CCN3 Protein Participates in Bone Regeneration as an Inhibitory Factor

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Background: We previously demonstrated that CCN3 inhibits BMP-2-induced osteoblast differentiation by in vitro experiments. CCN3 is up-regulated in bone regeneration and acts as a negative regulator for bone regeneration. This study provides the first evidence that CCN3 inhibits bone regeneration and contributes to develop new strategies for bone regeneration therapy.

Results: CCN3 is up-regulated in bone regeneration and acts as a negative regulator for bone regeneration. This study may contribute to the development of new strategies for bone regeneration therapy.

Conclusion: CCN3 is a negative regulator for bone regeneration.

Significance: This study provides the first evidence that CCN3 inhibits bone regeneration and contributes to develop new strategies for bone regeneration therapy.

Bone has a robust intrinsic capacity to regenerate, and this process is accomplished by intimate interactions among various factors, including cytokines/chemokines, transcription factors, and extracellular matrices. In this process, complex interplay between activators and suppressors that regulate diverse signaling pathways and target gene expression in osteogenesis is involved in successful bone healing (1). Indeed, the expression of bone morphogenetic protein (BMP), one of the factors that stimulate bone regeneration (2–4), is associated with the expression of BMP antagonists at an early stage of bone regeneration (5–9). To understand these intricate processes, extensive analysis of the gene expression profile during bone regeneration will be helpful.

Bone formation is mediated by osteogenic transcription factors, such as Runx2 and Sp7 (Osterix), and osteoblast-related molecules, such as alkaline phosphatase (Alpl), type 1 collagen (Col1), and osteocalcin (bone γ-carboxyglutamate protein, Bglap), which are regulated by BMP (10–13). In addition to

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2 The abbreviations used are: BMP, bone morphogenetic protein; micro-CT, microcomputed tomography; pQCT, peripheral quantitative computed tomography; MAR, mineralization apposition rate; BMD, bone mineral density; BV, bone volume; TV, total volume; Tb.N, trabecular number; MS, mineralized surface; BS, bone surface; BFR, bone formation rate; Ob.S, osteoblast surface; ES, eroded surface; N.Oc, osteoclast number; B.Pm, bone perimeter; cfu-Alp, colony-forming unit-Alp; cfu-F, colony-forming unit-fibroblastic.
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these, many factors involved in fetal skeletogenesis are recapitulated to mediate regeneration in adulthood, as observed in the regeneration processes of other organs (1), but such molecules implicated in bone regeneration in adults have not been well elucidated. To explore the expression profile of genes involved in bone regeneration, we conducted microarray analysis using a bone regeneration model in mice and identified CCN3 as one of the most up-regulated genes at the early stage of bone regeneration. CCN3 is a matricellular protein that belongs to the CCN family of genes. CCN family proteins are intercellular signaling proteins that are composed of six homologous members: cysteine-rich 61(Cyr61)/CCN1, connective tissue growth factor (Ctgf)/CCN2, nephroblastoma overexpressed gene (Nov)/CCN3, Wnt-induced secreted protein 1 (Wisp1)/CCN4, Wisp2/CCN5, and Wisp3/CCN6. They mediate many biological processes, such as angiogenesis, wound healing, and tumorigenesis, by regulating the proliferation, migration, and differentiation of the target cells (14, 15). Ccn3 was originally identified as a gene that is responsible for retrovirus-induced avian nephroblastoma (16). Human nephroblastoma (Wilms' tumor) synthesizes a truncated, abnormal CCN3 protein (17), and this tumor shows mixed components of metanephric, epithelial, and stromal derivatives, such as muscle, fat, cartilage, and bone, suggesting the important role of CCN3 in the generation of various types of tissues. CCN3 is expressed in notochord and presomitic mesoderm in early stage development (18), and it is also expressed in diverse tissues in adults, including the nervous system, muscle, cartilage, and bone (19–22). CCN3 positively modulates cartilage formation (23) and suppresses the myogenic differentiation of C2/4 cells (24). We previously demonstrated that CCN3 suppresses the differentiation of MC3T3-E1 and Kusa-A1 cells to osteoblasts by interacting with the BMP or Notch signaling pathways (25–27). Recently, Ouellet et al. (28, 29) demonstrated that CCN3 is a modulator for bone turnover and regulated bone metastasis by breast cancer, but the biological significance of CCN3 in bone regeneration has not been elucidated. In this study, we identified Ccn3 as an up-regulated gene during bone regeneration by microarray analysis and investigated the role of CCN3 in bone regeneration by analyzing the healing process of drill hole injury created in femurs of wild-type mice and Ccn3-mutated mice. We demonstrate that CCN3 participates in bone regeneration as a negative regulator.

EXPERIMENTAL PROCEDURES

Generation of Ccn3 Mutant Mice—Ccn3 transgenic (Ccn3 Tg) mice were generated and maintained in the C57BL/6j background, in which murine Ccn3 was overexpressed under the control of the 2.3-kb Col1a1 promoter. The sequences of PCR primers used for genotyping were 5′-GTT CCT CCC AGG TCT CCA AGA and 5′-GCT CTG GTC AGA CGG TCT CAT CTC, which detects the fused fragment of the Col1a1 promoter and Ccn3 exon 2.

