Skeletal Overexpression of Connective Tissue Growth Factor Impairs Bone Formation and Causes Osteopenia

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Connective tissue growth factor (CTGF), a member of the CCN family of proteins, is expressed in skeletal cells, and the *ctgf* null mutation leads to neonatal lethality due to defects in skeletal development. To define the function of CTGF in the postnatal skeleton, we created transgenic mice overexpressing CTGF under the control of the human osteocalcin promoter. CTGF transgenic female and male mice exhibited a significant decrease in bone mineral density, compared with wild-type littermate controls. Bone histomorphometry revealed that CTGF overexpression caused decreased trabecular bone volume due to impaired osteoblastic activity because mineral apposition and bone formation rates were decreased. Osteoblast and osteoclast number and bone resorption were not altered. Calvarial osteoblasts and stromal cells from CTGF transgenics displayed decreased alkaline phosphatase and osteocalcin mRNA levels and reduced bone morphogenetic protein (BMP) signaling mothers against decapentaplegic, Wnt/β-catenin, and IGF-I/Akt signaling. In conclusion, CTGF overexpression *in vivo* causes osteopenia, secondary to decreased bone formation, possibly by antagonizing BMP, Wnt, and IGF-I signaling and activity. (*Endocrinology* 149: 4374–4381, 2008)

Precursor mesenchymal cells can differentiate into cells of various lineages, including osteoblasts, chondrocytes, and adipocytes (1). The fate of mesenchymal cells and their differentiation toward cells of the osteoblastic lineage is tightly controlled by extracellular and intracellular signals. Bone morphogenetic proteins (BMPs) are important determinants of cell fate, and play a central role in the regulation of osteoblastogenesis and endochondral bone formation (2). BMPs, in conjunction with Wnt, induce the differentiation of mesenchymal cells toward the osteoblastic lineage and enhance the pool of mature osteoblasts (3–5). The effects of BMPs and Wnt are controlled by a large group of secreted polypeptides that prevent BMP or Wnt signaling by binding to BMPs or Wnt, or to their receptors/coreceptors, precluding ligand-receptor interactions (2, 5–7). IGFs do not direct the differentiation of immature cells toward cells of the osteoblastic lineage, but enhance the function of the mature osteoblast and increase bone formation (8).

Members of the CCN family of cysteine-rich (CR) secreted proteins include cysteine-rich 61 (Cyr 61), connective tissue growth factor (CTGF), nephroblastoma overexpressed (Nov),

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and Wnt-inducible secreted proteins 1, 2, and 3 (9, 10). CCN proteins are highly conserved and share four distinct modules: an IGF-binding domain, a von Willebrand type C domain containing the CR domain, a thrombospondin-1 domain, and a C-terminal domain, important for protein-protein interactions (9, 10). CCN proteins are structurally related to certain BMP antagonists, such as twisted gastrulation and chordin, and can have important interactions with regulators of osteoblast cell growth and differentiation (11).

CTGF is expressed in a variety of tissues, including bone and cartilage. In osteoblasts, CTGF expression is induced by BMP, TGFβ, Wnt, and cortisol, suggesting a possible role in the activity of these agents in bone cell function (12–14). CTGF regulates different cellular functions including adhesion, proliferation, migration and differentiation. The function of CTGF in skeletal cells is not well understood, and *in vitro* studies have yielded controversial results (13, 15). By mechanisms that would resemble the activity of certain BMP or Wnt antagonists, CTGF binds to BMP-2 and -4 through its CR domain, and to Wnt coreceptors through its C-terminal domain, and inhibits osteoblastic differentiation (15, 16). *In vivo* studies indicate that CTGF is necessary for endochondral bone formation, and deletion of *ctgf* in mice results in newborn lethality and skeletal abnormalities (17). Overexpression of CTGF in chondrocytes under the control of type XI collagen promoter has suggested that CTGF in excess can lead to osteopenia (18). However, the function of CTGF in the adult skeleton is not known.

The intent of this study was to investigate the direct effect of CTGF on bone remodeling and the mechanisms involved. For this purpose, we created transgenic mice overexpressing CTGF under the control of the osteoblast-specific osteocalcin promoter, and determined their skeletal phenotype. Cultures of osteoblastic and stromal cells from CTGF transgenics were performed to establish mechanisms responsible for the phenotype.

Materials and Methods

Osteocalcin/CTGF construct and generation of transgenic mice

After introduction of the Kozak consensus sequence upstream of the translation initiation codon, a 1046-bp fragment coding for murine *ctgf* (R.P. Ryseck, Princeton, NJ) was cloned downstream of a 182-bp artificial intron and a 3.8-kb fragment of the human osteocalcin promoter (E. Gardiner; Sydney, Australia), and upstream of polyadenylation sequences and a 3.5-kb fragment of the 3′-untranslated region and flanking DNA of the osteocalcin gene (19). Nucleotide sequence analysis confirmed the absence of mutations and the correct orientation of the construct. Microinjection of linearized DNA into pronuclei of fertilized oocytes from FVB (for tropism to Friend Leukemia Virus Strain B) inbred mice, and transfer of microinjected embryos into pseudopregnant FVB mice were carried out by the transgenic facility at the University of Connecticut Health Center (Farmington, CT). Positive founders were identified by Southern blot analysis of tail DNA (20). Founder mice were bred to wild-type FVB mice to generate transgenic lines. Intermatings of heterozygous transgenics were used to create a homozygous offspring. All animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

X-ray analysis and bone mineral density (BMD)

Radiography was performed on mice anesthetized with tribromoethanol (Sigma Chemical Co., St. Louis, MO) on a Faxitron x-ray system (model MX 20; Faxitron X-Ray Corp., Wheeling, IL). The x-rays were performed at an intensity of 35 kW for 25 sec. Total bone mineral content (BMC; grams), skeletal area (cm^2) and bone mineral density (BMD; grams per cm^2) were measured on anesthetized mice using the PIXImus small animal DEXA system (GE Medical System/LUNAR, Madison, WI) (21). Calibrations were performed with a phantom of a defined value, and quality assurance measurements were performed before each use. The coefficient of variation for total BMD is less than 1% (n = 9).

