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## Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development

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### SUMMARY

Coordinated production and remodeling of the extracellular matrix is essential during development. It is of particular importance for skeletogenesis, as the ability of cartilage and bone to provide structural support is determined by the composition and organization of the extracellular matrix. Connective tissue growth factor (CTGF, CCN2) is a secreted protein containing several domains that mediate interactions with growth factors, integrins and extracellular matrix components. A role for CTGF in extracellular matrix production is suggested by its ability to mediate collagen deposition during wound healing. CTGF also induces neovascularization in vitro, suggesting a role in angiogenesis in vivo. To test whether CTGF is required for extracellular matrix remodeling and/or angiogenesis during development, we examined the pattern of *Ctgf* expression and generated *Ctgf*-deficient mice. *Ctgf* is expressed in a variety of tissues in midgestation embryos, with highest levels in vascular tissues and maturing chondrocytes. We confirmed that CTGF is a crucial regulator of cartilage extracellular matrix remodeling by generating *Ctgf*<sup>-/-</sup> mice. *Ctgf* deficiency leads to skeletal dysmorphisms as a result of impaired chondrocyte proliferation and extracellular matrix composition within the hypertrophic zone. Decreased expression of specific extracellular matrix components and matrix metalloproteinases suggests that matrix remodeling within the hypertrophic zones in *Ctgf* mutants is defective. The mutant phenotype also revealed a role for *Ctgf* in growth plate angiogenesis. Hypertrophic zones of *Ctgf* mutant growth plates are expanded, and endochondral ossification is impaired. These defects are linked to decreased expression of vascular endothelial growth factor (VEGF) in the hypertrophic zones of *Ctgf* mutants. These results demonstrate that CTGF is important for cell proliferation and matrix remodeling during chondrogenesis, and is a key regulator coupling extracellular matrix remodeling to angiogenesis at the growth plate.

## Keywords

CCN; CTGF; Chondrogenesis; Angiogenesis; Mutant

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## INTRODUCTION

Connective tissue growth factor (CTGF, CCN2) is a member of the CCN family of secreted proteins, which also includes Cyr61, NOV, WISP1, WISP2 and WISP3 (Bork, 1993; Moussad and Brigstock, 2000; Perbal, 2001). CTGF is a major inducer of extracellular matrix (ECM) production in fibrotic diseases, which are characterized by excessive collagen deposition. CTGF is overexpressed in fibrotic lesions, and the degree of overexpression correlates with severity of disease (Blom et al., 2001; Dammeier et al., 1998; Frazier et al., 1996; Grotendorst, 1997; Lasky et al., 1998; Mori et al., 1999; Stratton et al., 2001). The ability of CTGF to induce collagen deposition in pathological conditions raises the possibility that it may be a mediator of ECM production in tissues such as cartilage and bone during development. However, nothing is known about its role in normal tissues.

CTGF may act in part as a mediator of transforming growth factors  $\beta$  (TGF $\beta$ s) and bone morphogenetic proteins (BMPs) during development. TGF $\beta$ s play roles in a wide variety of developmental events, and TGF $\beta$  induces CTGF expression in many cell types because the CTGF promoter contains a TGF $\beta$  response element (Holmes et al., 2001). Moreover, CTGF contains a von Willebrand type C domain, which is thought to mediate physical interactions with growth factors such as TGF $\beta$  (Wong et al., 1997). Consistent with this, CTGF binds to BMPs and TGF $\beta$ , leading to inhibition of BMP and enhancement of TGF $\beta$  signaling (Abreu et al., 2002).