Ccn3 knock-out (Ccn3 KO) mice were generated and maintained in the C57BL/6j background. Exons 1 and 2 and the distal portion of exon 3 were replaced with the neomycin resistance (NeoR) gene cassette (30). The sequences of the PCR primers used for genotyping were 5′-TGA ATG AAC TGC AGG ACG AG and 5′-AAT ATC AGG GGT AGC CAA CG, which detects the NeoR cassette, and 5′-GCC TTC CTG TTC CAT CTC TTA and 5′-CCT CTA GTC GGC GGC AAG TGA CCT, which detects the targeted region of the Ccn3 gene.

Antibodies—A rabbit polyclonal antibody against CCN3 was raised against the synthetic peptide CPQNEAFQDLLEK, which corresponded to the amino acid residues 246–260 of murine CCN3 (31), and purified antibodies were obtained from antisera by immunoaffinity chromatography. Rabbit monoclonal antibodies against mouse Smad1 and phosphorylated Smad1/5 were purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibody against actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit monoclonal antibody against vimentin was purchased from Epitomics (Burlingame, CA).

Cell Culture—Parietal bones were dissected from 1-day-old mice and digested with a mixture of 0.1% collagenase type 2 (Worthington) and 0.2% dispase II (Godo Shusei Co., Tokyo, Japan) in Hank's balanced salt solution (Sigma-Aldrich). The dissociated cells were collected by centrifugation and cultured in α-modified minimum essential medium containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin G and 100 mg of streptomycin) at 37 °C. Subconfluent cells were treated with 0.1% trypsin and 0.02% EDTA in calcium- and magnesium-free phosphate-buffered saline, and these first passage cells were used for the experiments. These cells were treated with human recombinant bone morphogenetic protein-2 (BMP-2; Osteogenetics GmbH, Wuerzburg, Germany) at a concentration of 500 ng/ml.

To assess the status of osteoprogenitors in bone marrow, we performed a colony formation assay. Bone marrow cells were flushed out from the diaphysis of femurs obtained from 8-week-old wild-type mice and Ccn3 KO mice, respectively. The collected marrow cells were inoculated into individual wells of 6-well plates at a cell density of 2 × 10⁵ cells/well and cultured with α-minimum Eagle’s medium containing 10% fetal bovine serum and antibiotics for 15 days. After fixation, the cells were stained for ALP activity as described previously (13) and von Kossa. The numbers of total colonies and Alpl-positive colonies (colony-forming unit-Alpl (cfu-Alpl)) were counted. To assess the number of total colonies, the cells were counterstained with nuclear fast red.

Surgery—Surgery was performed under pentobarbital anesthesia on 8-week-old male mice. Prior to surgery, the bilateral legs were shaved and sterilized with 70% ethanol. A 5-mm incision was made in the front skin of the mid-femur. After splitting the muscle, the periosteum was separated to expose the femoral surface. A drill hole injury was made by a drill bit with a diameter of 1.2 mm in the anterior portion of the diaphysis of bilateral femurs, 5 mm above the knee joint. The surgical field was irrigated with saline, and the incision line was sutured (32). The animals were sacrificed at days 5, 10, and 15 after the injury, and the femoral portion surrounding the drill hole was dissected with a 1-mm margin. As a normal control, femurs at the same site were taken from mice that did not undergo surgery, which were designated as day 0 specimens. These samples were used for further morphological and gene expression analyses. The
experimental procedures were reviewed and approved by the Animal Care and Use Committee at Tokyo Medical and Dental University.

**RNA Extraction, Microarray Analysis, and Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)**—The marrow of the left femur was flushed out with saline, and the portion surrounding the drill hole was dissected with a 1-mm margin. The specimen was frozen in liquid nitrogen and crushed into powder using a Multi-beads Shocker (Yasui Kikai, Osaka, Japan). Total RNA was extracted using TRIZol (Invitrogen) and the Total RNA Isolation Kit (Macherey-Nagel, Duren, Germany).

Microarray analysis was conducted using Affymetrix GeneChip Murine Genome U74A version 2 arrays containing probes for ~12,424 sequences. Biotin-labeled cRNA probes were generated from the total RNA isolated from the diaphyseal bone fragments obtained at days 0, 5, and 10 after the surgery. The cRNA probes were individually hybridized on the arrays, and the signals were detected according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Hybridization data were analyzed using the MAS5.0 (Affymetrix).

For real-time RT-PCR, aliquots of 1 µg of RNA were reverse transcribed to cDNA using a random primer (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Waltham, MA). The expression was quantified by real-time PCR using a Light Cycler System (Roche Applied Science). The relative amount of each mRNA was normalized to β-actin expression. The primer sequences are listed in Table 1.

**Microcomputed Tomography (Micro-CT)**—For the investigation of skeletal changes in Ccn3 Tg and Ccn3 KO mice, micro-CT analysis was performed on proximal tibiae using micro-CT-40 (SCANCO Medical AG, Brüttisellen, Switzerland) with a resolution of 12 µm, and microstructure parameters were calculated three-dimensionally as described previously (33). The definitions of parameters used are according to the American Society for Bone and Mineral Research standards. The bone regeneration process of femurs was analyzed by a micro-CT imaging system (SMX-100CT, Shimadzu (Kyoto, Japan)). Morphometric indices were calculated using the TRI/3D-BON software package (RATOC System Engineering, Tokyo, Japan) (34). Mineral content and mineral density at trabecular bones and cortical bones were measured by peripheral quantitative computed tomography (pQCT) using XCT Research SA+ (Stratec Medizintechnik GmbH, Pforzheim, Germany).