Bone histomorphometric analysis

Static and dynamic histomorphometry was carried out on transgenic mice and wild-type littermate controls at one month of age. Mice were injected with calcein, 20 mg/kg, and demeclocycline, 50 mg/kg, at an interval of 2 d, and killed by CO_2 inhalation 2 d after the demeclocycline injection. Femurs were dissected, fixed in 70% ethanol, dehydrated and embedded undecalcified in methyl methacrylate. Longitudinal sections, 5 μm thick, were cut on a microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI) and stained with toluidine blue or Von Kossa. Static parameters of bone formation and resorption were measured in a defined area between 181 μm and 1086 μm from the growth plate, using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA) (22). For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured in unstained sections under UV light, using a B-2A set long pass filter, and bone formation rate was calculated. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (23).

Serum C-terminal cross-linked telopeptide of type I collagen (CTX)

The serum bone remodeling marker CTX was measured by ELISA using RatLaps ELISA kits (Nordic Bioscience Diagnostics, Herlev, Denmark), according to manufacturer's instructions.

Primary osteoblast and bone marrow stromal cell cultures

Osteoblastic cells were isolated from parietal bones of 3-d-old CTGF heterozygous transgenic and wild-type control littermate mice, following the mating of CTGF heterozygous and wild-type FVB mice. Cells were obtained by five sequential digestions of the parietal bones using bacterial collagenase (CLS II, Worthington Biochemical, Freehold, NJ) (24). Cell populations harvested from the third to the fifth digestions were cultured as a pool, and were previously shown to have osteoblast characteristics (24). Osteoblastic cells were cultured in DMEM (Life

Technologies, Inc., Grand Island, NY) supplemented with nonessential amino acids, 20 mM HEPES, 100 μg/ml ascorbic acid, and 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), at 37 C in a humidified 5% CO_2 incubator. Subconfluent cells were trypsinized and these first passage cells were used for subsequent experiments. To test for effects on BMP or Wnt signaling, cells were plated at a density of 25,000 cells/cm^2, cultured to subconfluence (~35,000 cells/cm^2) and transfected with BMP/Signaling mothers against decapentaplegic (Smad) or Wnt/β-catenin reporter constructs and treated with BMP-2 (Wyeth Research, Collegeville, PA) or Wnt 3a (R&D Systems, Minneapolis, MN), as described below. To assess for effects on the phosphorylation of Smad or serine/threonine protein kinase B (Akt), or on cytosolic β-catenin levels, cells were plated at a density of 40,000 cells/cm^2 and cultured to confluence (~50,000 cells/cm^2) or for 10 d after confluence, in the presence of 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO), serum-deprived overnight and exposed to vehicle, BMP-2 for 20 min, Wnt 3a for 6 h or IGF-I (PeproTech Inc., Rocky Hill, NJ) for 15 min, as indicated in text and legends. Alternatively, cells cultured for 10 d after confluence were serum deprived overnight and analyzed for gene expression.

Bone marrow stromal cells were isolated from femurs of 4-wk-old CTGF heterozygous transgenic and wild-type littermate control mice, by bone marrow flushing, after CO_2 asphyxiation, as described (22). Cells were plated at a density of 5×10^6 cells/cm^2 and cultured in α-MEM (Invitrogen, Carlsbad, CA) containing 15% FBS at 37 C in a 5% CO_2 incubator. All adherent cells without further cell isolation were grown to confluence (~10^5/cm^2; 6–7 d of culture), the medium was changed to α-MEM supplemented with 10% FBS, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate. Cells were cultured for an additional 10-d period, serum deprived overnight, and analyzed for gene expression. To estimate the number of viable cells, mitochondrial dehydrogenase activity was measured using the Cell Titer 96 Aqueous One cell proliferation assay in accordance with the manufacturer's instructions (Promega, Madison, WI). Metabolically active cells were estimated by their ability to reduce the tetrazolium compound 3-(4,5-dimethyl-thiazol-2-yl)-5(-3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt to a formazan product, which was quantified at an absorbance of 490 nm.

Real-time RT-PCR

Total RNA was extracted from calvariae or from cell cultures, and CTGF, alkaline phosphatase, osteocalcin, bone sialoprotein (BSP), and type I collagen mRNA levels determined by real-time RT-PCR (25, 26). For this purpose, 1–10 μg of RNA were reverse-transcribed using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen), according to manufacturer's instructions and amplified in the presence of primers specific for these genes or primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ribosomal protein L38 (RPL38) (Table 1), and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 54–60 C for 45 cycles. Gene copy number was estimated by comparison with a standard curve constructed using CTGF (R. P. Ryseck), alkaline phosphatase, osteocalcin or BSP (all from American Type Culture Collection [ATCC (Manassas, VA)] and corrected for RPL38 (ATCC) copy number. CTGF copy number from calvarial extracts was normalized for GAPDH (R. Wu, Cornell University, Ithaca, NY) expression (27, 28). Reactions were conducted in a 96-well spectrofluorometric thermal iCycler (Bio-Rad, Hercules, CA), and fluorescence was monitored during every PCR cycle at the annealing step.

