Connective Tissue Growth Factor (CTGF) Is Regulated by Wnt and Bone Morphogenetic Proteins Signaling in Osteoblast Differentiation of Mesenchymal Stem Cells*

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Osteoblast lineage-specific differentiation of mesenchymal stem cells is a well regulated but poorly understood process. Both bone morphogenetic proteins (BMPs) and Wnt signaling are implicated in regulating osteoblast differentiation and bone formation. Here we analyzed the expression profiles of mesenchymal stem cells stimulated with Wnt3A and osteogenic BMPs, and we identified connective tissue growth factor (CTGF) as a potential target of Wnt and BMP signaling. We confirmed the microarray results, and we demonstrated that CTGF was up-regulated at the early stage of BMP-9 and Wnt3A stimulations and that Wnt3A-regulated CTGF expression was β-catenin-dependent. RNA interference-mediated knockdown of CTGF expression significantly diminished BMP-9-induced, but not Wnt3A-induced, osteogenic differentiation, suggesting that Wnt3A may also regulate osteoblast differentiation in a CTGF-independent fashion. However, constitutive expression of CTGF was shown to inhibit both BMP-9- and Wnt3A-induced osteogenic differentiation. Exogenous expression of CTGF was shown to promote cell migration and recruitment of mesenchymal stem cells. Our findings demonstrate that CTGF is up-regulated by Wnt3A and BMP-9 at the early stage of osteogenic differentiation, which may regulate the proliferation and recruitment of osteoprogenitor cells; however, CTGF is down-regulated as the differentiation potential of committed pre-osteoblasts increases, strongly suggesting that tight regulation of CTGF expression may be essential for normal osteoblast differentiation of mesenchymal stem cells.

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Osteoblast lineage-specific differentiation from the pluripotent mesenchymal stem cells is a well orchestrated process (1–3). Although not well understood, bone morphogenetic proteins (BMPs)1 play an important role in regulating osteoblast differentiation and subsequent bone formation (4, 5). At least 15 types of BMPs have been identified in humans (6–8). BMPs belong to the TGF-β superfamily and play an important role in development (6, 9). Genetic disruptions of BMPs have resulted in various skeletal and extraskeletal abnormalities during development (10). Through a comprehensive analysis of the osteogenic activity of the 14 types of human BMPs, we have recently demonstrated that BMP-2, BMP-6, and BMP-9 are the most potent inducers of osteogenic differentiation both in vitro and in vivo (11, 12). BMPs fulfill their signaling activity by interacting with the heterodimeric complex of two transmembrane serine/threonine kinase receptors, BMPR type I and BMPR type II (13, 14). The activated receptor kinases phosphorylate the transcription factors Smads 1, 5, or 8, which in turn form a heterodimeric complex with Smad4 in the nucleus and activate the expression of target genes in concert with other co-activators (13, 14). We have recently analyzed the gene expression profiles of the pre-osteoblast precursor cells that were stimulated with osteogenic BMPs versus non-osteogenic BMPs (15). Our results demonstrated that osteogenic BMPs (e.g. BMP-2, BMP-6, and BMP-9) regulated a distinct set of downstream targets that may play a role in regulating BMP-induced osteoblast differentiation (15, 16).

Cross-talk between BMP and Wnt signaling plays a critical role in development (17, 18). Both synergistic and antagonistic roles of BMPs and Wnt signaling have been observed during embryonic development (19–23). For example, Wnt3A and LEPF1-deficient mice display similar phenotypes with the latter developing limb deformities (24). The Wnt family consists of a large number of secreted glycoproteins that are involved in embryonic development, tissue induction, and axis polarity (25, 26). The Wnt ligands initiate the signaling pathway by binding to the frizzled receptors and co-receptors LRP5/6, leading to