**Bone Histomorphometry**—The unilateral proximal tibiae fixed with ethanol were embedded in glycolmethacrylate and cut into 5-µm-thick longitudinal sections. The structural parameters were analyzed at the secondary spongia. For the assessment of dynamic histomorphometric indices, calcein (at a dose of 20 mg/kg body weight) was injected twice (72-h intervals) into wild-type and Ccn3 mutant mice, respectively. The sections were stained with toluidine blue and analyzed using a semiautomated system (Osteoplan II, Zeiss (Oberkochen, Germany)). In the cortical bone, mineralization apposition rate (MAR) was measured at the mid-shaft of tibia. The nomenclature, symbols, and units used are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (35).

**Histological and Immunohistochemical Analyses**—The femurs that received the drill hole injury were decalcified in 20% EDTA at 4 °C for 14 days and then embedded in paraffin. Four-micrometer-thick sections were cut and stained with hematoxylin and eosin. For immunohistochemical staining, deparaffinized sections were incubated with primary antibodies (anti-CCN3 and anti-phosphorylated Smad1/5) overnight at 4 °C. After washing with PBS, the sections were incubated with peroxidase-conjugated secondary antibody (Envision System—HRP-labeled Polymer Anti-rabbit, Dako (Glostrup, Denmark)) for 1 h. Anti-rabbit IgG Alexa Fluor 594 (Invitrogen) was used as the secondary antibody. Counterstaining was done using DAPI.

**Western Blot Analysis**—Tissues isolated from drill hole regions at day 5 after the surgery were used. The specimen was frozen in liquid nitrogen and crushed into powder using a Multi-beads Shocker. Protein was extracted using Laemmli buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol) containing protease inhibitor mixture (Nakarai Tesque, Kyoto, Japan) and phosphatase inhibitor (Sigma-Aldrich). The samples were applied to 10% SDS-polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare). The proteins were identified by Western blotting using the following rabbit anti-mouse monoclonal antibodies with a 1:1000 dilution of Smad1, phospho-Smad1/5, and a 1:2000 dilution of vimentin at 4 °C overnight. The proteins were incubated with peroxidase-conjugated secondary antibody (Envision + System—HRP-labeled Polymer Anti-rabbit, Dako) and phosphatase inhibitor (Sigma-Aldrich). The samples were applied to 10% SDS-polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare). The proteins were identified by Western blotting using the following rabbit anti-mouse monoclonal antibodies with a 1:1000 dilution of Smad1, phospho-Smad1/5, and a 1:2000 dilution of vimentin at 4 °C overnight. The proteins were incubated with peroxidase-conjugated secondary antibody (Envision + System—HRP-labeled Polymer Anti-rabbit, Dako).

**Ectopic Bone Formation Induced by BMP-2**—To assess ectopic bone formation activity in wild-type and Ccn3 Tg mice,
we conducted a BMP-2-induced ectopic bone formation assay. The pellets composed of type I collagen gels containing 5 μg of rhBMP-2 were transplanted into the subfascial region of the back muscle of mice. After 2 weeks of the transplantation, the pellets were removed. The bone mineral density (BMD) was measured by using a dual-energy x-ray absorptiometer (DCS-600R, Aloka (Tokyo, Japan)).

Statistical Analysis—The results are expressed as means ± S.E. The data were analyzed using Student’s t test. p values less than 0.05 were considered significant.

RESULTS

CCN3 Expression in Bone Regeneration—Fig. 1A summarizes representative histology of untreated control (day 0) and bone-healing processes on days 5, 10, and 15 after drill hole injury at diaphysis of femurs. On day 5, the hole was filled with granulation tissue accompanied by a few woven bones. On day 10, the surface of the drill hole was covered with thin newly formed bone. On day 15, more matured trabecular bones were observed, and these bones covered the bone defect.

We compared gene expression by microarray analysis in diaphysis of femurs from untreated control mice (day 0) and injured mice at day 5 and day 10 after the surgery. The expression levels of 156 of 12,424 genes were increased on both day 5 and day 10 compared with that on day 0. The genes whose expression levels were more than 10 times higher on day 5 than on day 0 are listed in Table 2, accompanied by their expression levels on day 10. Among these, Ccn3 was one of highly up-regulated genes. This prompted us to focus on the role of CCN3 in
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### Table 2: Genes that exhibited 10 times higher expression levels on day 5 as compared with day 0

| Gene symbol | Gene name                      | Fold change day 5/day 0 | Fold change day 10/day 0 |
|-------------|--------------------------------|-------------------------|--------------------------|
| TnnC1       | Troponin C, cardiac/slow skeletal | 74.85                   | 81.89                   |
| Mfap5       | Microfibrillar associated protein 5 | 34.49                   | 22.62                   |
| Col5a1      | Mus musculus COL5A1 gene for collagen α-1 | 30.41                   | 18.98                   |
| Myh7        | Myosin, heavy polypeptide 7, cardiac muscle, β | 23.07                   | 28.72                   |
| Adam12      | A disintegrin and metalloproteinase domain 12 (meltrin α) | 19.25                   | 9.85                    |
| Ccn3/Nov    | Nephroblastoma overexpressed gene | 17.87                   | 20.00                   |
| Igfbp3      | Insulin-like growth factor binding protein 3 | 16.18                   | 10.96                   |
| Bmp1        | Bone morphogenetic protein 1 | 16.02                   | 12.78                   |
| Emcn        | Endomucin                       | 11.04                   | 7.85                    |
| Fgfr1       | Fibroblast growth factor receptor 1 | 11.00                   | 6.63                    |