Transient transfections

To determine changes in BMP-2 signaling, a construct containing 12 copies of a Smad 1/5 consensus sequence linked to an osteocalcin minimal promoter and a luciferase reporter gene (12xSBE-Oc-pGL3; M. Zhao, San Antonio, TX) was tested in transient transfection experiments (29). To determine changes in Wnt/β-catenin transactivating activity, a construct containing 16 copies of the lymphoid enhancer binding factor/T-cell-specific factor (Lef1/Tcf-4) recognition sequence, cloned upstream of a minimal thymidine kinase promoter and a luciferase reporter gene (16xTCF-Luc; J. Billiard, Wyeth Research) was tested in transient transfection experiments (30). To determine changes in osteocalcin promoter activity, a 1080-bp fragment of the rat osteocalcin promoter, cloned into pGL3 upstream of the luciferase reporter gene (I. Kalajzic, Farmington, CT), was

TABLE 1. Primers used for real-time RT-PCR amplification

Gene	Accession no.	Strand	3' Locus	Sequence (5'–3')
Ctgf	NM_010217	F	772	CACTCCGGGAAATGCTGCGAGGAG[FAM]G
		R	840	GTTGGGTCTGGGCCAAATGT
Alkaline phosphatase	BC065175	F	371	CGGTTAGGGCGTCTCCACAGTAAC[FAM]G
		R	444	CTTGGAGAGGGCCACAAAGG
Osteocalcin	L24429	F	496	CACTTACGGCGCTACCTTGGGTAAGT[FAM]G
		R	523	CCCAGCACAACTCCTCCCTA
Collagen type 1	NM_007742	F	920	GACCAGTGCCAAAGGAGATGCTGGT[FAM]C
		R	990	GTCCAGGGCGACCTCTCTCA
Bone sialoprotein	BC045143	F	408	CGAGACCACACTTTCCACACTCT[FAM]G
		R	469	CTTCTTGGGCAGTTGGAGTGC
Rpl38	BC055346	F	160	CGAACCGGATAATGTGAAGTTCAAGGTT[FAM]G
		R	203	CTGCTTCAGCTTCTCTGCCTTT
Gapdh	NM_017008	F	1425	CACGCTCTGGAAAGCTGTGGCG[FAM]G
		R	1507	AGCTTCCCGTTCAGCTCTGG

tested in transient transfection experiments. Osteoblast cells were cultured to 70% confluence and transiently transfected with the indicated constructs using FuGENE6 (3 μl FuGENE/2 μg DNA), according to manufacturer's instructions (Roche, Indianapolis, IN). A cytomegalovirus (CMV) directed β-galactosidase expression construct (Clontech, San Jose, CA) was used to control for transfection efficiency. Cells were exposed to the FuGENE-DNA mix for 16 h and transferred to serum-free medium for 8 h, treated with BMP-2, Wnt 3a or vehicle for 24 h, as indicated in the text and legends, and harvested. Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity.

Western immunoblot analysis

To determine the level of the phosphorylation of Smad 1/5/8 and Akt, the cell layer of control and CTGF overexpressing cells was washed with cold PBS and extracted in cell lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton, and 1 mM EDTA] (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors, as described (22, 31). Protein concentration was determined by DC protein assay (Bio-Rad), and 10–20 μg of total cellular protein was fractionated by gel electrophoresis in 12.5% polyacrylamide gels under reducing conditions, and transferred to Immobilon P membranes, which were blocked with 3% BSA in PBS. For Smad 1/5/8, membranes were exposed to a rabbit polyclonal antibody, which recognizes Smads 1, 5, and 8 phosphorylated at Ser 463/465 (Smad 1 and 5), and Ser 426/428 (Smad 8) (Cell Signaling Technology), or exposed to a rabbit polyclonal antibody to unphosphorylated Smad 1 (Santa Cruz Biotechnology, Santa Cruz, CA) (32, 33). For Akt phosphorylation, membranes were exposed to a rabbit polyclonal antibody, which recognizes Akt phosphorylated at Ser 473, or a polyclonal antibody to unphosphorylated Akt (both, Cell Signaling Technology). To determine cytosolic β-catenin levels, the cell layer was extracted in 10 mM Tris, 140 mM NaCl, 5 mM EDTA, and 2 mM dithiothreitol buffer at pH 7.6, in the presence of protease inhibitors, and the cytosolic fraction was separated by ultracentrifugation, as described (34). Twenty micrograms of protein were fractionated by gel electrophoresis in 7.5% polyacrylamide gels and transferred to Immobilon P membranes. The membranes were blocked with 3% BSA in PBS and exposed to a monoclonal antibody to unphosphorylated β-catenin or a polyclonal antibody to human actin (both from Santa Cruz Biotechnology). All blots were exposed to antirabbit or antimouse IgG antiserum conjugated to horseradish peroxidase and developed with a chemiluminescence detection reagent (PerkinElmer Life Sciences, Boston, MA).

Statistical analysis

Data are expressed as means ± SEM. Statistical differences were determined by Student's *t* test or ANOVA.