1 The abbreviations used are: BMP, bone morphogenetic proteins; CTGF, connective tissue growth factor; RNAi, RNA interference; TGFβ, transforming growth factor-β; FCS, fetal calf serum; GFP, green fluorescent protein; qPCR, quantitative real time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; ECM, extracellular matrix; ALP, alkaline phosphatase.
phosphorylation of the disheveled protein, which, through its association with axin and the adenomatous polyposis coli tumor suppressor, prevents glycogen synthase kinase 3β from phosphorylating β-catenin. Antagonist Dkk1 competes with Wnt for binding to LRP5/6. Unphosphorylated β-catenin is stabilized by escaping recognition by β-TrCP, a component of a ubiquitin-protein isopeptide ligase. Free β-catenin translocates to the nucleus, where it engages transcription factors LEF/TCF-4 to activate expression of downstream genes (27), such as c-Myc, peroxisome proliferator-activated receptor-δ, cyclin D1, and WISP1s (28–33). Increasing evidence suggests that Wnt signaling plays an important regulatory role in bone formation. Several Wnt genes (e.g. Wnt3A) are expressed in the developing limb and are implicated in mesenchymal chondrogenesis (34, 35). Some Wnt proteins may play an important role in osteoblast differentiation and osteogenesis (36, 37). Recently, Wnt co-receptor LRP5 has been shown to participate in bone mass accrual and that loss-of-function mutations in the LRP5 gene cause the low bone mass phenotype of the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (38). Conversely, activating mutations of LRP5 result in the autosomal dominant high bone density trait (39, 40). These findings suggest that Wnt/β-catenin signaling could lend insights into the molecular mechanisms that contribute to normal bone mass accrual and osteogenesis (36, 37). Unphosphorylated β-catenin was induced at the early stage of BMP-9 and Wnt3A. Our findings demonstrate that CTGF may play an important role in osteoblast progenitor cells, and that its activity has to be downregulated during the terminal differentiation of the committed osteoblasts. This indicates that a balanced regulation of the CTGF gene expression may be essential to osteogenic differentiation and normal bone formation.

**Experimental Procedures**

**Cell Culture and Chemicals—** HCT116 parental line and its CTNNB1 knockout derivatives were provided by Ken Kinzler and Bert Vogelstein of The Johns Hopkins Medical Institutions, Baltimore, and were maintained and propagated previously. C3H10T1/2 cells were maintained in complete DMEM supplemented with 10% fetal calf serum (FCS, Mediatech, Herndon, VA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. C3H10T1/2 cells were maintained in basal medium Eagle in Earle’s balanced salt solution, supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. Unless indicated otherwise, all chemicals were purchased from Sigma or Fisher.

**Recombinant Adenoviral Vectors Expressing BMPs, Wnt3A, Oncogenic β-Catenin, and CTGF—** Recombinant adenoviruses (AdBMPs) expressing human BMP-2, -6, -9 (also known as GDF-2) were generated as described previously (11, 50). For construction of the adenoviral vectors expressing Wnt3A, the coding region of mouse Wnt3A (kindly provided by Roel Nusse of Stanford University) was PCR-amplified and subcloned into pAdTrack-CMV, resulting in pAdTrack-Wnt3A. An expression cassette containing an oncogenic S33Y mutation of human β-catenin was subcloned into pAdTrack, resulting in pAdTrack-β-Cat*. The coding region of mouse CTGF was PCR-amplified and subcloned into pAdTrack-CMV, resulting in pAdTrack-CTGF. These shuttle vectors were used to generate recombinant adenoviruses (i.e. AdWnt3A, Adβ-Cat*, and AdCTGF) as described previously (50). All PCR-amplified fragments and cloning junctions were verified by DNA sequencing.

For a control, we used an analogous adenovirus expressing only GFP (i.e. AdGFP) as described previously (28, 50). Details about vector construction are available upon request.

**Isolation of Total RNA—** Subconfluent C3H10T1/2 cells and/or HCT116 derivative lines were seeded onto 75-cm2 culture flasks for 12 h in complete medium supplemented with 0.5% FCS, and infected with a pre-determined optimal titer of AdBMP-2, AdBMP-6, AdBMP-9, Adβ-Cat*, AdWnt3A, or AdGFP. At the indicated time after infection, total RNA was isolated using RNASEQ Total RNA isolation kit (Promega, Madison, WI) according to the manufacturer’s instructions.

**Microarray Analysis—** Fully characterized RNA samples were used for target preparation and subjected to hybridizations to Affymetrix mouse gene chips 430a (containing ~22,000 known genes and ESTs). The acquisition and initial quantitation of array images were performed using the Affymetrix MAS 5.0 with the default parameters. The acquired microarray raw data were further filtered and normalized to remove noise, although retaining true biological information by filtering out the genes with signal intensity in all samples ≥100 intensity units, and by removing the genes that received an “absent” call for all hybridizations. The clustering analysis was carried out by using the dChip 1.3 software (51).

**Reverse Transcription and Quantitative Real Time PCR (qPCR) Analysis—** Ten micrograms of total RNA were used to generate cDNA templates for reverse transcriptase PCR. The first strand cDNA synthesis was performed using a hexamer (Promega) and Superscript II reverse transcriptase (Invitrogen). The first strand cDNA products were further diluted 10-fold and used as qPCR templates. Expression level of CTGF was determined by qPCR analysis using oligonucleotides (18-mers) to amplify the 3′-end (~150 bp) of the mouse or human CTGF gene. A complete list of the primers is available at www.boneandcancer.org/cctgenes.htm. The SYBR Green-based qPCR analysis was carried out by using the Option DNA Engine thermocycler (MJ Research, Waltham, MA). The cycling program was as follows: 94 °C for 2 min for 1 cycle, 4 cycles at 92 °C for 20 s, 68 °C for 30 s, and 72 °C for 30 s with a decrease of 0.5°C/cycle, and 30 cycles at 92 °C for 20 s, 57 °C for 30 s, and 72 °C for 30 s. A standard curve was generated with serially diluted cDNA from mouse or human CTGF gene. For a control, we used an analogous adenovirus expressing only GFP (i.e. AdGFP) as described previously (28, 50). Details about vector construction are available upon request.