In addition to its potential role in TGF $\beta$  and BMP pathways, several lines of evidence indicate that CTGF acts independently of TGF $\beta$  superfamily members. For example, CTGF and the related protein Cyr61 have effects on gene expression that often oppose those of TGF $\beta$  (Chen et al., 2001a), and the induction of CTGF expression occurs through both TGF $\beta$ -dependent and -independent pathways (reviewed by Blom et al., 2002). In addition, a distinguishing feature of CTGF and other CCN proteins is the presence of several domains that participate in protein interactions (Bork, 1993). In addition to the von Willebrand type C domain required for TGF $\beta$  and BMP binding (Abreu et al., 2002), CCNs contain a thrombospondin (TSP) module, which enables TSP to bind to ECM proteins, matrix metalloproteinases (MMPs) and integrins (Bornstein, 2001; Lau and Lam, 1999). CTGF promotes effects on cell survival, adhesion and migration through interactions with integrins (Babic et al., 1999; Chen et al., 2001a; Jedsadayanmata et al., 1999; Leu et al., 2002). CTGF also binds to low density lipoprotein receptor-related protein (LRP), but it is as yet unclear whether this interaction facilitates CTGF signaling and/or clearance (Babic et al., 1999; Jedsadayanmata et al., 1999; Segarini et al., 2001). In addition, CTGF binds to MMPs, and inactivates VEGF through direct physical interactions (Inoki et al., 2002). Finally, the C-terminal domain of CTGF promotes cell proliferation (Brigstock, 1997). Although the constellation of proteins with which CTGF interacts in vivo is not known, the presence of multiple domains is consistent with a role for CTGF as an integrator of multiple growth factor-, integrin- and ECM-derived signals.

Because the ECM transduces signals from the microenvironment, and regulates the release of growth factors, alterations in ECM composition during development lead to dynamic changes in its signaling properties. ECM remodeling is achieved by regulating the production and degradation of specific ECM components. MMPs, which comprise a large family of enzymes with differential abilities to degrade specific ECM components, play a

vital role in this process (Sternlicht and Werb, 2001). MMPs also cleave growth factors and their binding proteins, thereby activating or inhibiting specific signaling pathways. Overexpression of CTGF in fibroblasts leads to increased expression of MMP1, MMP2 and MMP3 (Chen et al., 2001b; Fan and Karnovsky, 2002), suggesting that CTGF coordinates ECM production and degradation.

The expression of CTGF in cartilage, and its ability to promote chondrogenic differentiation in vitro (Nakanishi et al., 2000), is consistent with a potential role for CTGF in ECM remodeling during skeletal development. During chondrogenesis, mesenchymal cells condense into characteristic shapes. Cells within these condensations subsequently differentiate into chondrocytes, which secrete ECM components, surrounded by a layer of perichondrial cells. As development proceeds, cells within the aggregates exit the cell cycle and mature, leading to stratified zones of cells at progressive stages of differentiation (resting, proliferative, prehypertrophic and hypertrophic). In cartilages destined to be replaced by bone through endochondral ossification, terminally differentiated hypertrophic chondrocytes undergo apoptosis as the growth plate is invaded by blood vessels and osteoblasts. The ability of the growth plate to support angiogenesis is dependent upon the activity of MMPs, although the targets of MMP action in hypertrophic chondrocytes are not known (Vu et al., 1998; Ensig et al., 2000).

Along with a potential role in the regulation of ECM composition, a role for CTGF in angiogenesis is likely, as CTGF expression is induced by vascular endothelial growth factor (VEGF) (Suzuma et al., 2000), is expressed in endothelial and vascular smooth muscle cells, and induces neovascularization (Babic et al., 1999; Moussad and Brigstock, 2000; Shimo et al., 1999). Although these studies imply a positive role for CTGF in angiogenesis, CTGF can bind to VEGF and inhibit the ability of VEGF to induce angiogenesis (Hashimoto et al., 2002; Inoki et al., 2002). These observations suggest that CTGF may have both positive and negative effects on angiogenesis.

Despite strong evidence that CTGF promotes ECM production and angiogenesis in fibrotic disease, nothing is known about its role during development. In particular, downstream targets of CTGF action in normal tissues have not been identified. Additional questions include the extent to which CTGF collaborates with TGF $\beta$  during development, and whether CTGF and the related molecule, Cyr61, share overlapping functions, as these proteins have related activities in vitro, are co-expressed in several tissues, and serve as ligands for the same set of integrins (Perbal, 2001). To address these issues, we examined the pattern of *Ctgf* expression, studied its effects on ECM production, and generated *Ctgf*-deficient mice.

