Cartilage–Specific Over-Expression of CCN Family Member 2/Connective Tissue Growth Factor (CCN2/CTGF) Stimulates Insulin-Like Growth Factor Expression and Bone Growth

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Abstract

Previously we showed that CCN family member 2/connective tissue growth factor (CCN2) promotes the proliferation, differentiation, and maturation of growth cartilage cells in vitro. To elucidate the specific role and molecular mechanism of CCN2 in cartilage development in vivo, in the present study we generated transgenic mice overexpressing CCN2 and analyzed them with respect to cartilage and bone development. Transgenic mice were generated expressing a ccn2/lacZ fusion gene in cartilage under the control of the 6 kb-Col2a1-enhancer/promoter. Changes in cartilage and bone development were analyzed histologically and immunohistologically and also by micro CT. Primary chondrocytes as well as limb bud mesenchymal cells were cultured and analyzed for changes in expression of cartilage-related genes, and non-transgenic chondrocytes were treated with culture with recombinant CCN2. Newborn transgenic mice showed extended length of their long bones, increased content of proteoglycans and collagen II accumulation. Micro-CT analysis of transgenic bones indicated increases in bone thickness and mineral density. Chondrocyte proliferation was enhanced in the transgenic cartilage. In vitro short-term cultures of transgenic chondrocytes, the expression of col2a1, aggrecan and ccn2 genes was substantially enhanced; and in long-term cultures the expression levels of these genes were further enhanced. Also, in vitro chondrogenesis was strongly enhanced. IGF-I and IGF-II mRNA levels were elevated in transgenic chondrocytes, and treatment of non-transgenic chondrocytes with recombinant CCN2 stimulated the expression of these mRNA. The addition of CCN2 to non-transgenic chondrocytes induced the phosphorylation of IGFR, and ccn2-overexpressing chondrocytes showed enhanced phosphorylation of IGFR. Our data indicates that the observed effects of CCN2 may be mediated in part by CCN2-induced overexpression of IGF-I and IGF-II. These findings indicate that CCN2-overexpression in transgenic mice accelerated the endochondral ossification processes, resulting in increased length of their long bones. Our results also indicate the possible involvement of locally enhanced IGF-I or IGF-II in this extended bone growth.

Citation: Tomita N, Hattori T, Itoh S, Aoyama E, Yao M, et al. (2013) Cartilage–Specific Over-Expression of CCN Family Member 2/Connective Tissue Growth Factor (CCN2/CTGF) Stimulates Insulin-Like Growth Factor Expression and Bone Growth. PLoS ONE 8(3): e59226. doi:10.1371/journal.pone.0059226

Editor: Frank Beier, University of Western Ontario, Canada

Received October 26, 2012; Accepted February 12, 2013; Published March 28, 2013

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Funding: This work was supported by the program Grants-in-Aid for Scientific Research (C) to TH and (S) to MT and Exploratory Research (to MT) from the Japan Society for the Promotion of Science and by internal grants from Okayama University (to TH) and by a grant from Senri Life Science Foundation (to TH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

CCN2/CCN family 2/CTGF (connective tissue growth factor) is a member of the CCN family of secreted proteins, which also includes Cyr61/CCN1, NOV/CCN3, WISP1/CCN4, WISP2/CCN5, and WISP3/CCN6. CCN2 regulates diverse cell functions including mitosis, adhesion, apoptosis, extracellular matrix (ECM) production, growth arrest, and cellular migration [1,2]. The multimodular character of CCN factors allows multiple interactions between them and other growth factors such as TGFß, BMPs, IGFs or VEGF and networking between growth factors, extracellular matrix, and cell-surface receptors such as integrins [3]. Thus, it is not surprising that CCN factors are involved in a multiplicity of effects during development, differentiation, wound healing, and disease states, including tumorigenesis and fibrosis [2]. Most prominently, CCN2 has emerged as a major regulator of chondrogenesis, angiogenesis, and fibrogenesis [4]. CCN2 induces the migration of endothelial cells [5,6,7] and stimulates the synthesis of matrix proteins including collagens and fibronectin [8,9]. It is expressed in various tissues, with highest levels found in prehypertrophic chondrocytes and vascular tissues in developing embryos [for reviews, see refs [4,10]. Previously we demonstrated in a series of in vitro studies that CCN2 stimulates both the proliferation and synthesis of type II collagen and proteoglycans of growth-plate chondrocytes [11], human chondrosarcoma-derived chondrocytic cells [11,12], articular chondrocytes [13], and auricular chondrocytes [14]. Moreover, it induces hypertrophy

PLOS ONE | www.plosone.org 1 March 2013 | Volume 8 | Issue 3 | e59226
and calcification of growth-plate chondrocytes, but not those of articular or auricular chondrocytes [11,14,15]. Also, osteoblast proliferation and maturation are stimulated by CCN2 [16]. These in vitro findings are consistent with studies on CCN2-deficient mice, which develop skeletal dysmorphisms including kinky bone and cartilage elements, due to impairment of chondrocyte proliferation and extracellular matrix deposition in the hypertrophic zone [17]. As a result of CCN2 deficiency, growth-plate angiogenesis and endochondral ossification are partially impaired, and CCN2-deficient mice die after birth because of respiratory failure caused by the skeletal defects [17]. Although multiple effects of CCN2 on differentiation, proliferation, and matrix synthesis of chondrocytes, fibroblasts, endothelial cells, and osteoblasts have been reported, the specific role of CCN2 synthesized by chondrocytes during cartilage and bone development in vivo remains unclear.

To elucidate the role of chondrocyte-derived CCN2, we generated CCN2-over-expressing mice with the gene expressed under the control of a 6 kb-Col2a1 promoter that included a cartilage-specific enhancer element in the first intron of the Col2a1 gene and obtained in vivo evidence for a key role of CCN2 in regulating chondrocyte gene expression and cartilage differentiation. Furthermore, our data suggest that CCN2 regulates the endochondral ossification process in long bones partially through increased expression of IGF-I and IGF-II.

**Materials and Methods**

**Generation of Transgenic Mice**

To express the ccn2 as transgene in chondrocytes, we cloned the cDNA encoding a HA-tagged mouse ccn2 gene into a vector containing 3 kb of the Col2a1 promoter and 3.02 kb of the intron 1 sequence [18,19]. The LacZ gene preceded by an internal ribosomal entry site was placed downstream of the ccn2 cDNA (Fig. 1A). This construct was microinjected into the pronuclei of fertilized C57BL/6CrSlc eggs to generate transgenic mice. Routine genotyping to identify the transgene was done by PCR on genomic DNA. The primer sequences used were 5-GCATCGAGCTGGGTAATAAGCGTTGGCAAT-3 and 5-GACACCAGACCAACTGGTAATGGTAGCGAC-3. All experimental procedures were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan and approved by the Animal Research Control Committee of Okayama University (Approval No.: OKU-20121113).

**LacZ Staining and Skeletal Preparation**

LacZ activity was detected by staining with X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside; Roche) for 3–6 hours following fixation with glutaraldehyde and formaldehyde as described earlier [20]. For staining of embryos older than 15.5 days, the skin and internal organs were removed before fixation. LacZ-stained embryos were postfixed overnight in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections were counterstained with eosin. Some LacZ-stained embryos were cleared with KOH–glycerol. Skeletal morphology was analyzed by alizarin red and alcian blue staining followed by clearing with 1% (w/v) KOH [21,22].