**RNAi-mediated Silencing of CTGF Gene Expression—** To generate siRNA pools, we employed the in vitro dicer-mediated digests of double-stranded RNA derived from the target genes. Briefly, a pair of PCR primers (anchored with a T7 promoter sequence at the 5′-end of each primer) was used to amplify mouse CTGF or GFP. The PCR primers used are as follows: for mouse CTGF, 5′-GCG TAA TAC GAC TCA CTA TAG GCG TAA AGC CAG CAG GAA GTA AG-3′ and 5′-GCG TAA TAG GAC TCA CTA TAG GCT CAG CCT ACG AAC AGG TG-3′; and for

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GFP, 5′-GGG TAA TAC GCA TCA TTA GTC GAG GAC 5′.GAG TGA AAG-3′ and 5′-GGG TAA TAC GCA TTA GTC GAG GAG GGC TAC AAG TCC AGC AGG-3′. The purified PCR products were subjected to 77 RNA polymerase-mediated in vitro transcription (Promega). The resultant double-stranded RNAs were subjected to recombinant dicer (or RNase III) digestion (New England Biolabs, Beverly, MA) following the manufacturer’s instructions. Transfection of the siRNA mixtures was carried out using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescence Staining—The cells were fixed with methanol at −20 °C for 15 min and washed with PBS 2–3 times. The fixed cells were permeabilized with 1% Nonidet P-40/PBS and blocked with fetal calf serum at room temperature for 30 min, followed by incubating cells with fetal calf serum containing a goat anti-CTGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. After being washed, the cells were incubated with biotin-labeled anti-goat IgG secondary antibody for 30 min, followed by incubating the cells with streptavidin-Alexa Fluor-594 (Molecular Probes, Eugene, OR) for 20 min at room temperature. The presence of CTGF protein was examined under a fluorescence microscope. Stains without the primary and/or secondary antibodies, or with control IgG, were used as negative controls.

 Determination of Alkaline Phosphatase Activity—Alkaline phosphatase activity was assessed by the colorimetric assay and/or histochemical staining as described previously (11, 12, 15).

Mixed Cell Plating Assay—HCT116 cells were seeded in 12-well plates at 10–20% confluence and infected with AdCTGF or AdGFP. At 15 h after infection, the infected HCT116 cells were examined under a fluorescence microscope, and all HCT116 cells were GFP-positive. In order to determine whether C3H10T1/2 cells were attracted by AdCTGF- or AdGFP-infected HCT116 cells, trypsinized C3H10T1/2 cells were infected with AdGFP or AdCTGF and added to the pre-infected HCT116 cells at various densities. At 3 h after plating C3H10T1/2 cells, cell migration was examined under both bright field and fluorescence microscopy (magnification, ×300). Representative results from three experiments are shown.

Cell Wounding Assay—C3H10T1/2 cells were seeded at subconfluency in 12-well cell culture plates and were infected with a pre-determined optimal titer of AdGFP or AdCTGF. At 15 h after infection, the monolayer cells were wounded with pipette tips. At 0, 1, 2, and 4 h after wounding, the wound healing at approximately the same fields was recorded under both bright field and fluorescence microscopy. Representative results from three experiments are shown.

Boyden Chamber Migration Assay—HCT116 cells were plated into the bottom wells of Transwell 12-well plates (Corning Costar, Cambridge, MA) and were infected with AdGFP, AdCTGF, AdBMP-9, or AdWnt3A at a comparable titer. At 4 h after infection, cells were washed twice with DMEM containing 0.1% BSA and incubated in the DMEM, 0.1% BSA medium (2 ml per well) at 37 °C, 5% CO2, for 12 h. In order to set up the migration assays, exponentially growing C3H10T1/2 cells were trypsinized and washed twice in DMEM, 0.1% BSA medium. Approximately 5 × 104 cells (resuspended in 1.0 ml of pre-equilibrated DMEM, 0.5% BSA) were added into each Transwell insert, which consisted of types I and III collagen-coated microporous membrane (3.0 μm). The cells were allowed to migrate for 4 h in a humidified 5% CO2, 37 °C incubator. The apparatus was disassembled, and the membranes were rinsed in PBS to remove unattached cells. The cells on the membranes were fixed in 10% formalin for 10 min and stained in hematoxylin for 30 min. Cells attached to the unmigrated (top) side were removed by gently wiping that side with a damp cotton swab. The membrane was then mounted onto a glass slide with Permount (Fisher) and allowed to dry. The average numbers of migrated cells were determined by counting the cells in 10 random high power fields (×300). The Transwell assays for each condition were performed in duplicate, and representative results are shown.