Results

Characterization of CTGF transgenic mice

Two transgenic lines overexpressing CTGF under the control of the osteocalcin promoter were established. Their initial identification by Southern blot analysis revealed approximately 7 and more than 100 copies of *ctgf* transgene in heterozygous from line 1 and 2, respectively. Overexpression of CTGF transcripts in transgenics was confirmed by real-time RT-PCR in calvarial extracts; the *ctgf/gapdh* copy number in homozygous transgenic males from line 2 was 170 ± 59 compared with 3.7 ± 1.3 in wild-type littermate controls, and in homozygous transgenic females was 82 ± 27 compared with 1.9 ± 0.4 in controls (both 45-fold greater than control, both $P < 0.05$). CTGF transgenic mice were compared with wild-type littermate controls of identical sex, at the age of 4 wk, a time of high activity of the osteocalcin promoter (35). Line 2 was studied in detail, and 1-month-old homozygous CTGF transgenic male mice displayed reduced weight by 24% (Table 2). In contrast, only minor changes in body weight were observed in homozygous CTGF transgenic female mice, relative to their wild-type controls (Table 2) and no changes in body weight were noted in either male or female mice of line 1 (not shown). Both male and female CTGF overexpressing mice from line 2 displayed a 15–28% decrease in BMC and 8% decrease in BMD, when compared with control wild-type mice (Table 2). Contact radiography did not reveal obvious skeletal abnormalities (Fig. 1).

Bone histomorphometric analysis of distal femurs from 1-month-old female heterozygous and homozygous CTGF transgenics from line 2 revealed a 50–70% decrease in trabecular bone volume, due to a reduced number of trabeculae (Table 3). A 40 to 70% decrease in bone volume was observed in homozygous and heterozygous male transgenics from line 2 (Table 4). The decrease in bone volume observed was not associated with changes in osteoblast number. Although the

TABLE 2. Weight, total BMC, bone area, and BMD of 1-month-old CTGF homozygous transgenics and wild-type control mice

	Weight	BMC	Bone Area	BMD
Male				
WT	15.7 ± 0.6	223 ± 11	6.4 ± 0.2	345 ± 7
CTGF	11.9 ± 0.7[a]	162 ± 13[a]	5.2 ± 0.4[a]	314 ± 1[a]
Female				
WT	15.2 ± 0.3	215 ± 7	6.1 ± 0.1	353 ± 5
CTGF	14.1 ± 0.6	184 ± 5[a]	5.6 ± 0.1[a]	329 ± 3[a]

Weight (g), BMC (g × 10^4), bone area (cm^2), and BMD (g/cm^2 × 10^4) were obtained from one month old CTGF homozygous transgenics from line 2 and wild-type (WT) controls. Values are means ± SEM (n = 5–8).
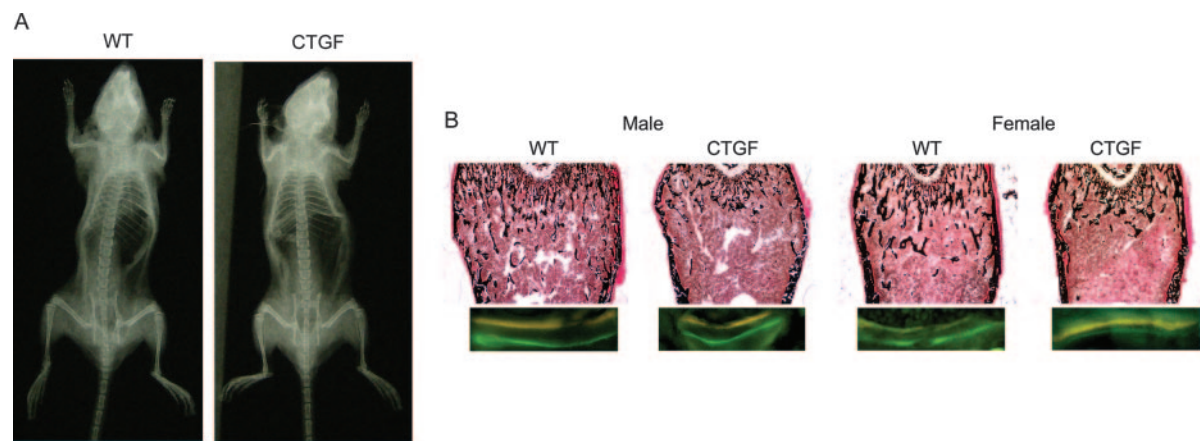[a] Significantly different from wild-type controls, $P < 0.05$.

FIG. 1. A, Skeletal radiograph of a 1-month-old homozygous male CTGF transgenic (line 2) and wild-type (WT) control. B, Representative femoral sections from 1-month-old male and female CTGF transgenic homozygous (line 2) and wild-type (WT) controls stained with von Kossa (*upper panel*) and visualization of calcein and demeclocycline labels (*lower panel*) by fluorescence microscopy at a final magnification of ×40 and ×400, respectively.

number of osteoblasts per area was decreased because of the lesser number of trabeculae, the number of osteoblasts per perimeter and osteoblast surface were not different from controls. Fluorescence microscopy revealed a decrease in mineralizing surface, mineral apposition rate, and in bone formation rate, which was statistically significant in homozygous and heterozygous female mice, indicating that the decreased bone volume in CTGF transgenics was secondary to a decrease in osteoblast function (Tables 3 and 5 and Fig. 1). Changes in trabecular bone volume were not associated with changes in bone resorption because eroded surface was not different between transgenics and controls. Moreover, the serum levels of CTX did not differ between control and transgenic mice, indicating normal bone remodeling in CTGF transgenics. Serum concentrations of CTX (means ± SEM; n = 4) were 111 ± 19 ng/ml in control male mice and 110 ± 32 ng/ml in CTGF homozygous transgenics and 139 ± 24 ng/ml in control female mice and 101 ± 19 ng/ml in CTGF homozygous transgenics. There was no difference between female (Table 3) and male (Table 4) phenotypes, except for the fact that the reduction in bone formation in male transgenics was less pronounced and a 30% decrease in bone formation observed in homozygous male transgenics did not reach statistical significance.