Bone regeneration. To verify the microarray results, we examined CCN3 expression during bone regeneration by real-time RT-PCR and Western blot analyses. *Ccn3* mRNA was significantly up-regulated on days 5 and 10 (Fig. 1B), and its expression decreased almost to the level of untreated control (day 0) on day 15 (Fig. 1B). The level of CCN3 protein dramatically increased on days 5 and 10 compared with that on day 0 (Fig. 1C). We next investigated the expression of *Ccn3* during bone regeneration by immunohistochemistry. As shown in Fig. 1D, few CCN3-positive cells appeared uninjured (day 0), but numerous CCN3-positive spindle cells appeared adjacent to bone regeneration on days 5 and 10, and their number decreased on day 15 (Fig. 1D), suggesting that CCN3 was mainly expressed in immature osteogenic cells near the newly formed bone in the early phase of bone regeneration. These results indicate that CCN3 is significantly induced at the early stage of bone regeneration.

To confirm whether *Ccn3* expression correlates with osteoblast differentiation, we investigated the expression profile of *Ccn3* during osteoblast differentiation using osteoblastic cells isolated from parietal bone of neonatal mice. BMP-2 treatment stimulated osteoblast differentiation in a time-dependent manner by increasing the expression of mRNAs for *Alpl* and *Bglap* compared with BMP-2-untreated cells (Fig. 1E). In contrast, the expression of *Ccn3* was significantly down-regulated on day 6 compared with that in BMP-2-untreated cells (Fig. 1E). These results indicate that *Ccn3* expression is down-regulated, depending on osteoblast differentiation, and support our hypothesis that CCN3 is induced mainly in immature osteogenic cells during bone regeneration.

**Skeletal Changes in Ccn3 Transgenic Mice**—The femurs of wild-type mice exhibited an extremely low level of *Ccn3* mRNA, but femurs of *Ccn3* Tg mice showed a greatly increased level of *Ccn3* mRNA by RT-PCR analysis (~3,000-fold increase) (Fig. 2A). In addition, immunohistochemistry revealed that osteoblastic cells, including mature osteoblasts covering bone surface showed immunoreactivity against CCN3 antibody in *Ccn3* Tg mice, whereas that in wild-type mice showed no immunoreaction (Fig. 2B).

Although the body weight of *Ccn3* Tg mice was less than that of WT mice at 2 weeks, no significant difference was seen thereafter (supplemental Fig. 1A). No significant differences were observed in the lengths of femur and tibia between wild-type and *Ccn3* Tg mice (supplemental Fig. 1C). Micro-CT analysis revealed a significant decrease in bone volume (VOX BV/TV) and trabecular number (Tb.N) in the tibia of *Ccn3* Tg mice compared with that in wild-type mice (Fig. 2, C and E). The pQCT analysis showed the lower mineral content and mineral density at trabecular bones and cortical bones in *Ccn3* Tg mice compared with those in wild-type mice (Fig. 2F). The decrease in bone volume (BV/TV) was also evident by bone histomorphometric analysis (Fig. 2, D and G). This analysis also revealed significant decreases in mineralized surface (MS/BS) and bone formation rate (BFR/BS) in *Ccn3* Tg mice compared with those in wild-type mice, whereas no significant changes were observed in MAR, osteoblast surface (Ob.S/BS), eroded surface (ES/BS), and osteoclast number (N.Oc/B.Pm) between wild-type and *Ccn3* Tg mice (Fig. 2G). The values of MAR at cortical bone were not significantly different between wild-type and *Ccn3* Tg mice (1.123 ± 0.403 versus 0.755 ± 0.338). These results indicate that *Ccn3* Tg mice exhibit osteopenia probably due to disturbance in the differentiation and function of osteoblastic cells.

To characterize the BMP response, we isolated osteoblastic cells from parietal bones of newborn mice of wild-type and *Ccn3* Tg mice. We first examined the expression level of *Ccn3* mRNA in these cells, because other groups reported that the activity of 2.3-kb Col1a1 promoter greatly decreased when osteoblastic cells were cultured in vitro (36, 37). As expected, *Ccn3* mRNA expression in the cells isolated from *Ccn3* Tg mice exhibited only a 2-fold increase on day 3, and it became almost the same level as wild type on day 6 in culture (Fig. 3A), although *Ccn3* expression was 3,000-fold higher in the bone of *Ccn3* Tg mice than in that of wild-type mice (Fig. 2A). These results indicated that the isolated osteoblastic cells from our *Ccn3* Tg mice are not suitable for assessment of cellular response to BMP-2 in vitro. Thus, we next applied a BMP-2-induced ectopic bone formation assay to investigate BMP response of osteogenic cells in *Ccn3* Tg mice. The soft x-ray analysis revealed the formation of mineralized bones 2 weeks after BMP-2 transplantation in wild-type (WT) and *Ccn3* Tg mice (Tg) (Fig. 3B). Radiopaque areas in wild-type mice are more prominent than in *Ccn3* Tg mice. In addition, BMD assessed by a dual-energy x-ray absorptiometer in *Ccn3* Tg mice exhibited significantly lower values than that in wild-type mice (Fig. 3C). These results indicate that BMP-2-induced bone formation is less active in *Ccn3* Tg mice compared with that in wild-type mice.