RESULTS

Expression Profiling Analysis of the Early Stage of BMP and Wnt3A-induced Osteoblast Differentiation of Mesenchymal Stem Cells—Through a comprehensive analysis of the osteogenic activity of the 14 types of human BMPs, we demonstrated previously (11, 12) that BMP-2, BMP-6, and BMP-9 exhibit the greatest ability to induce osteoblast differentiation of mesenchymal progenitor cells in vitro as well as in vivo. As shown in Fig. 1A, BMP-9 and Wnt3A induced a drastic increase in alkaline phosphatase activity, a well established marker of early osteogenic differentiation, in C3H10T1/2 cells. The C3H10T1/2 cells are mouse pluripotent embryonic fibroblasts that can differentiate into multiple lineages, including osteocytes, chondrocytes, adipocytes, and myoblast, upon appropriate stimulations.

In order to understand the mechanisms behind BMP- and Wnt3A-mediated osteogenic differentiation in mesenchymal stem cells, we determined the gene expression profiles in C3H10T1/2 cells stimulated with three osteogenic BMPs (i.e. BMP-2, BMP-6, and BMP-9), Wnt3A, and the GFP control. By using the 430A Affymetrix GeneChips (containing ~22,000 gene probes per chip), we found that the overall expression pattern of the osteogenic BMPs was similar to each other but was distinct from that of the GFP control. These findings are consistent with the results of our recent gene profiling analysis in the pre-osteoblast C2C12 cells (15, 16). We also found that although the overall expression pattern of the osteogenic BMPs was distinct from that of the Wnt3A-stimulated cells, there was a group of genes that was regulated by both BMPs and Wnt3A. As shown in Fig. 1B, when the dChip clustering analysis was conducted on the most significantly differentially expressed 33 transcripts, we identified a subcluster of five genes that were significantly up-regulated by both BMPs and Wnt3A. Among the five, three were less characterized ESTs, and the other two genes were CTGF and diphertheria toxin receptor. Because both Wnt3A and BMPs have been shown to effectively induce the osteogenic marker alkaline phosphatase in mesenchymal stem cells, we hypothesized that functional elucidation of the potential mutual target of BMPs and Wnt3A signaling may provide insights into the mechanisms of osteogenic differentiation. Thus, we chose to further investigate and characterize the functional role of CTGF in Wnt3A- and BMP-9-induced osteoblast differentiation.

CTGF Is Among the Most Significantly Up-regulated Target Genes by BMPs and Wnt3A in Mesenchymal Stem Cells—Several recent studies (52–57) suggest that CTGF may be regulated by members of the TGFβ superfamily, including BMPs. CTGF belongs to the CCN family, and three members (i.e. WISP-1, -2, and -3) have been shown to be regulated by Wnt and/or β-catenin (32, 33). However, our microarray results were the first to suggest that CTGF may be regulated by Wnt signaling. Furthermore, it remains largely undefined what functional role(s), if any, CTGF may play in BMP and/or Wnt signaling pathways. Here we sought to determine the possible functional role of CTGF in BMP-9 and Wnt3A-induced osteogenic differentiation.

We first verified that CTGF expression was indeed up-regulated by BMP-9 and Wnt3A. In this study, we focused on BMP-9 as we have demonstrated that BMP-9 is one of the most potent osteogenic BMPs (11, 12); yet it is one of the least characterized BMPs. As shown in Fig. 2A, after the normalization of the microarray hybridizations, the signal intensity of the CTGF gene probe was induced ~7.8- and 4–5-fold by Wnt3A and the three osteogenic BMPs, respectively. To verify the microarray data, we infected C3H10T1/2 cells with AdBMP-9, AdWnt3A, or AdGFP at a pre-determined optimal titer, and total RNA was isolated at days 1–3, 5, and 7 after infection. The expression level of the CTGF gene was analyzed by qPCR. As shown in Fig. 2B, C and D, the expression of CTGF was effectively induced by both BMP-9 and Wnt3A in C3H10T1/2 cells. The induction of CTGF peaked at day 3 and then decreased and returned to near basal levels at day 7. Specifically, CTGF expression was induced 2.1-, 9.6-, 5.6-, and 2.2-fold at 1–3, 5, and 7 days after BMP-9 stimulation, respectively. Accordingly, CTGF expression was up-regulated 2.7-, 7.5-, 19.8-, 3.1-, and 2.2-fold at 1–3, 5, and 7 days after Wnt3A stimulation. We also found that the expression of GAPDH was not significantly changed (<1.5-fold) in all samples (data not shown). Most interestingly, consistent with our microarray findings...
was the fact that Wnt3A was shown to induce CTGF expression to a much greater magnitude than BMP-9 in most samples. Nevertheless, our qPCR analysis confirmed that CTGF is an early target in both Wnt3A and BMP-9 signaling pathways.