Both transgenic lines studied displayed analogous skeletal phenotypes, although the phenotype was less pronounced in line 1, probably because of the lesser degree of *ctgf* transgene expression. In accordance, one month old female CTGF homozygous transgenics from line 1 expressed an approximately 20-fold increase in CTGF transcripts in calvarial extracts and exhibited a modest decrease in trabecular bone volume with an approximately 40% decrease in bone formation ($P > 0.05$) (Tables 5). One-month-old male CTGF homozygous transgenics from line 1 also were osteopenic, and trabecular bone volume/tissue volume was 9.2 ± 0.8 in controls and 7.3 ± 0.5 in CTGF transgenics ($P < 0.05$) due to a decreased trabecular number, but bone formation was not suppressed significantly. CTGF heterozygous from line 1 did not exhibit a phenotype possibly due to the low level of transgene expression (not shown). Mice from line 1 showed only a 5 to 9% decrease in BMC ($P > 0.05$) without a significant change in BMD.

Primary osteoblast and stromal cell cultures

To investigate the impact of CTGF on osteoblastic cell function, calvarial osteoblastic cells from heterozygous transgenics (line 2) and wild-type controls were cultured. In accordance with the phenotype observed in CTGF transgenics *in vivo*, CTGF did not have an effect on cell number at confluence, and up to 14 d after confluence (data not shown). After 10 d of culture, alkaline phosphatase and osteocalcin mRNA levels were suppressed by about 50% in osteoblastic

TABLE 3. Femoral static and dynamic bone histomorphometry of 1-month-old CTGF transgenics and wild-type control females, line 2

Line 2	WT	$CTGF^{+/-}$	$CTGF^{+/+}$
Bone volume/tissue volume (%)	10.6 ± 0.7	5.4 ± 0.7[a]	3.5 ± 0.7[a]
Trabecular separation (μm)	110 ± 8	237 ± 36[a]	400 ± 84[a]
Trabecular number (mm^{-1})	8.5 ± 0.7	4.4 ± 0.6[a]	2.9 ± 0.5[a]
Osteoblasts/bone perimeter (mm^{-1})	38.0 ± 1.1	36.7 ± 1.5	38.8 ± 2.8
Osteoblasts/tissue area (mm^{-2})	506 ± 35	250 ± 26[a]	179 ± 39[a]
Osteoclasts/bone perimeter (mm^{-1})	7.0 ± 0.3	7.1 ± 0.4	8.6 ± 1.1
Eroded surface/bone surface (%)	21.0 ± 0.8	20.7 ± 0.9	23.0 ± 1.8
Mineralizing surface/bone surface (%)	6.7 ± 0.4	5.2 ± 0.3[a]	5.2 ± 0.6[a]
Mineral apposition rate (μm/d)	0.94 ± 0.02	0.79 ± 0.03[a]	0.79 ± 0.02[a]
Bone formation rate/bone surface (μm^3/μm^2/d)	0.063 ± 0.003	0.041 ± 0.002[a]	0.041 ± 0.004[a]

Bone histomorphometry was performed on femurs from 1-month-old female CTGF homozygous ($CTGF^{+/+}$), CTGF heterozygous ($CTGF^{+/-}$) and wild-type (WT) controls for line 2. For static histomorphometry, sections were stained with toluidine blue, and for dynamic histomorphometry unstained sections were analyzed by fluorescence microscopy. Values are means ± SEM; n = 6 to 9.

[a] Significantly different from wild type controls, $P < 0.05$.

TABLE 4. Femoral static and dynamic bone histomorphometry of 1-month-old CTGF transgenics and wild-type control males, line 2

	WT	$CTGF^{+/-}$	WT	$CTGF^{+/+}$
Bone volume/tissue volume (%)	10.7 ± 0.5	6.2 ± 0.5^a	12.4 ± 0.96	3.8 ± 0.6^a
Trabecular separation (μm)	92 ± 5	175 ± 15^a	88 ± 7	308 ± 50^a
Trabecular number (mm^{-1})	9.8 ± 0.5	5.6 ± 0.4^a	10.3 ± 0.6	3.5 ± 0.6^a
Osteoblasts/bone perimeter (mm^{-1})	58.0 ± 7.6	52.4 ± 3.3	33.3 ± 1.6	34.2 ± 2.8
Osteoblasts/tissue area (mm^{-2})	889 ± 105	458 ± 43^a	531 ± 23	192 ± 38^a
Osteoclasts/bone perimeter (mm^{-1})	12.1 ± 0.8	13.3 ± 0.6	6.5 ± 0.4	7.1 ± 0.4
Eroded surface/bone surface (%)	23.6 ± 1.5	25.6 ± 1.3	18.8 ± 1.2	18.0 ± 1.7
Mineralizing surface/bone surface (%)	4.1 ± 0.6	4.8 ± 0.6	5.08 ± 0.47	3.74 ± 0.5
Mineral apposition rate (μm/d)	0.87 ± 0.06	0.90 ± 0.04	0.812 ± 0.009	0.723 ± 0.057
Bone formation rate/bone surface (μm^3/μm^2/d)	0.036 ± 0.006	0.042 ± 0.004	0.041 ± 0.004	0.028 ± 0.006

