced p38 phosphorylation to a greater extent in C2C12/Wnt-4 cells when compared with C2C12/V cells. We also observed that the basal level of p38 activity in MSC/Wnt-4 cells was higher than that in MSC/V cells. Upon the addition of differentiation-inducing medium, p38 phosphorylation in MSC/Wnt-4 cells was further enhanced as compared with MSC/V cells (Fig. 4D).

Our results suggest that Wnt-4 might directly stimulate p38 activation to promote osteogenic differentiation. Given that the purified recombinant Wnt-4 proteins are not available, we could not directly stimulate MSCs with Wnt-4 to examine p38 activation. To overcome this problem, we immunodepleted Wnt-4 proteins from the conditioned media from C2C12/Wnt-4 cells with anti-HA monoclonal antibodies, since Wnt-4 was HA-tagged (Fig. 4E). As shown in Fig. 4F, although the Wnt-4-conditioned media immunodepleted with control IgG retained the capacities to strongly induce p38 phosphorylation, the removal of Wnt-4 from the conditioned media abolished p38 phosphorylation in MSCs. These results suggest that Wnt-4-conditioned media did not contain the inducible secreted factors that could stimulate p38. Since the activation of p38 was rapidly induced in 5–10 min by the Wnt-4-conditioned media, it was likely that Wnt-4 directly activated p38. Additionally, we found that the conditioned media from MSC/Wnt-4 cells also strongly induced p38 phosphorylation in MSCs. The removal of Wnt-4 from the conditioned media also eliminated p38 activation in MSCs (data not shown). These results suggest that Wnt-4 can activate the noncanonical p38 pathway in MSCs.

Previously, it has been reported that the overexpression of Axin induced the activation of JNK (26). Because JNK and p38 are two closely related MAPK pathways (42, 43), although...
Wnt-4 did not activate JNK at MSCs, we explored whether Axin played a role in Wnt-4-mediated p38 activation. As shown in Fig. 4G, overexpression of Axin strongly induced p38 phosphorylation. Since C2C12 cells were more readily transfected with small interference RNA (siRNA) than MSCs, we also utilized siRNA to knock down Axin to determine if endogenous Axin played a role in p38 activation induced by Wnt-4. As shown in Fig. 4H, we were able to partially reduce Axin expression over 60%. This partial reduction of Axin significantly reduced p38 phosphorylation induced by Wnt-4-conditioned media (Fig. 4I). Taken together, our results suggest that Wnt-4 signals through Axin to activate p38.

To determine if the activation of p38 played a role in the potentiation of osteogenic differentiation induced by Wnt-4, we utilized a specific p38 inhibitor, SB-203580, to suppress p38 activity. Western blot analysis demonstrated that SB-203580 not only inhibited low p38 activity induced by BMP4 but also abolished enhancing p38 activity stimulated by both BMP-4 and Wnt-4 (Fig. 5A). The ALP activity assay revealed that the inhibition of p38 by SB-203580 significantly attenuated the increased ALP activity induced by BMP-4 and Wnt-4 (Fig. 5B). Moreover, SB-203580 also abolished p38 phosphorylation induced by Wnt-4-conditioned media in MSCs (Fig. 5C). Similarly, the treatment with SB-203580 suppressed ALP activity and mineralization in MSC/Wnt-4 cells upon the stimulation of differentiation-inducing media (Fig. 5D). Of note, SB-203580 did not significant affect cell proliferation and cell death during the induction of cell differentiation. Taken together, our results suggest that Wnt-4 activated a new noncanonical p38 signaling pathway to enhance osteogenic differentiation independent of β-catenin.

Wnt-4 Promotes Healing of Periodontal Bone Defects—Chronic periodontitis is a common inflammatory disease that often destroys alveolar bone and periodontal tissues, resulting in loss of tooth support (44–46). To date, there are no effective therapies to restore alveolar bone loss. Since our MSCs were originally isolated from human periodontal ligament, we sought to determine whether MSCs engineered to express Wnt-4 could promote the repair of periodontal bone defects. As described in Fig. 3, we have found that Wnt-4-expressing MSCs generated significantly more bonelike tissue in mice than MSC/V cells after mixing with hydroxyapatite/tricalcium phosphate. Toward clinical application, we tested whether using polymer scaffolds seeded with transplanted MSCs, which can be conveniently handled in the clinical setting to deliver MSCs, could also generate bonelike tissues. A nude rat model of periodontal bone defect was utilized as previously described (44–46). The periodontal ligament and alveolar bone were removed from the mesial and distobuccal roots of the mandibular first molar and the mesiobuccal root of the mandibular second molar teeth within the defect area. Polylactic co-glycolide polymer scaffolds were seeded overnight with MSC/Wnt-4 or MSC/V cells and placed within the respective defects. Five weeks after operation, rats were sacrificed, and mandibulae were subjected to μCT and histological analysis. Minimal healing was observed in the control group, whereas extensive periodontal bone regeneration was found in the group implanted with MSC/Wnt-4 cells (Fig. 6A). Specifically, alveolar bone healing with complete bridging was seen only in the MSC/Wnt-4 group. Importantly, the newly formed bone tissues were well vascularized, and no significant inflammatory reaction was detected in these tissue areas. μCT analysis revealed that MSC/Wnt-4 cells generated 3–5-fold greater alveolar bone than MSC/V cells (Fig. 6B).
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**DISCUSSION**