**Wnt3A-regulated CTGF Expression Is Mediated by the Canonical β-Catenin Pathway**—We next tested if Wnt3A-induced expression of CTGF is dependent on functional β-catenin. We took advantage of the recently reported human cell line HCT116 that has been genetically manipulated (49). Specifically, HCT116 is a human colorectal cancer line that harbors a heterozygous oncogenic mutation of the β-catenin gene (i.e. CTNNB1 mut/wt), leading to the constitutive activation of β-catenin/Tcf4 activity. Somatic deletion of the mutant allele (i.e. CTNNB1mut/−/wt) rendered the cells to the basal level of β-catenin/Tcf4 activity in the absence of Wnt stimulation, although the CTNNB1mut/wt line behaved like the parental line (CTNNB1−/−/wt) (49). As shown in Fig. 3A, the Tcf4 reporter was inactive in CTNNB1−/−/wt cells infected with AdGFP, but the reporter was readily activated when the cells were infected with a recombinant adenovirus expressing Wnt3A (AdWnt3A) or the constitutively active form of β-catenin (Adβ-Cat*). We sought to investigate how CTGF was regulated in the HCT116 CTNNB1−/−/wt cells upon Wnt3A or β-Cat* expression. By using quantitative real time PCR analysis, we demonstrated that CTGF expression was readily induced 4–7-fold in HCT116 CTNNB1−/−/wt cells expressing a constitutively active form of β-catenin (Adβ-Cat*) or exogenous Wnt3A, although the CTGF expression maintained a high level, independent of Wnt3A or mutant β-catenin expression (Fig. 3B). The results from the quantitative real time PCR were also confirmed by immunofluorescence staining analysis using a CTGF-specific antibody. As shown in Fig. 3C, Wnt3A but not GFP induced CTGF expression in HCT116 CTNNB1−/−/wt cells, whereas a high level of CTGF expression was readily detected in CTNNB1mut/−/wt cells. Taken together, these results indicate that Wnt3A likely regulates CTGF expression through the canonical β-catenin pathway.

**BMP-9-induced but Not Wnt3A-induced Osteogenic Differentiation Is Inhibited by RNAi-mediated Silencing of CTGF Expression in Mesenchymal Stem Cells**—Although three members of the CCN family WISP-1, -2, and -3 have been identified as downstream targets of Wnt1/β-catenin signaling (32, 33), the above findings were the first to demonstrate that CTGF is regulated by Wnt3A. The above results and several recent studies (52–57) have indicated that CTGF is regulated by BMPs. However, the actual role of CTGF in BMP- or Wnt3A-mediated osteoblast differentiation is poorly understood. To that end, we sought to determine whether or not the RNA interference-mediated depletion or knockdown of the CTGF gene expression would have any effect on BMP-9- or Wnt3A-induced osteogenic differentiation. In order to generate small interfering double-stranded RNAs (siRNAs) that efficiently target mouse CTGF transcripts, we synthesized double-stranded RNAs corresponding to the coding region of mouse CTGF as a Mutual Target of Wnt/β-Catenin and BMP Signaling
We next tested if the RNAi-mediated knockdown of CTGF expression affected BMP-9- or Wnt3A-induced osteogenic differentiation. We transfected the siRNA mixtures into subconfluent C3H10T1/2 cells, which were subsequently infected with either AdBMP-9 or AdGFP. To assess the status of osteogenic differentiation, we determined the ALP activity in the transfected/infected C3H10T1/2 cells, as ALP is a well established marker of early osteogenesis and significantly elevates in committed osteoblasts (1–3). Five days after transfection/infection, cells were collected for a quantitative analysis of the ALP activity. As shown in Fig. 4B, introduction of the siRNA mixture targeting CTGF resulted in a 77% decrease in BMP-9-induced ALP activity, whereas there was no effect by the control siRNA mixture targeting GFP expression. However, the RNAi-mediated knockdown of CTGF did not affect the Wnt3A-induced ALP activity (Fig. 4B). Similar results were obtained when the transfected/infected cells were fixed, and the ALP activity was determined histochemically. As illustrated in Fig. 4C, the introduction of the siCTGF mixture into C3H10T1/2 cells reduced the BMP-9-induced ALP activity, but the Wnt3A-induced ALP activity was not affected by the transfection of the siCTGF mixture. These results were highly reproducible in at least three independent batches of experiments. Taken together, these findings strongly suggest that CTGF may play a critical role in BMP-9-induced osteogenic differentiation, whereas Wnt3A may also regulate osteoblast differentiation of mesenchymal stem cells through the CTGF-independent pathway, which remains to be further investigated.