## MATERIALS AND METHODS

### In situ hybridization

In situ hybridization was performed as described (Hogan et al., 1994). The *Ctgf* probe was generated by subcloning a partial mouse cDNA IMAGE clone (ID 551901) into pBluescript (Stratagene), linearizing with *EcoRI*, and reverse transcribing with T7 polymerase. The *Tgfb1* probe was obtained from American Type Culture Collection. The *ColIII* and *ColX* probes were a generous gift from Vicki Rosen. A. McMahon kindly provided the *Ihh* probe.

### Gene targeting

*Ctgf* clones were isolated from a strain 129Sv/J mouse BAC library (Incyte). The targeting construct was generated by replacing a 500 bp *SmaI* fragment containing exon 1, the TATA box and the transcription start site with the neomycin resistance gene under the control of a PGK promoter (PGKneopA). The targeting vector was electroporated into RW-4 ES cells (Incyte) as described (Ramírez-Solis et al., 1993). Targeted clones were injected into

blastocysts by the UCLA Transgenic Mouse Facility. Chimeras were bred to Balbc/J females to test for germline transmission. The mutation has been maintained on a hybrid 129Sv/J × Balbc/J background. Genotyping was performed by Southern blot analysis of *Hind*III-digested genomic DNA using the external probe indicated in Fig.2A.

### Semi-quantitative RT-PCR

Embryonic fibroblasts (EFs) were prepared as described (Abbondanzo et al., 1993) and grown to confluence in DMEM containing 10% FBS. Cells were then grown in serum-free DMEM containing 50 µg/ml ascorbic acid, with or without 5 ng/ml TGFβ1 (R&D Systems) for 24 hours. RNA was harvested using the Qiashreder and Rneasy kits (Qiagen). RNA synthesis was performed using Superscript II (Gibco BRL). RT-PCR was performed using total RNA from EFs derived from mutant or wild-type neonates. The CTGF primers were 5'CTGTCAAGTTTGAGCTTTCTGG 3' and 5' GGAAGTCAAAGATGTCATTGTCC 3'. Control primers for GAPDH were 5' ACCCAGAAGACTGTGGATGG 3' and 5' ATCATACTTGGCAGGTTTCTCC 3'.

Long bones at E14.5, or growth plates at later stages from individual genotyped wild-type and mutant littermates were dissected out and total RNA was prepared using TRIzol (Gibco-BRL). Levels of *ColIII*, *ColIX*, *Cbfa1* (*Runx2* – Mouse Genome Informatics), *Vegf* (*Vegfa* – Mouse Genome Informatics) and *Mmp9* were examined by semi-quantitative RT-PCR, on oligo (dT)-primed cDNA (Superscript, Gibco-BRL) from growth plate total RNA using the following primers: *ColIII*, 5'-CACACTGGTAAGTGGGGCAAGAC and 3'-GGATTGTGTTGTTTCAGGGTTCGGG; *ColIX*, 5'-CCTGGGTTAGATGGAAA and 3'-AATCTCATAAATGGGATGGG; *Vegf*, 5'-GGGTGCACTGGACCCTGGGTTTAC and 3'-CCTGGCTCACCGCCTTGGCTTGTC; *Cbfa1*, 5'-TGACTGCCCCACCCTCTTAG, 3'-GGCAGCACGCTATTAATCCAAA; *Mmp9* 5'-AACCTGTGTGTTCCCGTT and 3'-GGATGCCGCTATGTCGTCT; and *Gapdh* 5'-CCCCTTCATTGACCTCAACT and 3'-TTGTCATGGATGACCTTGGC. Typical reactions were performed with cycles conditions of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute. The following numbers of cycles were used for each primer pair: *Gapdh*, 20, 22, 24; *ColIII*, 22, 24, 26; *ColIX*, 26, 30, 24; *Mmp9*, 26, 28, 30; *Runx2*, 30, 2, 34; *Vegf*, 28, 30, 32. RNA samples from five wild-type and five mutant littermates were examined. Each RNA sample was analyzed twice. Quantification of expression relative to *Gapdh* was performed using NIH image.