**Skeletal Changes in Ccn3 Knock-out Mice**—Our *Ccn3* KO mice expressed no DNA coding *Ccn3* exons 1 and 2 and the
The distal part of exon 3 and expressed a neomycin resistance gene (Fig. 4A). Ccn3 mRNA expression was abolished in these mice (Fig. 4A).

The Ccn3 KO mice were fertile and appeared normal. They showed no significant difference in body weight (supplemental Fig. 1B) and the length of femur and tibia between wild-type

**FIGURE 2. Skeletal changes in Ccn3 Tg mice.**

A, mRNA expression of Ccn3 in wild-type (WT) and Ccn3 Tg (Tg) mice by RT-PCR analysis. n = 7. B, immunohistochemistry for expression of CCN3 at metaphyseal bone in WT and Ccn3 Tg mice. Note that CCN3-positive cells covering the trabecular bone (brown) are observed in Ccn3 Tg mice, but no such cells are seen in WT mice. C, typical micro-CT image of the metaphyseal region of WT and Ccn3 Tg mice. D, typical histology of the metaphyseal region of WT and Ccn3 Tg mice. Toluidine blue stain of undecalcified sections is shown. E, quantitative analyses of bone volume (BV/TV) and trabecular number (Tb.N) assessed by micro-CT. *, p < 0.05, n = 4 in Ccn3 Tg mice and n = 5 in WT mice. F, mineral content and mineral density of trabecular bone and cortical bones in WT and Ccn3 Tg mice measured by pQCT. n = 5 in WT mice, and n = 4 in Tg mice. *, p < 0.05, compared with WT mice. G, bone histomorphometric analyses at the metaphyseal region of WT and Ccn3 Tg mice. Bone volume (BV/TV), eroded surface (ES/BS), osteoclast number (N.Oc/B.Pm), osteoblast surface (Ob.S/BS), MAR, mineralized surface (MS/BS), and bone formation rate (BFR/BS) are shown. *, p < 0.05, compared with WT mice. n = 5. Error bars, S.E.
deficiency induced no apparent skeletal changes in long bones. Bone regeneration processes among wild-type, Ccn3 KO mice. We investigated the time-dependent changes in expression of Ccn3 mRNA during bone regeneration in wild-type and Ccn3 Tg mice (Fig. 6A). Ccn3 Tg mice always exhibited extremely higher levels of Ccn3 mRNA during bone regeneration (Fig. 6A). In contrast, fold increases in Ccn3 mRNA expression over day 0 (uninjured control) were much higher in wild-type mice (~10-fold increase) than those in Ccn3 Tg mice (~2–3-fold increase) (Fig. 6B). As shown in Fig. 6C, the expression levels of osteoblast-related mRNAs (Runx2, Sp7, Col1a1, Alpl, and Bglap) were not significantly different between wild-type and Ccn3 Tg mice during bone regeneration except for Alpl mRNA on day 15.

In contrast, we found accelerated bone regeneration in Ccn3 KO mice compared with wild-type mice (Fig. 7). Micro-CT analysis demonstrated that the radiopaque area was wider in the drill hole of Ccn3 KO mice than in that of the wild-type mice on day 10, and this area covered all of the drill hole in Ccn3 KO mice but not in wild-type mice on day 15 (Fig. 7A). Two-dimensional longitudinal images taken by BMD color mapping confirmed a wider spread of bony mineralization at drill holes on days 10 and 15 in Ccn3 KO mice than in wild-type mice (Fig. 7B). Quantitative analyses of BV/TV and BMD on drill holes in Ccn3 KO mice revealed the significant increase in BV/TV on day 15 and BMD on days 10 and 15 compared with those in wild-type mice (Fig. 7C). Histological examination also confirmed accelerated bone regeneration in Ccn3 KO mice compared with wild-type mice (Fig. 7D). Collectively, overexpression of Ccn3 in osteoblastic cells induced no apparent changes in bone regeneration, but Ccn3 depletion accelerated bone regeneration. These results indicate that Ccn3 exerts an inhibitory effect on bone regeneration.

Fig. 8A summarizes the time-dependent changes in expression of osteoblast-related mRNAs (Runx2, Sp7, Col1a1, Alpl, and Bglap) during bone repair in wild-type and Ccn3 KO mice. These studies demonstrated that all of these mRNAs were up-regulated in Ccn3 KO mice preceding day 5 compared with wild-type mice (Fig. 8A). These results suggest that osteogenesis was more accelerated at an early stage of bone regeneration in Ccn3 KO mice than in wild-type mice.