**Constitutive CTGF Expression Inhibits the BMP-9- and Wnt3A-induced Osteogenic Differentiation of Mesenchymal Stem Cells**—We next sought to investigate the effect of constitutively expressed CTGF on BMP-9- and Wnt3A-induced osteoblast differentiation. Based on our time course study reported in Fig. 2B, we hypothesized that a prolonged expression of the CTGF gene could inhibit BMP-9- or Wnt3A-induced osteogenic differentiation. To establish an efficient means of expressing CTGF exogenously, we constructed a recombinant adenoviral vector that expressed mouse CTGF (i.e. AdCTGF) by using our recently developed AdEasy system (50). Adenovirus-mediated expression of the CTGF gene was confirmed by Western blotting analysis (data not shown). As mentioned, we previously constructed AdBMP-9, AdWnt3A, and the control AdGFP recombinant adenoviral vectors (11, 12, 28, 50). In addition to the expression of the transgenes, AdCTGF, AdBMP-9, and AdWnt3A also expressed GFP as a marker of gene transfer. As shown in Fig. 5A, all four viral vectors were shown to transduce the C3H10T1/2 cells with equally high efficiency.

To determine the effect of AdCTGF on BMP-9- or Wnt3A-induced osteogenic differentiation, we co-infected subconfluent C3H10T1/2 cells with AdBMP-9/AdCTGF, AdBMP-9/AdGFP, AdWnt3A/AdCTGF, AdWnt3A/AdGFP, AdCTGF/AdGFP, or AdGFP only. At 5 days after infection, cells were collected, and the ALP activity was quantitatively measured using a colorimetric assay. As shown in Fig. 5B, overexpression of CTGF alone exhibited a negligible effect on ALP activity. However, overexpression of CTGF decreased BMP-9- and Wnt3A-induced ALP activity by 90 and 75%, respectively. Similar results were obtained when the ALP activity was determined histochemically (Fig. 5C). These results were reproducible in at least three independent batches of experiments. Taken together, these findings were supportive of our hypothesis that a constitutive expression of CTGF inhibits BMP-9- or Wnt3A-induced osteoblast differentiation thus suggesting the following: 1) CTGF may play an important role in transducing the osteogenic signaling of BMP-9 and Wnt3A pathways; and 2) a balanced tight regulation of CTGF expression may be essential for BMP-9- or
Wnt3A-induced osteoblast differentiation of mesenchymal stem cells.

**CTGF Promotes the Motility of Mesenchymal Stem Cells**—Although the biological functions of CTGF and other CCN family members are currently unknown, several lines of studies suggest that the CCN proteins may regulate multiple cellular processes, such as cell proliferation, migration, differentiation, angiogenesis, and skeletal development (43–48). Here we conducted three different but complementary experiments to investigate whether CTGF exerted any effect on the migratory features of mesenchymal stem cells. First, we performed mixed cell plating experiments to determine whether the exogenously expressed CTGF could recruit mesenchymal stem cells. Specifically, the sparsely plated human colon cancer HCT116 cells were infected with the pTOP-Luc reporter and infected with AdWnt3A, Adβ-Cat**, or AdGFP. At 30 h after transfection/infection, cells were collected for luciferase assays using the luciferase assay kit (Promega). Each assay condition was done in triplicate. Transfection efficiency was normalized by measuring the β-galactosidase activity of the co-transfected pCMV-β vector.