**RNA Preparation and Northern Hybridization**

RNA was prepared either directly from cartilage or from chondrocyte cultures. For the direct RNA preparation, rib cages of E18.5 or 19.5 embryos were separated from soft tissues, and single ribs were isolated. The isolated ribs were separated from bone, and the cartilage was soaked in Isogen (Nippon Gene) and homogenized until the tissue clumps had disappeared. The cartilage RNA were purified according to the Isogen instructions, and the purified RNA were further cleaned by using the RNasea kit (Qiagen). For the RNA preparation from chondrocytes, the cells from rib cartilage were cultured as described below, harvested, and then subjected to RNA purification using the RNasea kit. For Northern hybridization, 10 μg of RNA from costal cartilage was resolved on an agarose gel, transferred onto a nylon membrane (Bio-Rad), and hybridized with 32P-labeled LacZ or ccn2 probes as described previously [23].

**Western Blotting**

Rib cartilage from E18.5 embryos was isolated as described above and homogenized with lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM PMSF). After centrifugation, the supernatant was collected; and 6 μg of protein per lane was loaded onto an SDS-PAGE gel. Western blotting was done as described previously [24] by using anti-HA (Covance), anti-actin (Sigma), anti-phospho IGF-1 receptor (Cell Signaling), and anti-IGF-1 receptor (Cell Signaling) antibodies.

**Histological Examination**

For histological analysis, tissues from E17.5 and E19.5 embryos and from 1- and 3-day postnatal mice were fixed with 10% formaldehyde/PBS, deamineralized with 0.5 M EDTA, and embedded in paraffin. Then 7 μm-thick-sections were stained with hematoxylin, eosin, and safranin-O. Immunohistochemical staining was performed by using a peroxidase-conjugated polymer (Nichirei, Japan) and anti-type II collagen MoAb (CII D3, [25] or anti-type X MoAb (X53, kindly provided by Dr. K. von der Mark, Germany, [26,27]). For cell proliferation analysis, a PCNA staining kit (Zymed) was used. For detection of apoptotic cells, TUNEL analysis was performed by using an In Situ Cell Death Detection Kit, POD (Roche).

**Cell Cultures**

For preparation of primary cultures, chondrocytes were isolated from the rib cages of 18.5- or 19.5-day embryos and/or newborn mice as described previously [28]. Briefly, the rib cages were digested with collagenase (0.1% collagenase P, Roche, in F12/DMEM containing 10% fetal calf serum) after adhering connective tissue and muscle had been thoroughly removed by trypsin pretreatment. The cells were grown to confluence for 1 month to hypertrophy in α-modification of minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and supplemented with 50 μg/ml of ascorbic acid with or without recombinant CCN2, and then harvested for RNA extraction. For preparation of CCN2 recombinant protein, human ccn2 cDNA was amplified by PCR and subcloned into the pET-13b vector (Novagen), which harbors a His-tag; and *E. coli* BL21(DE3)pLysS Rosetta strain cells were subsequently transformed with this vector. Expressed His-tagged CCN2 protein was purified by the use of Ni-NTA agarose.

For inhibition of autophosphorylation of IGF-1 receptor, the IGF-1 receptor inhibitor PPP (Calbiochem) was used, at a concentration of 60 nM. Anti-CCN2 monoclonal antibody (11H3, kindly provided by Dr. Seto, Nippn Flour Mills Co., LTD.), which had an inhibitory effect on the CCN2-mediated enhancement of aggrecan gene expression was also used to inhibit this autophosphorylation.
Quantitative real-time PCR

Reverse transcription (RT) was performed with 0.5 μg of total RNA as described above, and the resulting cDNA was amplified in triplicate by using the SYBR-Green PCR assay (TOYOBO SYBR Green PCR Master Mix; TOYOBO, Osaka, Japan), after which the products were detected with a LightCycler™ system (Roche, Basel, Switzerland). PCR reaction mixtures were incubated for 15 min at 95°C, followed by 50 amplification cycles of 30 s annealing at 60°C, 40 s extension at 72°C, and 30 s denaturation at 95°C. GAPDH was used to standardize the total amount of cDNA, as described previously [29].

The primers designed for real-time PCR were the following: ccn2 (forward, 5'-GGTAAGGTCGATTTCACTCAC-3'; reverse, 5'-CTAGAAAGGTGCAAACATGTAAC-3'); gapdh (forward, 5'-GCCAAAAGGGTCATCATCTC-3'; reverse, 5'-GTCTTCTGGGTGGCAGTGAT-3'); aggrecan (forward, 5'-ACCATCACTGAGGGCGAAGC-3'; reverse, 5'-AGCTGAGGGCGAAGC-3'); Col2a1 (forward, 5'-GACCGGCTCCTGCTCCAC-3'; reverse, 5'-CACTGAGGGCGAAGC-3'); vegf (forward, 5'-ATGAACTGATCAAGTTC-3'; reverse, 5'-GACTTACTCTGGACCTGCC-3'); Col10a1 (forward, 5'-GCCAGGGTTACGAGGACAA-3'; reverse, 5'-GGTCAGGTCTGGGACCTGCG-3'); IRES-LacZ (top) and ccn2 (middle) probes were used to detect transgenic and endogenous ccn2, respectively. (F) Western blot (WB) analysis using cell lysates fromtg and wt cartilage and anti-HA antibody recognizing only the CCN2-HA transgene products (left blot). The HA-tagged CCN2 was expressed in cartilage oftg mice. A Western blot of the same cell lysate reacted with anti-actin antibody as a loading control is also shown (right blot).

Figure 1. Generation of Col2a1-ccn2 transgenic mice. (A) Schematic representation of the construct of the expression of HA-tagged CCN2 and IRES-LacZ in chondrocytes driven by the 6-kb Col2a1 promoter-enhancer. The original initiation codon of Col2a1 was mutated to CTG to facilitate translation from downstream cDNA. (B) Genotyping of transgenic mice (tg) by PCR to detect the transgene. wt, wild type. The location of the primers used are indicated in "A" by arrows. (C) Skeletal preparation of a newborn mouse after whole-mount X-gal staining, showing cartilage-specific expression of the transgene. (D) Sagittal sections of ulnae from wt andtg after whole-mount X-gal staining. All of the cartilaginous cells showed X-gal staining. The sections were counter-stained with Safranin-O. (F) Analysis of transgene expression by Northern hybridization using total RNA fromtg and wt cartilage. LacZ (top) and ccn2 (middle) probes were used to detect transgenic and endogenous ccn2, respectively. (F) Western blot (WB) analysis using cell lysates fromtg and wt cartilage and anti-HA antibody recognizing only the CCN2-HA transgene products (left blot). The HA-tagged CCN2 was expressed in cartilage oftg mice. A Western blot of the same cell lysate reacted with anti-actin antibody as a loading control is also shown (right blot). doi:10.1371/journal.pone.0059226.g001
Enhanced IGF-IGFR Pathway in CCN2 Transgenic Mice

(A) Images of mice showing the enhanced IGF-IGFR pathway in CCN2 transgenic mice.