Bone histomorphometry was performed on femurs from 1-month-old male CTGF heterozygous ($CTGF^{+/-}$) and CTGF homozygous ($CTGF^{+/+}$), of line 2 and their respective wild-type (WT) controls. For static histomorphometry, sections were stained with toluidine blue, and for dynamic histomorphometry unstained sections were analyzed by fluorescence microscopy. Values are means $\pm$ SEM; $n = 5$ to 8 for static histomorphometry, and $n = 3$ to 6 for dynamic histomorphometry.
^{a} Significantly different from wild-type controls, $P < 0.05$.

cells from transgenics when compared with control cells, confirming an inhibitory effect of CTGF on osteoblast function (Table 6). BSP expression was decreased by 40% ($P < 0.08$) and only a modest, approximately 20%, decrease in type I collagen was observed in cells from CTGF transgenics ($P > 0.05$, not shown). The activity of a transfected osteocalcin promoter construct was decreased from 14.9 ± 1.4 luciferase/β-galactosidase in control wild-type cells to 10.2 ± 0.3 luciferase/β-galactosidase in transgenic cells ($P < 0.05$).

To examine possible mechanisms underlying the osteopenia and decreased bone formation of CTGF transgenics, we examined whether CTGF overexpression affected BMP/Smad, Wnt/β-catenin or IGF-I signaling in cells of the osteoblastic lineage. Confirming previous results, BMP-2 induced the transactivation of the 12xSBE-Oc-pGL3 reporter construct, where 12 Smad binding sites direct luciferase expression, and enhanced Smad1/5/8 phosphorylation (Fig. 2) (29, 36, 36). CTGF transgenic cells displayed decreased response to BMP-2 on the transactivation of the transfected 12xSBE-Oc-pGL3 reporter construct and on Smad 1/5/8 phosphorylation (Fig. 2, A and C).

TABLE 5. Femoral static and dynamic bone histomorphometry of 1-month-old CTGF transgenics and wild-type control females, line 1

Line 1	WT	$CTGF^{+/+}$
Bone volume/tissue volume (%)	13.2 ± 1.1	9.6 ± 0.9^a
Trabecular separation (μm)	85 ± 7	116 ± 6^a
Trabecular number (mm^{-1})	10.6 ± 0.7	7.9 ± 0.4^a
Osteoblasts/bone perimeter (mm^{-1})	46.9 ± 3.6	48.7 ± 2.3
Osteoblasts/tissue area (mm^{-2})	766 ± 52	605 ± 38^a
Osteoclasts/bone perimeter (mm^{-1})	10.6 ± 0.5	10.5 ± 0.8
Eroded surface/bone surface (%)	25.3 ± 1.5	24.1 ± 1.3
Mineralizing surface/bone surface (%)	3.4 ± 0.7	2.1 ± 0.6
Mineral apposition rate (μm/d)	1.10 ± 0.07	1.11 ± 0.05
Bone formation rate/bone surface (μm^3/μm^2/d)	0.040 ± 0.011	0.023 ± 0.006

Bone histomorphometry was performed on femurs from 1-month-old female CTGF homozygous ($CTGF^{+/+}$), and wild-type (WT) controls for line 1. For static histomorphometry, sections were stained with toluidine blue, and for dynamic histomorphometry unstained sections were analyzed by fluorescence microscopy. Values are means $\pm$ SEM; $n = 6$ to 9, except for dynamic histomorphometry, where $n = 4$ to 6.
^{a} Significantly different from wild-type controls, $P < 0.05$.

Wnt 3a caused a stimulatory effect on the transactivation of a 16xTCF-Luc reporter, where 16 TCF/Lef binding sites direct luciferase expression. The effect was modest, possibly because osteoblast-enriched cell cultures and not cultures of early osteoblast precursor cells were studied. Wnt 3a also increased the levels of cytosolic β-catenin (Fig. 2). The effect of Wnt 3a on the transactivation of the 16xTCF-Luc reporter and on β-catenin levels was decreased in cells from CTGF transgenics (Fig. 2, B and D).

To explore additional mechanisms involved in the inhibitory effect of CTGF on osteoblast function, we tested whether CTGF modified IGF-I signaling. IGF-I signals through the phosphatidyl-inositol-3 kinase, resulting in the activation of Akt. IGF-I at 10 and 30 nM increased phosphorylation of Akt in osteoblastic cells from control mice, but not in cells from CTGF transgenics, indicating an inhibitory effect of CTGF on IGF signaling (Fig. 2E).

In accordance with the results obtained in calvarial osteoblastic cells, CTGF overexpression did not modify cell number (not shown), and tempered the rise in alkaline phosphatase and osteocalcin mRNA levels observed in control marrow stromal cells cultured in the presence of differentiation medium for 10 d (Table 7). BSP expression was decreased in cells from CTGF transgenics at confluence and to a lesser extent 10 d after confluence. CTGF overexpression tempered the increase in type I collagen expression so that after 14 d of culture type I collagen mRNA levels were 40% lower in cells from transgenics than in control cells ($P < 0.05$).