**FIG. 3.** Wnt3A-induced CTGF expression is β-catenin-dependent. A, activation of Tcf-4 luciferase reporter in HCT116 CTNNB1 knockout lines. HCT116 CTNNB1mut/− and HCT116 CTNNB1−/− allelic deletion cells were transfected with the pTOP-Luc reporter and infected with AdWnt3A, Adβ-Cat**, or AdGFP. At 30 h after transfection/infection, cells were collected for luciferase assays using the luciferase assay kit (Promega). Each assay condition was done in triplicate. Transfection efficiency was normalized by measuring the β-galactosidase activity of the co-transfected pCMV-β vector. B, Wnt3A- and oncogenic β-catenin-induced CTGF expression in HCT116 CTNNB1−/− knockout cells. The allelic knockout lines were maintained under a low serum condition and infected with AdWnt3A, Adβ-Cat**, or AdGFP. At 30 h after infection, total RNA was collected and subjected to qPCR analysis using primers corresponding to the 3’-end of human CTGF mRNA, as described in Fig. 2. Fold of change was determined by dividing the mean transcript level of Wnt3A- or oncogenic β-catenin-treated samples with that of the GFP control. C, immunofluorescence staining of CTGF expression in HCT116 CTNNB1 knockout lines. The allelic mutant lines were infected with AdWnt3A or AdGFP for 30 h. Cells were fixed and stained with an anti-CTGF antibody (Santa Cruz Biotechnology). A control IgG was used to stain the Wnt3A-stimulated cells. The presence of CTGF was detected with an Alexa Fluor-594-conjugated secondary antibody (Molecular Probes) under a fluorescence microscope. Representative results of three independent experiments are shown.

Wnt3A-induced osteoblast differentiation of mesenchymal stem cells.
tant feature of CTGF functions in osteoblast differentiation.

We next conducted a cell wounding experiment to determine whether CTGF could promote the cell migration. The cell wounding experiment is one of the commonly used methods to assess cell adhesion and migration under in vitro conditions (58). Experimentally, we infected subconfluent C3H10T1/2 cells with AdCTGF or AdGFP for 15 h. The infected monolayer cells were wounded with pipette tips. At 0, 1, 2, and 4 h after wounding, cell migration was recorded under both bright field and fluorescence microscopy (magnification, ×100). As shown in Fig. 6B, the cells were efficiently transduced by AdCTGF or AdGFP. At 1 and 2 h after wounding, migrating cells were observed in AdCTGF-infected cells, but not in AdGFP-infected controls. The cell migration and spreading were even more pronouncedly different 4 h after wounding (Fig. 6B). Although all wounds were healed after 15–24 h, the AdCTGF-infected cells always closed the gaps at much earlier time points (data not shown). These results were reproducible in multiple batches of experiments. Taken together, these findings suggest that exogenous expression of CTGF could indeed promote migration of C3H10T1/2 mesenchymal stem cells.

Finally, by using the Boyden chamber assays (59, 60), we sought to determine whether CTGF could function as a chemoattractant to mesenchymal stem cells. BMPs and Wnts are generally considered to be morphogens and chemoattractants (18). Here we included BMP-9 and Wnt3A as positive controls. Experimentally, HCT116 cells were plated into the bottom wells of Transwell 12-well plates and were infected with AdBMP-9 or AdWnt3A infection. Four h after infection, HCT116 cells were washed and incubated in the
DMEM, 0.1% BSA (serum-free) medium for 12 h. Approximately 5 x 10^4 C3H10T1/2 cells (resuspended in pre-equilibrated DMEM, 0.1% BSA) were added into each Transwell insert that contained types I and III collagen-coated microporous membrane (3.0 μm). The cells were allowed to migrate for 4 h. After the membranes were rinsed in PBS to remove unattached cells, the cells on the membrane were fixed in 10% formalin and stained in hematoxylin. The membrane was then mounted onto a slide and examined under bright field microscopy (magnification, ×300). As shown in Fig. 6C, a significant number of migrated cells were observed in the CTGF-expressing wells, as well as in BMP-9 and Wnt3A-expressing wells, whereas only a few cells were detected in the GFP controls. We next determined the average number of migrated cells by counting the cells in 10 random high power fields (×300). CTGF, BMP-9, and Wnt3A induced ~8-, 5-, and 9-fold increase in cell migration over the GFP controls, respectively (Fig. 6D). Transwell assays for each condition were performed in duplicate. These findings suggest that one aspect of the CTGF biological functions may be involved in attracting and recruiting mesenchymal stem cells.

**DISCUSSION**

The molecular mechanisms behind osteoblast lineage-specific differentiation of mesenchymal stem cells remain to be elucidated. We have demonstrated recently (11, 12) that BMP-2, BMP-6, and BMP-9 are the most potent osteogenic factors among the 14 types of BMPs. Recent studies (38–40) suggest that Wnt/β-catenin signaling may be involved in osteoblast differentiation and bone formation. We found previously that Wnt/β-catenin signaling is deregulated in ~70% of human osteosarcoma (42), suggesting that Wnt/β-catenin signaling may play a role in primary bone tumor development. Although several targets of Wnt/β-catenin signaling have been reported (61–64), the diverse biological functions initiated by Wnt/β-catenin signal in mesenchymal stem cells remain to be under-
stood. In this report, we have demonstrated that CTGF may be a mutual target of the Wnt and BMP signaling pathways by comparing the gene expression profiles in mesenchymal stem cells stimulated with Wnt3A and several osteogenic BMPs. Although several recent studies suggest that CTGF may be regulated by members of the TGF-β superfamily, including BMPs (52–57), our findings were the first to demonstrate that CTGF may be a downstream target of Wnt/β-catenin signaling.