(B) Graph showing the expression levels of ccn2/gapdh in transgenic (tg) and wild-type (wt) mice.

(C) Bar chart comparing the tibia length (mm) in transgenic (tg) and wild-type (wt) mice.

(D) Micrographs of bone sections from transgenic (tg) and wild-type (wt) mice.

(E-1) Bar chart showing the total mineral content in transgenic (tg) and wild-type (wt) mice.

(E-2) Graph showing the trabecular mineral content in transgenic (tg) and wild-type (wt) mice.

(F) Diagram illustrating the cortical bone and cancellous bone with distal growth plate cartilage.

(G) Bar chart showing the cortical thickness in transgenic (tg) and wild-type (wt) mice.
Figure 2. Skeletal analysis of Col2a1-ccn2 transgenic mice. (A) Skeletal preparation of representative tg and wt littermates at E15.5 after alizarin red and Alcian blue staining. Skeletal development in tg mice appeared normal at this stage. (B) At 8 weeks the transgenic mice consistently showed an ~12% increase in body size. (C) Quantitative analysis by real-time PCR of ccn2 mRNA levels in primary cultures of tg and wt rib chondrocytes revealed high-expressing transgenic mice (e.g., #tg76/#tg74) in each litter, besides low-expressing littermates (#tg72), the latter of which expressed ccn2 at about the same level as the wt littermates (see also Fig. 2D). Real time-RCR analysis was repeated at least 2 times for each RNA preparation, and the 2 founder lines showed similar variations, but basically the same results. (D) Hematoxylin-eosin (HE) staining of transgenic and wild-type P1 tibiae from the same litters as shown in Fig. 2C. Tibiae from transgenic littermates showed a relatively extended lengths of the diaphyses in the high-expressing transgenic littermates. Tg and wt with a number indicate transgenic and non-transgenic littermates, respectively. Six litters from 2 different founder lines were investigated. (E-1) Diaphysis length of tibiae from transgenic and wild-type littermates of a P3 litter. Tibial diaphysis lengths of only pups that showed significantly enhanced levels of ccn2 mRNA, measured in primary cultures of rib chondrocytes were measured. Serial sections (5-7 slides) were randomly selected every 3 slides from a single tibia, and stained with HE. The images were incorporated into a computer, and the length of diaphyses were measured. Bars indicate the mean length and standard deviations of diaphyses of tibia from wild-type and transgenic littermates (e.g., 2 wt, 8 tg). (E-2) Mean length of diaphyses of tibiae from the wild-type and transgenic mice indicated in E-1: *p<0.0001. Two different founder lines with 3 litters each were analyzed and similar results were obtained. (F) Left: Representative micro-CT image (cross section) of femora of 8-week-old tg and wt littermates. Right: Positions of measurement in femur. (G) Peripheral quantitative computed tomography (pQCT) analysis (XCT Research SA, Aartselaar, Belgium). For generation of transgenic mice over-expressing CCN2 in cartilage, the distal epiphysial end by peripheral quantitative computed tomography (micro-CT) analysis, the same position was measured in cartilage of postnatal transgenic mice, tibiae of transgene and wild-type mice were scanned over the region from 1.2 mm to 4.0 mm from the growth plate, as indicated in "F" (right). Bars represent the mean ± SD (n=9, males). In transgenic bones significant enhancement was seen in total mineral content (tg: 1.36±0.08 mg/mm vs. wt: 1.10±0.12 mg/mm), in trabecular mineral content (tg: 0.49±0.01 mg/mm vs. wt: 0.38±0.01 mg/mm), and in cortical thickness (tg: 0.060±0.013 mm vs. wt: 0.049±0.021 mm); but only in the femora at site #1 were the differences significant (*P<0.05), doi:10.1371/journal.pone.0059226.g002

GATCTGCAATGG-3'; mnp9 (forward, 5'-GAAACTCACAG-GACATCTTCCA-3'; reverse, 5'-GAAGAATTT-3'); IGFI-1 (forward, 5'-GTTGGGACC-GAGGGGCTTTTACTTC-3'; reverse, 5'-GCCTTCAGTGGGGCACAGTACATCTC-3'); and IGFI-II (forward, 5'-TGGGATCGTGGGAAGAGTGTC-3'; reverse, 5'-GGGTTGGGATGGAAACAGG-3'); lacZ (forward, 5'-GGTTAACGATGGCCGATCTA-3'; reverse, 5'-ACGGCG-GATTGACCGTAAAT-3').

Micromass Culture
For preparation of micromass cultures, limbs from E11.5 embryos were digested in 0.05% trypsin for 1 hour on ice. After the cells had been suspended by pipetting, they were concentrated in 10% FCS-containing DMEM/F12 to 1 x 10^5 cells/ml. Ten microliters of cell suspension containing 1 x 10^5 cells was placed in the center of each well of a 24-well plate; and the cells were allowed to adhere to the bottom of the well for 1 h after the plate had been placed in an incubator (5% CO_2, 37°C). Thereafter, 1 ml of culture medium was added to each well; and the medium was replaced every 24 hours. Cell condensation in the cultures was visible after 1 or 2 days, and cartilage nodules appeared after 3 days. Some cells were stained with Alcian blue (pH 1) to visualize cartilage, and others were harvested for extraction of total RNA.

Analysis of Bone Mineralization
The femora from 8-week-old mice were removed, and the bones were scanned over the region from 1.2 mm to 4.0 mm from the distal epiphysial end by peripheral quantitative computed tomography [pQCT] analysis ([XCT Research SA]+Stratec Medizintechnik GmbH, Pforzheim, Germany]). For the micro-computed tomography (micro-CT) analysis, the same position was scanned by using a Skyscan 1072 micro-CT machine (Skyscan, Aartselaar, Belgium).

Results
Cartilage-specific Over-expression of ccn2 in Chondrocytes of Transgenic Mice Caused Increased Bone Size
For generation of transgenic mice over-expressing CCN2 in cartilage, HA-tagged ccn2 cDNA was cloned into a vector containing 3 kb of the Col2a1 promoter, 3.02 kb of the Col2a1 intron 1 sequence, and IRES-LacZ (Fig. 1A). The purified vector DNA was injected into oocytes, and 2 founders tested positive for the ccn2-LacZ transgene by PCR (Fig. 1B) and were kept to establish transgenic lines. X-gal staining of newborn transgenic mice showed intense, cartilage-specific lacZ expression in all cartilage elements (Fig. 1C). In tissue sections of newborns, all growth-plate and resting chondrocytes were positive after X-gal staining, indicating that the expression domains of the transgene overlapped with those of endogenous ccn2 (Fig. 1D; and see also [30]).

Over-expression of the ccn2 transgene in chondrocytes of the transgenic mice was confirmed by Northern and Western blot analyses. Northern blot hybridization of total RNA extracted from rib cage chondrocytes of E18.5 embryos with probes for LacZ and ccn2 showed a reaction with the same 6-kb transcript in transgenic, but not wt, chondrocyte RNA (Fig. 1E, LacZ and ccn2). The intensity of the transgene signal obtained with the ccn2 probe was about 75% of that of the endogenous ccn2 mRNA (Fig. 1E, middle panel). Endogenous ccn2 mRNA was also up-regulated (~110% of wild type) in transgenic cartilage (Fig. 1E, ccn2), possibly due to an autocrine mechanism. The HA-tagged CCN2 protein was detected in cell lysates from transgenic rib cartilage by Western blot analysis using an anti-HA antibody (Fig. 1F).