Our results reported here are the first demonstration that Wnt-4, a noncanonical member of Wnt family proteins, potently enhanced osteogenic differentiation of MSCs isolated from human adult craniofacial tissues in vitro and bone formation in vivo. Intriguingly, whereas Wnt-4 did not increase the cytosolic level of β-catenin, we revealed that Wnt-4 activated a novel noncanonical signaling pathway, p38 MAPK, which is known to positively regulate osteogenic differentiation induced by BMPs and other growth factors (42, 43). The siRNA knockdown of Axin significantly reduced p38 activation induced by Wnt-4, suggesting that Wnt-4 signals through Axin. The inhibition of p38 abolished osteogenic differentiation of MSCs promoted by Wnt-4. Moreover, using two different models of craniofacial bone injury, MSCs genetically engineered to express Wnt-4 significantly regenerated more bone tissues and promoted the repair of craniofacial bone defects in vivo. Our results suggest that Wnt-4 may have a novel application in improving bone regeneration and repair for craniofacial bone defects.

Recent work from several groups has elucidated the importance of the canonical Wnt/β-catenin signaling pathway in the skeletal development and the postnatal maintenance of bone new bone nodules were observed (Fig. 7B). Moreover, μCT analysis revealed that the bone mineral density values of new bone at the calvarial defect generated by MSC/Wnt-4 cells were significantly higher than that by MSC/V cells (178.00 ± 13.18 versus 138.20 ± 15.10 mg/cm³; p < 0.01), suggesting that Wnt-4 can strongly enhance mineralization of MSCs in large calvarial defects (Table 1).
undifferentiated state, thereby inhibiting their osteogenic differentiation. Nevertheless, these studies strongly suggest that targeting the canonical Wnt/β-catenin signaling pathway to improve osteogenic potential of MSCs may require further investigation.

A number of Wnts have been identified to be expressed in bone or to be associated with bone development. For example, Wnt-1, Wnt-4, Wnt-7, and Wnt-14 have been found to be expressed by osteoblasts, and Wnt-1 and Wnt-3a are induced by BMP in MSCs (12, 16, 29). We also found that Wnt-4 was induced during osteogenic differentiation of MSCs (supplemental Fig. 3). However, it should be noted that endogenous Wnt-4 expression was induced at a fairly late stage during MSC differentiation. Thus, it is likely that other endogenous Wnt(s) may regulate osteogenic differentiation of the MSCs. Although the canonical Wnt/β-catenin signaling pathway has been extensively examined in bone, little is known about whether or not the noncanonical Wnt signaling pathway plays a role in skeletal development and the postnatal maintenance of bone mass. Moreover, results from studies with Dkk2 knockout mice suggest that regulation of bone formation by Wnt signaling is more complex (37). Dkk2 is known to be an inhibitor of Wnt signaling and is induced by Wnts as one of the feedback mechanisms to attenuate Wnt stimulation. Interestingly, Dkk2−/− mice have increased secreted bone matrix but impaired mineralization, resulting in an osteopenic phenotype (37). Our studies demonstrated that Wnt-4 enhanced osteogenic differentiation of MSCs, suggesting that noncanonical Wnts may also play a role in osteoblast differentiation and bone formation. We observed that Wnt-4 induced p38 activation to promote osteogenic differentiation. p38 activation is a known pathway that is associated with osteogenic differentiation (42, 43). Using the combination of the conditioned media and an immunodepletion approach, we demonstrated that Wnt-4 could directly activate p38 through its receptor. First, the Wnt-4-conditioned medium rapidly stimulated p38 activation in 5–10 min, indicating that it was unlikely that Wnt-4 induced a new protein(s) to stimulate p38 activation through autocrine mechanisms. Unfortunately, we were unable to use
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FIGURE 6. Wnt-4 promotes healing periodontal alveolar bone defects mediated by MSCs in vivo. A, Wnt-4 promoted the repair of alveolar bone defect mediated by MSCs in vivo. Polyactic co-glycolide polymer scaffolds were seeded overnight with MSC/V cells or MSC/Wnt-4 cells and transplanted into mandibular osseous defects. Five weeks following surgery and cell transplantation, the biopsies were harvested for histology and μCT analysis. The left panel shows coronal slide orientations of MSC/Wnt-4 and MSC/V cell treatments of the defect at low power (2×) magnification; the middle panel shows high power (10×) magnification of corresponding treatment groups; and the right panel shows μCT images of corresponding treatment groups. After 5 weeks, minimal healing was observed in the control group, whereas extensive periodontal bone regeneration was found in the group with MSC/Wnt-4 cell treatment. Specifically, alveolar bone healing with complete bridging was seen only in the MSC/Wnt-4 group. The arrows indicate the borders of the osseous defects. In the μCT panel, the red color indicates tooth crowns (yellow, tooth roots; blue, newly regenerated alveolar bone). B, linear measures of alveolar bone repair by μCT. Linear measures of alveolar bone repair on the exposed root surfaces were assessed by μCT. The values reveal the amount of alveolar bone regeneration in mm on the bone surfaces as measured from μCT scans of the biopsies. Results from analysis of variance showed that the fractions were significantly different between the two groups (*, p = 0.011).