In order to understand the functional roles of CTGF in BMP-9- and Wnt3A-induced osteoblast differentiation, we first confirmed the microarray findings, and we demonstrated that CTGF may be up-regulated at the early stage (e.g., the first 3 days) of BMP-9 and Wnt3A stimulations but is nearly at basal level at around day 7. We next demonstrated that Wnt3A-regulated CTGF expression is β-catenin-dependent. By using RNAi-mediated gene knockdown experiments, we found that knockdown of CTGF expression significantly diminished BMP-9-induced osteogenic differentiation, whereas Wnt3A-induced osteogenic differentiation was not affected by RNAi knockdown of CTGF expression. These results suggest that CTGF may function as a critical mediator of BMP-9-induced osteogenic signaling, whereas Wnt3A may also regulate osteoblast differentiation in a CTGF-independent fashion. However, a constitutive expression of CTGF was shown to inhibit BMP-9- and Wnt3A-induced osteogenic differentiation, suggesting that a tightly regulated CTGF expression may be critical during osteoblast lineage-specific differentiation of mesenchymal stem cells. Furthermore, we demonstrated that the exogenous expression of CTGF was able to promote cell migration and recruitment of mesenchymal stem cells in various in vitro assays.

**FIG. 6.** CTGF promotes the migration of mesenchymal stem cells. **A**, mixed cell plating assay. HCT116 cells were seeded in 12-well plates at a low confluence and infected with AdCTGF or AdGFP. 15 h after infection, trypsinized C3H10T1/2 cells were added to the pre-infected HCT116 cells at various densities. 3 h after plating C3H10T1/2 cells, cell migration was recorded under both bright field (BF) and fluorescence microscopy (FF) (magnification, ×300). The GFP-positive cells (identified by yellow arrowheads in BF) were AdGFP- or AdCTGF-infected HCT116 cells. Representative results from three batches of experiments are shown. **B**, cell wounding assay. C3H10T1/2 cells were seeded in 12-well plates and infected with AdGFP or AdCTGF. 15 h after infection, the monolayer cells were wounded with pipette tips. At the indicated time points, the wound healing in approximately similar fields was recorded under both bright field and fluorescence microscopy (magnification, ×100). Representative results from three batches of experiments are shown. **C and D**, Boyden chamber migration assay. HCT116 cells were plated into the bottom wells of Transwell 12-well plates and were infected with AdGFP, AdCTGF, AdBMP-9, or AdWn3A. 4 h after infection, cells were washed and incubated in the DMEM, 0.1% BSA medium (2 ml/well) for 12 h. Approximately 5 × 10⁴ cells (resuspended in 1.0 ml of pre-equilibrated DMEM, 0.1% BSA) were added into each Transwell insert, which consisted of types I and III collagen-coated microporous membrane (3.0 μm). The cells were allowed to migrate for 4 h. The membranes were rinsed in PBS to remove unattached cells. The cells on the membrane were fixed in 10% formalin and stained in hematoxylin. Cells attached to the unmigrated (top) side were removed by gently wiping that side with a damp cotton swab. The membrane was then mounted onto a slide with Permount and examined under bright field microscopy (magnification, ×300). **D**, the average numbers of migrated cells were determined by counting the cells in 10 random high power fields (HPF, ×300). The Transwell assays for each condition were performed in duplicate, and representative results are shown. See “Experimental Procedures” for details.
Based on the current findings we propose a working model of possible CTGF action in BMP- and Wnt-induced osteoblast differentiation. As illustrated in Fig. 7, when pluripotent mesenchymal stem cells are stimulated with Wnt3A or BMPs, CTGF is up-regulated at the early stage of osteogenic differentiation, which may play a role in promoting the proliferation and recruitment of early osteoprogenitor cells. Although CTGF may play an important role in BMP-induced osteogenic differentiation, Wnt3A may also induce osteogenic differentiation of mesenchymal stem cells in a CTGF-independent fashion. However, CTGF has to be down-regulated when the differentiation potential of the committed pre-osteoblasts increases, as indicated by the activation of the osteogenic marker alkaline phosphatase. Although CTGF may play an important role in BMP-induced osteogenic differentiation, our findings from the RNAi-mediated knockdown of CTGF expression also suggest that Wnt3A may induce osteogenic differentiation of mesenchymal stem cells in a CTGF-independent fashion.

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