At day E15.5 of embryonic development, no major abnormalities in cartilage or bone development were detected in the transgenic animals (Fig. 2A). At 8 weeks, however, the majority of the transgenic mice were about 12% larger than their wild-type littermates (Fig. 2B).

For detailed analysis of the morphological alterations in the skeleton of postnatal transgenic mice, rib of transgenic and wild-type newborns were sectioned, and their length was measured. The levels of ccn2 mRNA in chondrocytes cultured from rib cartilage of the same animal were also monitored. Quantitative real-time PCR analysis of ccn2 mRNA levels in rib chondrocytes in primary culture revealed high-expressing transgenic mice (e.g., #tg76/#tg74), as well as low-expressing transgenic littermates (#tg72) in the same litter, which expressed ccn2 at about the same level as the wt littermates (Fig. 2C). Comparison of tibial length and the ccn2 mRNA expression level of chondrocytes prepared from rib cartilage of the same animal showed a positive correlation (Fig. 2C and D). The length of diaphyses of tibiae from wt and transgenic littermates at the P3 stage was also measured. The expression level of ccn2 mRNA in primary cultures of rib chondrocytes from littermates was monitored, and tibiae from pups with significantly higher levels of ccn2 mRNA compared with wt levels were used for
Enhanced IGF-IGFR Pathway in CCN2 Transgenic Mice

(A) tg wt

(B) tg wt

(C) tg wt

(D) tg wt
Figure 3. CCN2 overexpression causes enhanced type II collagen and proteoglycan deposition, enhanced chondrocyte proliferation and shortening of the hypertrophic cartilage zone. Tibiae from P1 littersmates were stained with safranin-O for proteoglycans (A, left) and with anti-type II collagen antibody (B, left). Whole littermates were analyzed and the color intensity of 3 different wt or tg individuals was measured densitometrically, and the mean values are presented. (A, right; and B, right). *: p<0.005. Typical images from tg and wt littermates are shown. (C, left) Comparison of hypertrophic cartilage zone of CCN2 transgenic littermates. Tibiae were stained with type X collagen antibody. (C, right) The hypertrophic zone of tg cartilage appeared shorter compared with that of the wt cartilage. (D) Immunohistochemical analysis of proliferative cell nuclear antigen (PCNA) in tibiae of ccn2 tg embryos at E19.5. Proliferative cells were observed in the whole epiphyseal cartilage of tg animals, whereas they were restricted to the proliferative zone of the wt littermates. The number of PCNA-positive cells inside of the boxed area was counted in 5 fields of 3 comparable wt and tg sections. Mean values indicate enhanced chondrocyte proliferation in the tg cartilage (graph at the lower right). *: p<0.05. doi:10.1371/journal.pone.0059226.g003

Over-expression of CCN2 Increased Bone Density, Extent of Mineralization of Cancellous Bone, and Thickness of Cortical Bone

Further evidence for a stimulation of bone growth by CCN2 in transgenic animals was obtained when bone density and mineral content of cancellous bone were monitored by using peripheral-quantitative computed-tomography (pQCT) analysis. For these studies, 8-week-old femora from 4 wt and 5 tg littermates were analyzed for mineral content and cortical bone thickness at 2 sites, one 1.2 mm, and the other more central 4.0 mm distal from the growth plate (Fig. 2F). Significant differences (p<0.05) in total mineral content (tg: 1.36±0.08 mg/mm vs. wt: 1.10±0.12 mg/mm), trabecular mineral content (tg: 0.49±0.01 mg/mm vs. wt: 0.30±0.01 mg/mm), and cortical thickness (tg: 0.060±0.013 mm vs. wt: 0.049±0.021 mm) were observed for the part of the femora closer to the growth plate (Fig. 2F and 2G), but not for the central site (data not shown).

Over-expression of CCN2 in Chondrocytes Caused Enhanced Accumulation of Extracellular Matrix and Shortened Hypertrophic Zones

To examine the possibility that the extended skeletal growth of ccn2 transgenic mice may have been due to enhanced production of cartilage matrix in the epiphysis, we analyzed the extracellular deposition of proteoglycans and type II collagen in the cartilage matrices by staining with safranin O and anti-type II collagen, respectively. Safranin-O staining indicated consistently an enhanced density of proteoglycans in the transgenic cartilage in comparison with cartilage of wt littermates (Fig. 3A). This observation is in accordance with our previous studies showing that CCN2 promotes proteoglycan synthesis in chondrocytes [11]. Also, the immunohistological analysis of type II collagen showed an enhanced reaction in resting chondrocytes and in the growth plate (Fig. 3B and Figure S1A). These results indicate that the over-expression of CCN2 enhanced the production and deposition of extracellular proteoglycans and type II collagen, which is in line with our previous in vitro findings. Surprisingly, however, the enhanced matrix deposition did not result in an increase in the size of the cartilaginous epiphysis; rather, the extended bone length was the result of an elongated bony shaft of the diaphysis.

Staining of the skeleton of transgenic embryos with type X collagen antibodies indicated that the hypertrophic zone was shorter in the transgenic embryos than in their wt littermates (Fig. 3C). This observation suggests an acceleration of chondrocyte proliferation and maturation, but possibly also accelerated cartilage resorption and chondrocyte apoptosis in these transgenic animals. Therefore, we next measured chondrocyte proliferation and apoptosis rates in the growing long bones of ccn2 transgenic animals and their wt littermates.

Over-expression of CCN2 Resulted in Enhanced Cell Proliferation and Slightly Elevated Apoptosis of Epiphyseal Chondrocytes

In order to assess whether the enhanced bone growth of CCN2 transgenic animals was due to enhanced cell proliferation, we stained sections of E19.5-day transgenic and wt embryos with an antibody against proliferative cell nuclear antigen (PCNA). The data show that over-expression of CCN2 stimulated chondrocyte proliferation predominantly in the proliferative zone, but also in the resting zones (Fig. 3D). This observation is in accordance with previous in vitro studies showing that CCN2 promotes chondrocyte proliferation [11].

Curiously, however, staining for apoptotic cells in the growth plate of P3 by using the TUNEL assay revealed slightly, but not significantly, enhanced accumulation of apoptotic cells at the cartilage-bone interface and in the adjacent subchondral zone in the transgenic embryos as compared with their numbers in the wild-type (Fig. S1B). The length of the cartilaginous epiphyses seemed unaffected, since chondrocyte proliferation, cartilage matrix deposition, maturation, cartilage resorption, apoptosis, and assembly of trabecular bone were accelerated by the over-expressed CCN2.