Discussion

Our findings demonstrate that transgenic mice overexpressing CTGF develop decreased trabecular bone volume and osteopenia. The degree of osteopenia appeared to be related to the level of transgene expression, and heterozygous from a line expressing low transgene levels (line 1) did not exhibit a phenotype. There were no sex-dependent differences in the skeletal phenotype, although males from one transgenic line had decreased body weight, whereas females did not. The reason for this difference remains unexplained, but could involve skeletal and nonskeletal effects of CTGF because it is a secreted protein. The decrease in trabecular bone volume in CTGF transgenic mice appears to be secondary to decreased osteoblast function because there was a

TABLE 6. Expression of osteoblast gene markers in osteoblastic cells from CTGF transgenics and wild-type controls

Calvarial osteoblasts 10 d after confluence	WT	CTGF
Alkaline phosphatase	2.7 ± 0.5	1.2 ± 0.2[a]
Osteocalcin	1.1 ± 0.1	0.6 ± 0.1[a]
Bsp	5.1 ± 0.7	3.1 ± 0.7 (*P* < 0.08)
Ctgf	0.39 ± 0.03	0.8 ± 0.1[a]

Calvarial osteoblastic cells from CTGF heterozygous transgenics (line 2) and wild type (WT) controls were cultured for 10 d after confluence. Total RNA was reverse-transcribed, and amplified by real time RT-PCR in the presence of specific primers. Data are expressed as *alkaline phosphatase*, *osteocalcin*, *bsp* and *ctgf* copy number, determined by real time RT-PCR, corrected for *rpl38* expression. Values are means ± SEM, *n* = 4.

[a] Significantly different from wild-type controls, *P* < 0.05.

decrease in mineral apposition and bone formation rate, whereas osteoblast number was not affected. There was no apparent evidence of increased bone resorption. The lack of an effect of CTGF on osteoblast number may be a reflection of the osteocalcin promoter used in the study because it becomes active after osteoblast cell maturation. Consequently, CTGF is not overexpressed before the formation of mature osteoblasts. However, CTGF is a secreted soluble factor and could have affected neighboring undifferentiated cells of the osteoblastic lineage.

The *in vivo* skeletal phenotype of CTGF transgenics is consistent with previous studies indicating that transgenic mice overexpressing CTGF in chondrocytes, under the control of the type-XI collagen promoter, showed decreased bone density (18), suggesting that CTGF in excess leads to osteopenia. On the other hand, *ctgf* null mice have impaired bone formation and mineralization due to defects in cartilage and vascular development, indicating that CTGF is necessary for endochondral bone formation (17).

The *in vivo* findings were confirmed by *in vitro* experiments in osteoblastic cells from CTGF transgenic mice. CTGF overexpression caused a decrease in osteoblast gene marker expression in osteoblasts and stromal cells, indicating an inhibition of osteoblast cell function. The mechanism of the impaired osteoblastic function in CTGF transgenics could involve the binding and sequestration of BMPs by CTGF in the bone environment because osteoblasts from CTGF transgenics exhibited a decreased responsiveness to BMP-2 on Smad signaling. In fact, previous studies have shown direct interactions between CTGF and BMP-2, and as a consequence an inhibitory effect on osteoblastogenesis (15, 16). Although, BMPs have been shown to induce the differentiation of mesenchymal cells toward cells of the osteoblastic lineage, they also enhance the function of the osteoblasts, and our results in CTGF transgenics suggest a more pronounced inhibitory effect on osteoblastic function than number, caused possibly by the expression of the osteocalcin promoter in mature cells (37). CTGF overexpression also has an inhibitory effect on Wnt/β-catenin signaling and activity. This could be explained by direct interactions between CTGF with Wnt coreceptors, although the suppression of BMP signaling can contribute to decreased Wnt activity (38, 39). This dual inhibitory effect of CTGF is shared by BMP antagonists, which also have been shown to decrease BMP and Wnt activity. Similarly, Wnt antagonists, such as Dickkopf 1 and sclerostin op-

pose BMP actions (38, 39). In addition, CTGF has been shown to bind IGF-I, although with low affinity, and as a consequence to reduce IGF-I effects on phosphatidyl-inositol-3 kinase (8). Because IGF-I enhances osteoblastic function, its inhibition by CTGF may contribute to the osteoblastic phenotype exhibited by CTGF transgenic mice.

The BMP, Wnt, and IGF-I antagonistic activity of CTGF would support our findings of its inhibitory role in osteoblastic function and possibly osteoblastogenesis. However, down-regulation of CTGF *in vitro* using RNA interference in C3H10T ½ cells has demonstrated that CTGF is necessary, under selected experimental conditions, for osteoblastogenesis (13, 40, 41). This dual permissive and inhibitory activity is not limited to CTGF, and it is shared by BMP antagonists of the twisted gastrulation/chordin families, which are structurally related to CCN proteins (11, 42–46). Furthermore, under certain *in vitro* experimental conditions, forced overexpression of CTGF can favor osteoblastogenesis by mechanisms independent of BMP and Wnt signaling (47–51). Recently, we found that the constitutive expression of CTGF can induce hairy and enhancer of split 1 transcription and the transactivation of nuclear factor of activated T-cells (NFAT) in ST-2 stromal cells, and these two signals may have a stimulatory effect on osteoblastogenesis (47–50). Furthermore, the inhibitory effect of CTGF on BMP signaling shown in the present studies was not observed in ST-2 cells under conditions of forced overexpression of CTGF (47–50). It is apparent that the signals activated *in vitro* were not consistently operational *in vivo*, but they may have been active at selected times in certain mice. Although there were no changes in bone resorption, and the number of osteoclasts/ perimeter were not changed in CTGF transgenics as a whole, we noted that sporadic CTGF transgenics exhibited increased osteoclast surface. The mechanism could have involved induction of NFAT activity in these mice, and as a result an increase in osteoclastogenesis (51, 52).