Because CCN3 is an antagonist of BMP, we investigated the time-dependent changes in the expression of mRNAs for other BMP antagonists, including Chordin (Chrd), Noggin (Nog), and Gremlin1 (Greml1) (Fig. 8A). All of these mRNAs showed a higher level of expression in Ccn3 KO mice than in wild-type mice (Fig. 8A). These results suggest that several BMP antagonists, including CCN3, are up-regulated in the early stage of bone regeneration.

Ccn3 Deficiency Increases Osteoprogenitors and Stimulates BMP Signaling.—To investigate the status of osteoprogenitors, we determined the numbers of total colonies and cfu-Alpl using bone marrow-derived cells isolated from the femurs of wild-type mice and Ccn3 KO mice. The numbers of total colonies and cfu-Alpl significantly increased in cultures of Ccn3 KO mice compared with the numbers in wild-type mice (Fig. 8, B–D). The percentage of Alpl-positive colonies in the total col-

and Ccn3 Tg mice (supplemental Fig. 1, D and F). Micro-CT analysis showed no significant differences in VOX BV/TV and Tb.N (Fig. 4, C and E). The pQCT analysis showed no significant difference in mineral content and mineral density at trabecular bones and cortical bones in Ccn3 KO mice compared with those in wild-type mice (Fig. 4F). Bone histomorphometric analyses also revealed no significant changes in BV/TV, MS/BS, BFR/BS, Ob.S/BS, ES/BS, and N.Oc/B.Pm between wild-type and Ccn3 KO mice (Fig. 4G). These results indicate that CCN3 deficiency induced no apparent skeletal changes in long bones.

Bone Regeneration in Ccn3 Mutant Mice—We compared bone regeneration processes among wild-type, Ccn3 Tg, and Ccn3 KO mice. Fig. 5A summarizes typical micro-CT images of bone regeneration at diaphysis of wild-type and Ccn3 Tg mice. In wild-type mice, no apparent radiopaque region appeared in the drill hole on day 5. Amorphous radiopaque region appeared in the drill hole on day 10, and this area covering the drill hole spread by day 15. Micro-CT images obtained from bone regeneration processes in Ccn3 Tg mice were almost same as those from wild-type mice (Fig. 5A). Fig. 5B demonstrates two-dimensional longitudinal images generated by a BMD color mapping image, and Fig. 4C summarizes the results of quantitative analyses on bone volume (BV/TV) and BMD at the drill hole between wild-type and Ccn3 Tg mice. We found no significant differences in these parameters between wild-type and Ccn3 Tg mice. Histological examination also revealed no apparent histological changes at the drill hole on days 5, 10, and 15 after the surgery between wild-type and Ccn3 Tg mice (Fig. 5D).

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In contrast, we found accelerated bone regeneration in Ccn3 KO mice compared with wild-type mice (Fig. 7). Micro-CT analysis demonstrated that the radiopaque area was wider in the drill hole of Ccn3 KO mice than in that of the wild-type mice on day 10, and this area covered all of the drill hole in Ccn3 KO mice but not in wild-type mice on day 15 (Fig. 7A). Two-dimensional longitudinal images taken by BMD color mapping confirmed a wider spread of bony mineralization at drill holes on days 10 and 15 in Ccn3 KO mice than in wild-type mice (Fig. 7B). Quantitative analyses of BV/TV and BMD on drill holes in Ccn3 KO mice revealed the significant increase in BV/TV on day 15 and BMD on days 10 and 15 compared with those in wild-type mice (Fig. 7C). Histological examination also confirmed accelerated bone regeneration in Ccn3 KO mice compared with wild-type mice (Fig. 7D). Collectively, overexpression of Ccn3 in osteoblastic cells induced no apparent changes in bone regeneration, but Ccn3 deletion accelerated bone regeneration. These results indicate that CCN3 exerts an inhibitory effect on bone regeneration.

Fig. 8A summarizes the time-dependent changes in expression of osteoblast-related mRNAs (Runx2, Sp7, Col1a1, Alpl, and Bglap) during bone repair in wild-type and Ccn3 KO mice. These studies demonstrated that all of these mRNAs were up-regulated in Ccn3 KO mice preceding day 5 compared with wild-type mice (Fig. 8A). These results suggest that osteogenesis was more accelerated at an early stage of bone regeneration in Ccn3 KO mice than in wild-type mice.

Because CCN3 is an antagonist of BMP, we investigated the time-dependent changes in the expression of mRNAs for other BMP antagonists, including Chordin (Chrd), Noggin (Nog), and Gremlin1 (Greml1) (Fig. 8A). All of these mRNAs showed a higher level of expression in Ccn3 KO mice than in wild-type mice (Fig. 8A). These results suggest that several BMP antagonists, including CCN3, are up-regulated in the early stage of bone regeneration.

Ccn3 Deficiency Increases Osteoprogenitors and Stimulates BMP Signaling.—To investigate the status of osteoprogenitors, we determined the numbers of total colonies and cfu-Alpl using bone marrow-derived cells isolated from the femurs of wild-type mice and Ccn3 KO mice. The numbers of total colonies and cfu-Alpl significantly increased in cultures of Ccn3 KO mice compared with the numbers in wild-type mice (Fig. 8, B–D). The percentage of Alpl-positive colonies in the total col-
onies was also significantly elevated in cultures of Ccn3 KO mice compared with that in wild-type mice (Fig. 8E), suggesting that the number of osteoprogenitors was greater in the bone marrow of Ccn3 KO mice than in that of wild-type mice. We found no colonies showing mineralization by von Kossa staining.