Over-expression of CCN2 in Chondrocytes Resulted in Enhanced Gene Expression of Col2a1 and aggrecan, and in Enhanced Chondrocyte Maturation in vitro

The increased accumulation of proteoglycan and type II collagen in the cartilage matrix of transgenic animals raised the question as to whether ccn2 over-expression in chondrocytes stimulated cartilage and bone growth by enhancing cell proliferation, by stimulating the production of extracellular matrix or by accelerating the differentiation and maturation of chondrocytes. To obtain high ccn2 transgene-expression, we crossed transgenic male and female mice and monitored the effects of over-expression of CCN2 in chondrocytes on the expression of extracellular matrix genes. RNA was extracted from short-term primary cultures of rib-cage chondrocytes from E18.5 transgenic or wild-type embryos and analyzed for lacZ, ccn2, and Col2a1 mRNA levels by quantitative real-time PCR. The data showed about equal levels of lacZ expression in chondrocytes of transgenes #72,74, 76, 77 and 79, and a 2–3 fold higher level of the lacZ expression in tg #73 and #75, indicating that offspring #73 and #75 may bear double copies of transgene (Fig. 4A). Accordingly, the ccn2 level in chondrocytes derived from these embryos (#73, 75 tg) was 2–3
fold enhanced as compared with the level for the wt chondrocytes (#78 wt, Fig. 4B). Also tg #76 and #77 showed enhanced levels of ccn2 expression, whereas ccn2 expression levels in tg #72, #74 and #79 were not much higher than endogenous ccn2 levels measured in the wt embryo #78, perhaps due to inactivation of the transgene. Tg chondrocytes with high over-expression of ccn2 mRNA (#73, 75 tg), but also tg chondrocytes of #76 and 77 showed enhanced levels of Col2a1 mRNA as compared with the wt level (#78 wt), as revealed by real-time PCR analysis (Fig. 4B and C), whereas tg cultures with low overexpression of ccn2 (#72, 74, and 79) showed also low col2a1 expression. To confirm the enhanced expression of Col2a1 as well to estimate that of aggrecan mRNA in tg chondrocytes, we pooled primary–cultured chondrocytes from tg and wt littermates, and determined their ccn2, col2a1, aggrecan mRNA levels (figure S2). The levels of all 3 mRNA were greater in the tg than in the wt pooled cells. The enhanced levels of Col2a1 mRNA in the transgenic chondrocytes were also retained after 1 month in culture. During that time, chondrocytes ceased to proliferate and started to mature, but the ccn2 transgene over-expression in tg cultures #86–88 remained at a high level compared with wt cultures #83–85 or low expressing tg cultures #80–82 (Fig. 5A). Primary cultures of chondrocytes with high levels of over-expressed ccn2 mRNA continued to show strongly elevated aggrecan (Fig. 5B, 15-20,000 fold enhancement) and Col2a1 (Fig. 5G, 100–1000 times enhancement) mRNA levels. The expression of Col10a1, a marker of hypertrophy, and that of vegf and of mmp-9, both vascular invasion factors expressed in the hypertrophic zone and boundary between cartilage and bone, were also enhanced; but not at the same extent as the enhancement of aggrecan and col2a1 expression (Fig. 5D, 3–10 fold; 5E, 1.5–3 fold; and 5F, 1.5–3 fold enhancement). These results are in accordance with in vitro studies on the effect of ccn2 on cultured chondrocytes [11] and are consistent with the notion that ccn2 over-expression stimulated chondrocyte maturation.

Figure 4. Gene expression analysis reveals enhanced Col2a1 and ccn2 in chondrocyte primary cultures of Col2a1-ccn2 transgenic mice. To obtain high ccn2 transgene-expressing littermates, we crossed transgenic male and female mice within same founder line; and expression of LacZ, ccn2, and Col2a1 mRNA was measured by real-time PCR from 5 d chondrocyte cultures prepared from E18.5 wt and tg embryos. LacZ analysis revealed that high and low lacZ-expressing tg littermates and 1 wt were obtained (A). On average, ccn2 expression levels in tg chondrocytes were significantly higher than those in wild-type littermates (B). Col2a1 mRNA levels in tg chondrocytes were 2–3 fold higher than those in wt chondrocytes (C). Primary cultures of rib chondrocytes from individual littermates were prepared 3 times from each of the 2 founder lines, and total RNA were prepared. Real-time-PCR analysis was repeated at least 2 times for each RNA preparation; and the 2 founder lines showed similar variations, but gave basically the same results. Primary-chondrocytes from ccn2 tg and wt littermates were also pooled; and gene expression was analyzed as shown in figure S2.

doi:10.1371/journal.pone.0059226.g004

Over-expression of ccn2 Under the Control of the col2a1 Promoter Accelerated Chondrogenesis

To investigate the effect of over-expression of ccn2 on chondrogenic differentiation, we prepared micromass cultures of mesenchymal cells from 11.5-day embryonic transgenic and wt mouse limb buds. Mesenchymal cells from transgenic embryos started to develop Alcian blue-positive cartilaginous nodules after 2 days in culture (data not shown). After 3 days the cartilaginous nodule formation was significantly enhanced in cultures prepared from ccn2-overexpressing limb-buds cells as compared with that wild-type cells (Fig. 5G). The gene expression of ccn2, Col2a1 and
aggrecan was also up-regulated in ccn2 transgenic micromass cultures as measured by quantitative RT-PCR (Fig. 5H, I and J, respectively).

Over-expression of CCN2 Enhanced Expression of IGF-I and IGF-II

In order to elucidate the mechanism of growth stimulation by the over-expressed CCN2, we analyzed changes in expression levels of growth factors known to be involved in skeletal growth. Remarkably, the RNA from tg chondrocytes contained clearly enhanced expression levels of IGF-I and IGF-II mRNA (Fig. 6A).

This finding was confirmed by examining primary-cultured chondrocytes pooled from tg and wt littermates (Figure S3). This finding suggests that, in addition to the possible direct effects of over-expressed CCN2, these enhanced levels of IGF-I or II might have been responsible for the stimulation of cortical bone growth, as well as for the enhanced Col2a1 and aggrecan expression observed in the CCN2-over-expressing mice. To confirm this notion, we treated primary cultures of chondrocytes from 18.5-day wt embryos for 5 days with recombinant CCN2. The result showed a several-fold increase in the levels of IGF-I and IGF-II mRNA as well as a strong increase in endogenous ccn2 expression (Fig. 6B).
Figure 6. CCN2 stimulates IGF-IGFR pathway. Enhanced expression of IGF-I and IGF-II in primary cultures was found in primary cultures of chondrocytes prepared from the cartilage of ccn2-over-expressing mice, and in wt chondrocytes after treatment with recombinant CCN2. (A) Real-time PCR analysis of total RNA from tg cartilage which showed higher expression of ccn2 (107 tg) than wt cartilage (105 wt) also showed enhanced expression of IGF-I and II, whereas 106 tg with low ccn2 overexpression showed no enhanced IGF-II, but enhanced IGF-I expression. *:p<0.05. (B) Addition of recombinant CCN2 (50 ng/ml) to primary cultures of wt mouse rib chondrocytes stimulated IGF-I and II mRNA as well as ccn2 mRNA expression. Primary cultures of chondrocytes were prepared from wt E18.5 embryos; and the cells were seeded at 2×10^5 cells in 3.5-cm dishes with or without CCN2 in the media, and incubated for 5 days. mRNA levels were standardized with gapdh; and all reactions were done in triplicate. Values for 1 wt and 4 wt are from 2 independently generated cultures. *:p<0.005. (C) Phosphorylation of IGF receptor induced by addition of rCCN2 (100 ng/ml) for 24 hours to primary cultures of wt rib chondrocytes, and inhibition of this phosphorylation of IGFR by PPP, an inhibitor of autophosphorylation of IGFR (upper panel). Aggrecan mRNA levels were measured (lower panel) and standardized to gapdh; and all reactions were done in triplicate. (D) Enhanced phosphorylation of IGFR in ccn2-overexpressing chondrocytes and inhibition of phosphorylation of IGFR by CCN2 antibody. Primary cultures of chondrocytes were pooled from P3 rib cages of ccn2 tg and wt littermates; and cells were seeded at 2×10^5 cells in 3.5-cm dishes and cultured for 2 days until the cells had reached the confluent state. CCN2 antibody or control IgG was added to the media, and the cultures were then incubated for 24 hours, after which the cells were collected with lysis buffer.