### Skeletal analysis and histology

Cleared skeletal preparations were made as described (Yi et al., 2000). For histology, specimens were fixed with 4% paraformaldehyde or 10% neutral formalin, decalcified with Immunocal (Decal Chemical) and embedded in paraffin. Deparaffinized sections (7 µm) were stained with Toluidine Blue or safranin-o/light green. Plastic sections were fixed in neutral-buffered formalin, dehydrated and embedded undecalcified in DDK-Plast methylmethacrylate resin. Sections (4 µm) were stained with von Kossa/tetrachrome.

Immunostaining was performed on deparaffinized sections with antibodies for link protein and aggrecan (8A4, IC6, Developmental Studies Hybridoma Bank), MMP9 and MMP13 (Chemicon) or type II collagen (Research Diagnostics) at a 1:100 dilution using the Histomouse kit (Zymed). Tissue sections were pretreated with chondroitinase ABC (0.05n u/ml; Sigma) for 8A4 and IC6, or with 2.5% hyaluronidase (Calbiochem) for MMP9, MMP13, and type II collagen. Immunostaining for PECAM (Bectin Dickinson) was carried out on cryosections. Briefly, cryosections were treated with 2.5% hyaluronidase for 30 minutes at room temperature and with 3% hydrogen peroxide/PBS for 10 minutes at room temperature. After washing with PBS and blocking with 2% dry milk, 5% goat serum in PBS, incubation with anti-PECAM antibody (Zymed; 1:100) was carried out overnight at 4°C. After washing

with blocking buffer, slides were incubated with rat secondary antibody for 2 hours at room temperature. Color was developed with DAB for MMP9 and PECAM, and with the Zymed kit chromogen for all other antibodies.

For analysis of osteoclasts, sections were stained for tartrate-resistant acid phosphatase (TRAP) positive cells using a TRAP staining kit (Sigma).

### Cell proliferation and apoptosis

Cell proliferation was assessed by BrdU incorporation as described (Yi et al., 2000), and by PCNA immunostaining. For PCNA, 7  $\mu$ m decalcified paraffin wax-embedded sections were used with a 1:100 dilution of anti-PCNA antibody as described above for PECAM staining. Cell proliferation was assessed as described (Yi et al., 2000). Cell death analysis by TUNEL analysis was performed using the Apoptosis Detection System, Fluorescein kit (Promega).

## RESULTS

### *Ctgf* expression in midgestation embryos

In situ hybridization revealed strong expression in skeletal, vascular and neural tissues. *Ctgf* mRNA was detected beginning at E9.5 in the nasal process, proximal regions of the second and third branchial arches, and the developing heart (Fig. 1A). Expression was also observed within the neural tube (Fig. 1B). At E10.5-E11.5, high levels of *Ctgf* expression persisted in the proximal branchial arches, dorsal nasal process, heart and floorplate (Figs. 1C,D). Lower levels were seen in the roofplate and dermomyotome (Fig. 1D).

*Ctgf* expression persists in the meninges, heart and major blood vessels from E13.5 to birth (Fig. 1E,F, and data not shown). Within the heart, *Ctgf* mRNA is present in ventricular myofibroblasts and in midline tissue within the fusing cushions of the outflow tract (Fig. 1E). *Ctgf* transcripts persist at least until birth in endothelial and smooth muscle layers of major blood vessels (Fig. 1F).