Axin was overexpressed (26). Since p38 also is downstream of MEKK1, it is possible that Wnt-4 may stimulate interaction between Axin and MEKK1 and thereby activate p38 in MSCs.

The isolation of MSCs from various tissue sources, including dental and craniofacial tissues, has provided an excellent therapeutic approach for bone tissue regeneration and repair (5–10). The bone loss in dental, oral, and craniofacial regions is very common due to injuries, infection, and cancer-associated damage. To date, restoration of these defects or damaged tissues has mainly relied on implanting structural substitutes, often with little or no reparative potential. Our previous work has suggested that MSCs isolated from periodontal ligament have a potential to repair periodontal tissue damage (9). Although we were able to demonstrate that MSCs attached to the surface of alveolar bone and teeth when implanted in the defect regions, minimal alveolar bone repair was detected in a nude rat model (9). In this study, we found that MSCs engi-
neered to express Wnt-4 regenerated more bone tissues and repaired large alveolar bone defects when compared with MSCs alone. Importantly, the new bone tissues generated by MSC/Wnt-4 cells were well integrated with the host bones at the wound margins in both periodontal and calvarial bone defect models. Although new bone was well vascularized, we did not observe that Wnt-4 induced inflammatory cell infiltrations. It has been reported that vascular endothelial growth factor enhanced bone formation and promoted recruiting mesenchymal stem cells (7). Currently, we do not know whether Wnt-4 also helps to recruit MSCs from the host to promote bone repair in vivo. Wnt-4 has been reported to play a role in blood vessel development. Therefore, it was possible that Wnt-4 might enhance bone regeneration by promoting angiogenesis in vivo, which could further enhance bone formation. We observed that there was abundant bone marrow present in the new bones generated by MSC/Wnt-4 cells, which was quite different from bone tissue generated by other approaches in which bone marrows was barely observed. Our results suggest that Wnt-4 may promote MSCs to form a suitable microenvironment resembling natural bone and therefore recruit hematopoietic cells to generate an entire bone/bone marrow structure.

Finally, it should be pointed out that Wnt signaling has been found to be associated with tumor development. The abnormal activation of the canonical Wnt/β-catenin signaling pathway causes several human cancers, including colorectal cancers and melanoma (21–23, 39). Although the canonical Wnt signaling pathway plays an essential role in skeletal development and postnatal maintenance of bone mass, the overactivation of this pathway for tissue engineering may have a risk in increasing tumorigenesis. According to our studies, noncanonical Wnts may also play a role in bone formation. Given the fact that noncanonical Wnts activate diverse signaling pathways, it is possible that they may utilize different mechanisms to stimulate osteogenic differentiation. It is worth investigating whether other noncanonical Wnts also activate p38 to stimulate osteogenic differentiation. Importantly, we have observed that MSCs engineered to express Wnt-4 did not form tumors in nude mice. Taken together, our results suggest that Wnt-4 may be utilized for improving craniofacial bone regeneration and repair.

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