doi:10.1371/journal.pone.0059226.g006
In order to elucidate whether CCN2 stimulated IGF-IGF receptor pathway, we examined the autophosphorylation of the IGF-1 receptor in response to the addition of CCN2. CCN2 enhanced the autophosphorylation of IGF-1 receptor (Fig. 6C and Figure S3), and the addition of PPP, IGF-R inhibitor, abolished it (Fig. 6C). The CCN2-enhanced expression of aggrecan mRNA was also abolished by the addition of the IGF-R inhibitor (Fig. 6C). The ccn2-overexpressing chondrocytes from ccn2 tg rib cartilage showed enhanced phosphorylation of IGF-R compared with wt chondrocytes; accordingly, the CCN2 neutralizing antibody, 11H3, repressed this autophosphorylation (Fig. 6D). The addition of 11H3 antibody down-regulated the expression of ccn2, igf1, and igf2 mRNA (Figure S4). This finding of enhanced expression of IGF-I and -II in CCN2-transgenic chondrocytes is consistent with our finding of enhanced cortical bone growth and mineralization (see discussion).

Discussion

Previous in vitro studies on the response of rabbit growth-plate chondrocytes in primary culture and human chondrosarcoma cells HCS-2/8 to CCN2 demonstrated not only a significant stimulation of proliferation, differentiation, and enhanced synthesis of hyaline cartilage matrix components such as type II collagen and aggrecan, but also enhanced expression of hypertrophic cartilage proteins such as type X collagen and alkaline phosphatase [11,13]. Since CCN2 is expressed by prehypertrophic and hypertrophic chondrocytes, these finding indicate that CCN2 acts both in an autocrine and in a paracrine manner to promote chondrocyte proliferation and differentiation events. Thus, it may regulate cartilage matrix synthesis and turnover leading to endochondral ossification [4,11,15].

Here we provide experimental evidence in support of a significant role of CCN2 in cartilage development and endochondral ossification in vivo in transgenic mice over-expressing CCN2 driven by the cartilage-specific Col2a1 promoter. Most remarkably, transgenic mice expressing high levels of transgenic CCN2 had greater bone length as compared with their wt littermates. By 8 weeks, some of the tg littermates had greater body mass (~12%), possibly caused by a better eating with tough skeleton. This morphological phenotype reflects several enhanced cellular activities observed in the transgenic cartilage: i) Chondrogenic differentiation of limb-bud mesenchymal cells from CCN2 transgenic animals was greatly enhanced as compared with that of their wild-type counterparts. ii) Histological analysis of tg cartilage revealed increased type II collagen and aggrecan deposition in the extracellular cartilage matrix, consistent with our in vitro data showing that chondrocytes isolated from transgenic animals had highly elevated levels of Col2a1 and aggrecan mRNA shortly after isolation; iii) In long-term cultures, CCN2 transgenic rib chondrocytes also expressed higher levels of Col10a1, vegf and mmp9 mRNA than wt chondrocytes, indicating accelerated maturation to hypertrophic chondrocytes. iv) PCNA staining revealed a significant increase in chondrocyte proliferation in resting and growth-plate cartilage of transgenic animals; and v) CCN2 over-expression also caused slightly enhanced apoptosis of hypertrophic chondrocytes.

One explanation for these effects of the over-expressed CCN2 may be the enhanced levels of IGF-I and IGF-II mRNA in the transgenic chondrocytes. IGF-I and –II and IGF-binding proteins are known to be most potent regulators of cartilage and bone growth [31,32,33]. IGF-I and –II are well known to stimulate proliferation and proteoglycan synthesis in cultured chondrocytes [32,33,34]. Transgenic mice with an IGF-I gene under the control of the metallothionein-I gene promoter weigh 1.3 times more than their non-transgenic littermates [35]. Furthermore, IGF-II is considered to be a fetal growth factor that promotes skeletal growth in young rats [36,37]. Therefore, it is likely that a substantial part, if not all, of the observed effects seen in the transgenic cartilage were due to the additional IGF-I and -II induced by the over-expressed CCN2.

These unexpected findings require revision of current views on the molecular mechanism of growth stimulation by CCN2 and may provide an explanation for our previous observations on the stimulation of proteoglycan and DNA synthesis by CCN2 in HCS-2/8 chondrosarcoma cells and rabbit chondrocytes [11,13]. Previous studies have shown an interaction between module 1 of CCN proteins and IGF, suggesting a regulatory role of CCN proteins on IGFs [38,39]. The data presented here, however, indicate that the up-regulation of IGF-I and -II by CCN2 in mouse chondrocyte cultures occurred at the transcriptional level. To which extent the stimulation of bone growth in the transgenic animals was caused by the up-regulated IGFs or by IGF-independent actions of CCN2 remains to be elucidated.

Surprisingly, the enhanced IGF-I and -II levels in the CCN2 transgenic animals did not cause significant elongation of cartilaginous tissues, for the cartilaginous epiphyses of long bones were about the same size in transgenic animals and their wt littermates. Rather, the increase in bone length was due to an extended length of the diaphyseal bony part of the long bones. A paracrine stimulation of periosteal bone cells by CCN2 overexpressed by adjacent chondrocytes, or by IGFs induced by CCN2, is plausible in light of several in vitro studies showing stimulation of osteoblast proliferation and differentiation and mineralization by CCN2 [4,16,40]. A significant effect of over-expressed CCN2 on bone growth was also evident from the enhanced thickness of cortical bone and increased bone mineralization seen in transgenic mice as compared with those found in their wild-type littermates.

The hypertrophic zone was shorter in the transgenic animals, even though the cartilaginous epiphyses of the long bones were about the same size as in the wild-type animals. There are possible explanations for this phenomenon: 1) The enhanced chondrocyte proliferation may have been compensated by the increase in chondrocyte hypertrophy. 2) The level of VEGF, which induces vascular invasion of hypertrophic cartilage, and that of MMP9, which degrades cartilaginous matrices, were enhanced; in addition apoptosis was slightly accelerated in transgenic hypertrophic chondrocytes. On the other hand, VEGF-CCN2 complexes as formed in vitro have been shown to be degraded by MMPs [41]; and this may be an internal autoregulatory mechanism controlling CCN2 levels in the growth plate.