Because we reported that CCN3 is a BMP antagonist and inhibits phosphorylation of Smad1/5, which is a BMP-specific signaling molecule, in osteoblastic cells (25, 26), the expression of phosphorylation of Smad1/5 during bone regeneration in wild-type mice and Ccn3 KO mice was investigated by Western blot and immunohistochemistry. Western blot analysis demonstrated higher expression of phosphorylated Smad1/5 in Ccn3 KO mice compared with that in wild-type mice (Fig. 8F). Although few cells exhibited nuclear localization of phosphorylated Smad1/5 in the bone regeneration site in
wild-type mice (Fig. 8G), fibroblastic/osteogenic cells in the bone regeneration site frequently showed nuclear localization of phosphorylated Smad1/5 on day 5 after injury in Ccn3 KO mice (Fig. 8H). Collectively, these results indicate that CCN3 is up-regulated at an early stage of bone regeneration and exerts inhibitory effects on bone regeneration by attenuating the number of osteoprogenitors and BMP signaling.

**DISCUSSION**

We showed the apparent expression of CCN3 by immunohistochemistry and RT-PCR analyses during bone regeneration, whereas normal adult bone tissue exhibited an almost undetectable level of CCN3. Kawaki et al. (38) demonstrated that CCN3 is expressed in osteoblast lineage cells of fetal calvaria by immunohistochemistry. Because some factors that are expressed in organogenesis, and not in adults, are recaptured in regeneration processes in adulthood, CCN3 is likely to be such a factor in bone regeneration. We identified Ccn3 as an up-regulated gene in bone regeneration by microarray analysis using samples of normal bones and regenerative bones. This approach will be helpful to identify genes that are preferentially up-regulated in bone regeneration but not in normal adult bones.

We previously demonstrated that CCN3 suppresses osteogenic differentiation in vitro through interaction with the BMP
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FIGURE 7. Bone regeneration in Ccn3 KO mice. A, micro-CT images of drill hole injury on days 5, 10, and 15 after the surgery. WT, wild-type mice; KO, Ccn3 KO mice. B, BMD color mapping images obtained from micro-CT analysis of the drill hole injury on days 5, 10, and 15 after the surgery. C, quantitative analysis of newly formed bone assessed by micro-CT. *, p < 0.05, n = 3–4 in wild-type mice, and n = 4–7 in Ccn3 KO mice. D, typical histology of bone regeneration in WT and Ccn3 KO mice. Error bars, S.E.

In this mouse was similar to that in Ccn3 Tg mice generated by another group using osteocalcin promter (39). Although these results suggest that overexpression of Ccn3 in osteoblast lineage cells inhibits osteogenesis, further studies are necessary to conclude the roles of CCN3 in osteoblast differentiation in vivo because we assessed the phenotype using only a single Ccn3 Tg line. In contrast, Ccn3 KO mice showed no apparent skeletal phenotype. A similar skeletal phenotype was also reported in Ccn3 KO mice generated by another group (40), in which exons 1–5 were deleted, suggesting that CCN3 is dispensable for bone homeostasis. However, other Ccn3 mutant mice (NOVallele−/−), in which exon 3 was deleted and produces no wild-type CCN3 but expresses a mutant CCN3 peptide, exhibited multiple skeletal changes, including overgrowth of skeletal elements with enlarged vertebrae, elongated long bones, and digits in fetal mice and overgrowth of the appendicular and axial skeleton, including abnormal joint formation, in adult mice (41). Because this study presented no quantitative data on bone metabolism, including bone histomorphometric analysis, it is difficult to conclude that the skeletal phenotype observed in this NOVallele−/− mouse was caused by abnormalities of bone metabolism or skeletal morphogenesis. It is also suggested that the presence of the mutant CCN3 peptide in NOVallele−/− leads to phenotypic differences between our Ccn3 KO mice and NOVallele−/− mice (30). Thus, these observations suggest that, although CCN3 has a definite role in skeletogenesis, the absence of CCN3 can be compensated for in normal skeletal development.

We applied the drill hole injury model to investigate bone regeneration in the present study. In this model, an injury was created in cortical bone at the frontal part of the diaphysis of the femurs. This procedure provides a mechanically stable environment by protecting regenerative tissues from physical disturbances and yields more consistent results compared with other bone regeneration models, such as amputation of the diaphysis (42). We also scraped the periosteum of the injury site during the surgery, and this procedure generated intramembranous ossification during bone regeneration by eliminating endochondral ossification. Thus, bone healing in this model mainly occurred by intramembranous ossification, providing more stable bone regeneration by eliminating chondrocyte differentiation (32, 42, 43).