CTGF is expressed by endothelial cells, and has complex effects on angiogenesis. CTGF induces endothelial cell replication and survival and is required for angiogenesis in cartilage, but not in classic models of angiogenesis (53–55). The expression of CTGF is induced by hypoxia-inducible factor (HIF) acting either directly by increasing CTGF, or indirectly by enhancing the expression of vascular endothelial growth factor (VEGF), which in turn can stimulate CTGF expression (56, 57). It is of interest that both HIF and VEGF act by posttranscrip-

TABLE 7. Expression of osteoblast gene markers in stromal cells from CTGF transgenics and wild-type controls

Stromal cells	Confluence		10 d after confluence	
	WT	CTGF	WT	CTGF
Alkaline phosphatase	6.3 ± 1.7	2.8 ± 0.1	8.6 ± 1.0	4.0 ± 0.4[a]
Osteocalcin	2.1 ± 0.4	1.9 ± 0.1	21.0 ± 3.0	7.7 ± 1.4[a]
Bsp	2.7 ± 0.4	0.9 ± 0.4[a]	3.7 ± 1.6	2.1 ± 0.8
Ctgf	1.3 ± 0.1	2.1 ± 0.1[a]	0.8 ± 0.1	1.8 ± 0.1[a]

Bone marrow stromal cells from CTGF heterozygous transgenics (line 2) and wild type (WT) controls were cultured for 10 d after confluence. Total RNA was reverse-transcribed, and amplified by real-time RT-PCR in the presence of specific primers. Data are expressed as *alkaline phosphatase*, *osteocalcin*, *bsp* and *ctgf* copy number, determined by real-time RT-PCR, corrected for *rpl38* expression. Values are means ± SEM, *n* = 4.

[a] Significantly different from wild-type controls, *P* < 0.05.

FIG. 2. Effect of CTGF overexpression on BMP-2, Wnt 3a and IGF-I signaling in calvarial osteoblastic cells from CTGF heterozygous transgenics (line 2) and wild-type controls. A and B, Calvarial osteoblasts were cultured to subconfluence, and transfected with 12xSBE-Oc-pGL3 or 16xTCF-Luc, and a CMV/β-galactosidase expression vector. After 16 h, cells were switched to serum-free DMEM and treated or not with BMP-2 or Wnt 3a at the indicated doses for 24 h. Data shown represent luciferase/β-galactosidase activity for wild-type control cells (*white bars*) or CTGF overexpressing cells (*black bars*). *Bars* represent means ± SEM for six observations. *, Significantly different from wild-type controls, $P < 0.05$. C–E, Osteoblasts were cultured to confluence and for 10 d after confluence, serum-deprived overnight and treated with BMP-2 at 3.3 nM for 20 min, Wnt 3a at 2.7 nM for 6 h, or IGF-I at 10 and 30 nM for 15 min for the determination of Smad 1/5/8 phosphorylation (C), β-catenin levels (D) or Akt phosphorylation (E). Total cell lysates (C and E) or cytosolic extracts (D) were resolved by gel electrophoresis and transferred to Immobilon P membranes, which were incubated with antibodies to phosphorylated Smad 1/5/8 or unphosphorylated Smad 1, with antibodies to β-catenin or actin, or antibodies to phosphorylated or unphosphorylated Akt.
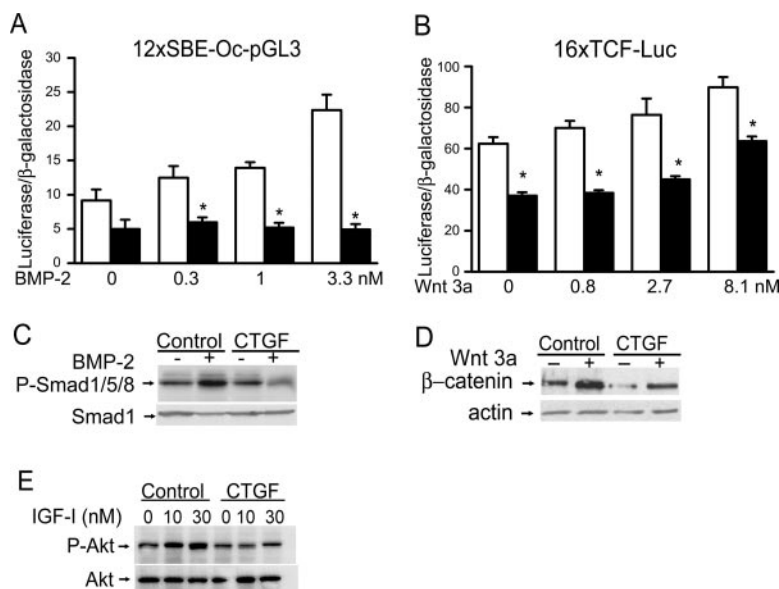
tional mechanisms to regulate CTGF expression. A feedback regulatory mechanism also plays a regulatory function because CTGF binds VEGF and suppresses HIF expression, and as a consequence decreases VEGF expression (58, 59). It is of interest that CTGF can induce matrix metalloproteinases (MMP) 2 and 9, and MMP-2 can free VEGF from CTGF, and as a result favor angiogenesis (60). Consequently, the impact of CTGF on angiogenesis in a specific tissue depends on its regulation and association with proteases and other factors present in the cellular environment. CTGF may have an important function in angiogenesis, under basal or hypoxia-induced conditions, in the skeletal microenvironment. However, immunohistochemistry of femoral sections of CTGF transgenics failed to reveal an increase in either VEGF or platelet endothelial cell adhesion molecule (PECAM-1, CD31), which is expressed on the surface of endothelial cells and plays a role in angiogenesis (data not shown) (61, 62). Additional studies in mice misexpressing *ctgf* are needed to define its role in skeletal angiogenesis.

In conclusion, our studies reveal that CTGF overexpression *in vivo* causes osteopenia and impaired osteoblast *i.e.* function, possibly by inhibiting BMP, Wnt, and IGF-I signaling and activity.

Acknowledgments

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