The results of our present gain-of-function experiment are for the most part in accordance with the findings of a loss-of-function study on CCN2-deficient mice [17], which develop skeletal dysmorphisms such as distorted cartilage and bone elements as a result of impaired chondrocyte proliferation and endochondral ossification. In line with the shortened hypertrophic zone observed in our CCN2 transgenic mice, the hypertrophic zone is extended in CCN2-deficient mice. Interestingly, however, CCN2 deficient mice do not show significant alterations in total bone size. Yet, this is in accordance with the notion that the major enhancing effect on bone growth in our CCN2 transgenic mice may have been caused by enhanced levels of IGFs. Thus, although the study on the CCN2-deficient mice confirmed the important role of CCN2 as a regulator of cartilage remodelling during endochondral ossification, the absence of more severe phenotypic alterations in these mice might have been due to redundant effects of other members of the CCN family [17].

Enhanced IGF-IGFR Pathway in CCN2 Transgenic Mice

PLOS ONE | www.plosone.org 11 March 2013 | Volume 8 | Issue 3 | e59226
In the CCN2 transgenic mouse lines presented here, the extent of bone elongation, as well as the extent of enhancement of Col2a1 and aggrecan mRNA levels correlated with the extent of CCN2 over-expression in transgenic chondrocytes of both founder lines. Besides high-expressing chondrocytes, also transgenic chondrocytes showing low levels of CCN2 expression comparable to those of wt rib chondrocytes were seen in each litter, even when derived from the same founder. Enhanced bone size as well as reduced length of the hypertrophic zone was only observed in tg mice with high levels of CCN2 expression. This was probably due to unpredictable somatic inactivation of the transgene in some embryos and reflects the limitation of this technique, which relies on a random integration of the transgene into the genome. Our previous CCN2-transgenic mice under the control of the Col9a1 promoter show dwarfism several months after birth and smaller testes, but not so much difference in body length [42]. The expression pattern and timing of Col9a1 expression, however, differ to some extent from those of Col2a1, which may explain the difference in phenotype.

Elucidation of the exact molecular mechanisms involved in IGF-independent, CCN2-regulated chondrocyte responses is still hampered by the fact that currently no specific cell-surface signalling cellular receptor for CCN2 has been identified so far in chondrogenic or osteogenic cells; instead, CCN2 seems to control cellular events by complex interactions with numerous growth factors such as IGFs, and perhaps through integrins and their signalling pathways [2,43,44].

In conclusion, our study demonstrates that the use of the Col2a1 promoter for specific over-expression of CCN2 or other members of the CCN family in chondrocytes may represent – together with ccn2-deficient chondrocytes - a powerful tool to provide further insight into the specific role of these growth factors in cartilage metabolism and skeletal development.

Supporting Information

Figure S1 Accumulation of type II collagen and slightly enhanced apoptosis in ccn2-overexpressing epiphyseal cartilage. (A) Comparison of accumulation of type II collagen in cartilage of ccn2-overexpressing and wt mice. Tibiae from P3 littersmates were stained with anti-type II collagen antibody. The color intensity was measured densitometrically. Four wt and 5 ccn2 mRNA levels correlated with the extent of CCN2 expression were repeated 3 times and showed similar results. (TIF)

Figure S2 Gene expression analysis in pooled primary chondrocytes from ccn2 tg and wt littermates. Expression analysis of ccn2, Col2a1, and Aggrecan mRNA of primary chondrocytes from pooled ccn2 tg and wt littermates. Real time-PCR analysis was done in duplicate, *: p<0.005. The experiments were repeated 3 times and showed similar results. (TIF)

Figure S3 Phosphorylation analysis of primary-cultured ccn2 tg and wt chondrocytes pooled from different transgenic line from figure 6A. Results of Western blot analysis of IGF-1R and phospho-IGF-1R (upper photos) and those of gene expression analysis (graphs at bottom) of the same cells as used in Western blot analysis are shown. Real time-PCR analysis was done in duplicate and repeated 3 times, *: p<0.005. (TIF)

Figure S4 Change in gene expression level of ccn2, igf1, and igf2 mRNA by the addition of CCN2 antibody (11H3) to primary cultures of mouse rib chondrocytes from P3 littersmates of ccn2 tg mice. Cells from these cultures were seeded at 2×10^5 cells in 5.5-cm dishes and cultured for 2 days until the cells had reached to confluence. CCN2 antibody or control IgG was added to the media. The cells were incubated for 24 hours, and total RNA was then extracted from them. Real-time PCR demonstrated that CCN2 antibody repressed gene expression of ccn2, igf1, and igf2 mRNA in the ccn2-overexpressing chondrocytes. Real time-PCR analysis was done in duplicate, *: p<0.005. The experiments were repeated for 3 times and showed similar results. (TIF)

Acknowledgments

We thank Drs. Shunichi Murakami and Benoît de Crombrugghe for their generous gifts of Col2a1 promoter and IaZ constructs, as well as Drs. Satoshi Kubota and Takashi Nishida for their valuable discussion. We are also grateful to Dr. Hiroshi Ikegawa, Ms. Ayako Ogo, Ms. Yoshiko Miyake and Ms. Tomoko Yamamoto for technical assistance and to Ms. Eri Yashiro for secretarial assistance.

Author Contributions

Conceived and designed the experiments: NT TH MT. Performed the experiments: NT SI TH. Analyzed the data: NT SI TH MY. Contributed reagents/materials/analysis tools: TY. Wrote the paper: TH MT.

References

1. Brigstock DR (2003) The CCN family: a new stimulus package. J Endocrinol 176: 169–178.
2. Takigawa M, Nishida T, Kubota S (2005) Roles of CCN2/CTGF in the control of growth and regeneration. In: Perbal B, Takigawa M, editors. CCN Proteins: A new family of cell growth and differentiation regulators. London: Imperial College Press. 19–59.
3. Kubota S, Takigawa M (2007) Role of CCN2/CTGF/He24 in bone growth. Int Rev Cytol 257: 1–41.
4. Takigawa M, Nakamichi T, Kubota S, Nishida T (2005) Role of CTGF/ HGS24/ecogenin in skeletal growth control. J Cell Physiol 194: 236–266.
5. Shimizu T, Nakamichi T, Kimura Y, Nishida T, Ishizaki K, et al. (1996) Inhibition of endogenous expression of connective tissue growth factor by its antiserine oligonucleotide and antiserine RNA suppresses proliferation and migration of vascular endothelial cells. J Biochem 124: 130–140.
6. Shimizu T, Nakamichi T, Nishida T, Azano M, Kanyama M, et al. (1999) Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. J Biochem 126: 137–143.
7. Babic AM, Chen CG, Lau LF (1999) Fasp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphabeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. Mol Cell Biol 19: 2938–2966.
8. Grotendorst GR, Duncan MR (2005) Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. Farh J 19: 729–738.
9. Twigg SM, Cao Z, McLeannan SV, Burns WC, Brammar G, et al. (2002) Renal connective tissue growth factor induction in experimental diabetes is prevented by aminoguanidine. Endocrinology 143: 4907–4915.
10. Kanaan RA, Aldwairi M, Al-Hanabi OA (2006) The role of connective tissue growth factor in skeletal growth and development. Med Sci Moni 12: RA177–281.
11. Nakamichi T, Nishida T, Shimizu T, Kubo Y, Kubo T, et al. (2000) Effects of CTGF/He24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. Endocrinology 141: 264–273.
12. Hattori T, Fujisawa T, Sasaki K, Yutani Y, Nakanishi T, et al. (1998) Isolation and characterization of a rheumatoid arthritis-specific antigen (RA-A47) from a human chondrocytic cell line (HC2-7/8). Biochem Biophys Res Commun 245: 679–683.
13. Nishida T, Kubota S, Nakamichi T, Kubo Y, Yosimichi G, et al. (2002) CTGF/He24, a hypertrophic chondrocyte-specific gene product, stimulates...
proliferation and differentiation, but not hypertrophy of cultured articular chondrocytes. J Cell Physiol 192: 53–63.