Up-regulation of CCN3 expression at an early stage of bone regeneration suggested that CCN3 plays some role in bone regeneration. To explore this possibility, we applied a drill hole injury model to Ccn3 Tg mice and Ccn3 KO mice. Although Ccn3 Tg mice exhibited osteopenia probably due to the disturbance of differentiation and function of osteoblasts in uninjured tibiae, we found no apparent changes in bone regeneration in this mouse. This might be due to a small induction ratio of Ccn3 expression between uninjured and injured bones in Ccn3 Tg mice. In the wild-type mice, Ccn3 expression exhibited an ~10-fold increase on days 5 and 10 after bone injury compared with that at a basal level before injury, whereas Ccn3 Tg mice showed only 2–3-fold increase during bone regeneration compared with that before injury (Fig. 6B). The small up-regulation of CCN3 during bone regeneration might be compensated for by CCN3 that had greatly elevated at basal level in

and Notch signaling pathways (25–27). CCN3 directly binds to BMP-2 and inhibits BMP signal transduction, indicating that CCN3 is an antagonist of BMP-2 (25–27). In this study, we further examined the effect of CCN3 on skeletal tissues using osteoblast-specific Ccn3 Tg mice and Ccn3 KO mice. Ccn3 Tg mice showed osteopenia, probably due to the disturbance of osteoblast differentiation and function. The skeletal phenotype

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Ccn3 Tg mice. In contrast, we clearly showed accelerated bone regeneration in Ccn3 KO mice. This might be due to a lack of intrinsic CCN3, which inhibits osteoblast differentiation and function, during bone regeneration. These results confirmed that CCN3 is involved in bone regeneration as an inhibitory factor. We previously reported that Ccn3 KO mice presented no apparent vascular phenotype under normal conditions but exhibited enhanced proliferation of vascular smooth muscle cells and intimal thickening after endothelial injury (30). These results favor the acceleration of bone regeneration in Ccn3 KO mice, because vascularization is a critical factor in the appropriate progress of bone regeneration.

BMP is an important factor in bone regeneration. Several reports have demonstrated that BMP antagonists, such as Noggin, Chordin, and Gremlin1, are induced during bone regeneration (5–8, 44). Noggin and Gremlin1 were up-regulated following osteogenic induction by BMP in primary calvarial cells (45, 46). These data suggest that a negative feedback loop is required to prevent the overfunction of BMP in bone regeneration. In our bone regeneration model, the expression levels of Noggin, Chordin, and Gremlin1 in Ccn3 KO mice were significantly higher than those in wild-type mice (Fig. 6A). The higher expression of these genes might be caused by a compensation effect due to the lack of CCN3, suggesting that CCN3 is an important BMP antagonist in bone regeneration.

To explore the mechanism underlying the stimulatory effects of CCN3 deficiency in bone regeneration, we compared cfu-F and cfu-Alpl between bone marrow cells isolated from wild-type mice and Ccn3 KO mice. This experiment indicated an increased number of cfu-F and cfu-Alpl in bone marrow cells isolated from Ccn3 KO mice compared with those from wild-type mice. Ccn3 KO mice also exhibited a higher percentage of cfu-Alpl in total cfu-F. These results suggest that bone marrow in Ccn3 KO mice contains more osteoprogenitors than that in wild-type mice, and these osteoprogenitors effectively participate in bone regeneration. These also suggest that CCN3 defi-
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Efficiency is involved in proliferation of osteoprogenitors in bone marrow. In addition, we compared the expression of phosphorylated Smad1/5 during the bone regeneration process in wild-type and Ccn3 KO mice by Western blot analysis, because we previously demonstrated that overexpression of Ccn3 in osteoblastic cells attenuated osteoblast differentiation by suppressing the phosphorylation of Smad1/5/8 (25–27). As expected, the expression of phosphorylated Smad1/5 was higher in Ccn3 KO mice than that in wild-type mice. Further, we confirmed that the nuclear localization of Smad1/5 was more frequently observed in fibroblastic/osteoblastic cells in Ccn3 KO mice than in wild-type mice by immunohistochemistry. These results suggest that the phosphorylation and the nuclear localization of Smad1/5 induced by CCN3 deficiency stimulated osteoblast differentiation at bone-regenerating sites in Ccn3 KO mice.

CCN3 has diverse roles in the generation of mesenchymal tissues. It positively regulates angiogenesis by promoting adhesion and movement of the endothelial cells via integrin signaling (47). CCN3 maintains the self-renewal of hematopoietic stem cells and supports the development of committed blood cells (48). CCN3 has a critical role in the direction of chondrocytes to articular cartilage (49). Chondrogenesis was also positively regulated by CCN3 in coordination with TGF-β1 signaling (23). Conversely, CCN3 inhibits myogenic (24) and osteoblastic differentiation (25, 26). These diverse roles of CCN3 can be attributed to its intrinsic ability to interact with many molecules and modulate their functions. CCN proteins (except for CCN5) have four structurally distinct domains: the insulin-like growth factor-binding protein-like, von Willibrand factor-like, thrombospondin 1-like, and C-terminal knot domains. Although the complete list of proteins that can associate with each domain is lacking, they are expected to interact with a large array of proteins, considering the polyreactive nature of these domains. In fact, CCN3 has been shown to directly bind to various proteins, including major signaling molecules such as integrin, BMP, and Notch (25–27, 47, 50). Thus, CCN3 might act as a modulator during healing that coordinates diverse intercellular and intracellular signaling pathways (51) and regulates the volume and localization of each mesenchymal component to maintain tissue integrity.

In summary, the present study demonstrates that CCN3 appears at an early stage of bone regeneration and that it participates in bone regeneration as an inhibitory factor. These results indicated that the use of counteracting agents against CCN3 is a potential strategy to develop new bone regeneration therapies.

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