14. Fujisawa T, Hattori T, Onda M, Uehara J, Kubota S, et al. (2008) CCN family 2/connective tissue growth factor (CCN2/CTGF) stimulates proliferation and differentiation of auricular chondrocytes. Osteoarthritis Cartilage.

15. Nishida T, Kubota S, Fukunaga T, Kondo S, Yosimichi G, et al. (2003) CTGF/Hcs24, hypertrophic chondrocyte-specific gene product, interacts with perlecan in regulating the proliferation and differentiation of chondrocytes. J Cell Physiol 196: 265–275.

16. Nishida T, Nakashima T, Asano M, Shimo T, Takigawa M (2000) Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. J Cell Physiol 184: 197–206.

17. Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Lhuda DE, et al. (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130: 2779–2791.

18. Murakami S, Balnes G, McKinney S, Zhang Z, Givol D, et al. (2004) Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfish and rescues the Fgfr3-deficient mouse phenotype. Genes Dev 18: 290–305.

19. Zhang R, Murakami S, Costrey F, Wang Y, de Crombrugghe B (2006) Constitutive activation of MKK6 in chondrocytes of transgenic mice inhibits proliferation and delays endochondral bone formation. Proc Natl Acad Sci U S A 103: 365–370.

20. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B (1999) Sox9 is required for cartilage formation. Nat Genet 22: 85–89.

21. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, et al. (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765–771.

22. Smits P, Li P, Mandel J, Zhang Z, Deng JM, et al. (2001) The transcription factors L-Sox3 and Sostx are essential for cartilage formation. Dev Cell 1: 277–290.

23. LeFebvre V, Huang W, Hazley VR, Goodfellow PN, de Crombrugghe B (1997) Sox9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(I) collagen gene. Mol Cell Biol 17: 2360–2366.

24. Hattori T, Eberspaecher H, Lu J, Zhang R, Nishida T, et al. (2006) Interactions between PIAS proteins and SOX9 result in an increase in the cellular concentrations of SOX9. J Biol Chem 281: 14417–14428.

25. Holmdahl R, Rubin K, Dahlen AD, Gilmore RC, et al. (1997) Osterix/Calvi-1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765–771.

26. Smith P, Li P, Mandel J, Zhang Z, Deng JM, et al. (2001) The transcription factors L-Sox3 and Sostx are essential for cartilage formation. Dev Cell 1: 277–290.

27. Boos N, Nerlich AG, Wiest I, von der Mark K, Ganz R, et al. (1999) Immunohistochemical analysis of type-X-collagen expression in osteoarthritis of the hip joint. J Orthop Res 17: 495–502.

28. von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, et al. (1992) Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 35: 806–811.

29. LeFebvre V, Garafalo S, Zhou G, Metzger M, Vuorio E, et al. (1994) Characterization of primary cultures of chondrocytes from type II collagen/ beta-galactosidase transgenic mice. Matrix Biol 14: 329–335.

30. Hattori T, Costrey F, Stephen S, Eberspaecher H, Takigawa M, et al. (2000) Transcriptional regulation of chondrogenesis by coactivator Tip60 via chromatin association with Sox9 and Sox5. Nucleic Acids Res 36: 3011–3024.

31. Nakashima T, Kimura Y, Tamura T, Ichikawa H, Yamaai Y, et al. (1997) Cloning of a mRNAs preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. Biochem Biophys Res Commun 234: 206–210.

32. Horlick A, Goutz W, Lichamska AM, Bieloobsy M, Tomshoff B, et al. (2007) Effects of insulin-like growth factor binding proteins in bone – a matter of cell and site. Arch Physiol Biochem 113: 142–153.

33. Schmid G (1995) Insulin-like growth factors. Cell Biol Int 19: 445–457.

34. Vetter U, Hellung G, Heit W, Prieü W, Sterza K, et al. (1985) Clonal proliferation and cell density of chondrocytes isolated from human fetal epiphysial, human adult articular and nasal septal cartilage. Influence of hormones and growth factors. Growth 49: 229–243.

35. Takigawa M, Okawa T, Pau H, Aoki C, Takahashi K, et al. (1997) Insulin-like growth factors I and II are autocrine factors in stimulating proteoglycan synthesis, a marker of differentiated chondrocytes, acting through their respective receptors on a clonal human chondrosarcoma-derived chondrocyte cell line, HCS-2/8. Endocrinology 138: 4390–4400.

36. Quiné C, Mathews IS, Pinkert CA, Hammer RE, Breister RL, et al. (1989) Histopathology associated with elevated levels of growth hormone and insulin-like growth factor I in transgenic mice. Endocrinology 124: 40–48.

37. Adams SO, Nisley SP, Handberger S, Rechler MM (1983) Developmental patterns of insulin-like growth factor-I and -II synthesis and regulation in rat fibroblasts. Nature 302: 150–153.

38. Land PK, Moats-Staats BM, Hynes MA, Simmons JG, Jansen M, et al. (1986) Somatomedin-C insulin-like growth factor-I and insulin-like growth factor-II mRNAs in rat fetal and adult tissues. J Biol Chem 261: 14339–14344.

39. Bork P (1993) The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett 327: 125–130.

40. Burren CP, Wilson EM, Hwa V, Oh Y, Rosenfeld RG (1999) Binding properties and distribution of insulin-like growth factor binding protein-related protein 3 (IGFBP-rP3/NovH), an additional member of the IGFBP Superfamily. J Clin Endocrinol Metab 84: 1096–1103.

41. Safadi FF, Xu J, Smock SL, Kanaan RA, Selim AH, et al. (2003) Expression of connective tissue growth factor in bone: its role in osteoblast proliferation and differentiation in vitro and bone formation in vivo. J Cell Physiol 196: 51–62.

42. Hashimoto G, Inoki I, Fujii Y, Aoki T, Ikeda E, et al. (2002) Matrix attachment region binding protein, a novel component of the human insulin-like growth factor I receptor, engages the receptor tyrosine kinase activity. J Biol Chem 277: 24685–24694.

43. Chen CC, Chen N, Lau LF (2001) The angiogenic factors Cyr61 and connective tissue growth factor (CTGF) are autocrine factors in collagen-induced angiogenesis in vivo. J Biol Chem 276: 36288–36295.

44. Chen CC, Chen N, Lau LF (2001) The angiogenic factors Cyr61 and connective tissue growth factor (CTGF) are autocrine factors in collagen-induced angiogenesis in vivo. J Biol Chem 276: 36288–36295.

45. Hashimoto G, Inoki I, Fujii Y, Aoki T, Ikeda E, et al. (2002) Matrix attachment region binding protein, a novel component of the human insulin-like growth factor I receptor, engages the receptor tyrosine kinase activity. J Biol Chem 277: 24685–24694.