In situ hybridization experiments revealed that *Ctgf* is highly expressed in cartilage in midgestation embryos. During skeletogenesis, *Ctgf* is first expressed at E12.5 in perichondrium (Fig. 1G,H). By E13.5, *Ctgf* persists at high levels in perichondrium and in adjacent chondrocytes (Fig. 1I). At this stage, *Ctgf* can also be detected at lower levels within maturing chondrocytes at the centers of developing long bones (Fig. 1J). At E14.5, *Ctgf* expression persists in chondrocytes adjacent to the perichondrium, and is upregulated in maturing chondrocytes (Fig. 1K); at this stage, *Ctgf* expression overlaps extensively with that of Indian hedgehog (*Ihh*), a marker for prehypertrophic and hypertrophic chondrocytes (Bitgood and McMahon, 1995) (Fig. 1L; see also Fig. 5A,B). At this and subsequent stages, the strongest site of *Ctgf* expression is within the most mature population of chondrocytes. For example, by E16.5, *Ctgf* is highly expressed in terminally differentiated hypertrophic chondrocytes, as demonstrated by its overlapping pattern of expression with that of type X collagen (*ColX*), a marker for hypertrophic chondrocytes (Fig. 5F,H). *Ctgf* expression persists at this stage in chondrocytes adjacent to the perichondrium within the prehypertrophic zone (Fig. 5F,G). The expression of high levels of *Ctgf* in hypertrophic cells continues at least until birth (Fig. 1M). In summary, within developing cartilage, *Ctgf* is expressed initially in the perichondrium. At later stages, *Ctgf* is expressed within maturing chondrocytes. Transcripts persist in chondrocytes adjacent to the perichondrium at least until E16.5. However, terminally differentiating prehypertrophic and hypertrophic chondrocytes are the major sites of *Ctgf* expression in developing cartilage.



## Generation of *Ctgf* mutants

The expression of *Ctgf* in cartilage and vascular structures throughout development suggested roles in the development of these tissues. To test this hypothesis, we generated *Ctgf*-deficient mice (Fig. 2A,B). *Ctgf* heterozygotes are viable and fertile. Semi-quantitative RT-PCR analysis confirmed that the targeted allele is null. Fibroblasts from wild-type embryos exhibited a low level of *Ctgf* expression, which increased 20-fold upon treatment with TGF $\beta$ 1 (Fig. 2C). By contrast, *Ctgf* transcripts were undetectable in mutant fibroblasts, even after exposure to TGF $\beta$ 1. Homozygous mutants are recovered among neonates in the expected Mendelian ratio. In spite of widespread *Ctgf* expression in vascular tissues, histological examination and gross dissections revealed no evidence for generalized angiogenesis defects or impairment of cardiac function in *Ctgf* mutants (data not shown). However, *Ctgf*<sup>-/-</sup> mice died within minutes of birth.

## Generalized chondrodysplasia in *Ctgf* mutants

*Ctgf*<sup>-/-</sup> mice die shortly after birth because of respiratory failure caused by skeletal defects. Within the axial skeleton, defects are observed along the entire vertebral column. By E14.5, vertebrae in mutants are broader than in wild-type littermates (Fig. 3A), and this phenotype persisted at birth (Fig. 3B). In newborns, the sterna of *Ctgf* mutants are short and bent inwards, and ossified regions of the ribs are kinked (Fig. 3C,D). The kinks in ossified regions are a consequence of prior defects in chondrogenesis, as the rib cartilage adjacent to sites of mineralization is already bent at E14.5 (Fig. 3E). The overall lengths of individual ribs are not significantly different, but the extent of ossification is reduced in mutants (Fig. 3F), and the zone of mineralizing cartilage is expanded (Fig. 3F; arrow in Fig. 3C), suggesting defective replacement of cartilage by bone during endochondral ossification. In addition, ~10% of mutants exhibit misaligned sternal fusion (Fig. 3G). Endochondral defects are also observed throughout the appendicular skeleton. By E13.5, deformation of the limb cartilage is apparent in *Ctgf* mutants (Fig. 3H), leading to kinks in the radius, ulna, tibia and fibula in *Ctgf* mutants at birth (Fig. 3I).

Within the craniofacial skeleton, the cranial vault had a domed appearance, the mandibles were shortened, and the ethmoid bones were deformed (Fig. 3J,K). All *Ctgf* mutants have a secondary cleft palate because of a failure in elevation of the palatal shelves (Fig. 3L), most likely as a secondary consequence of defects in the formation of endochondral elements at the base of the skull and in nasal cartilages (Fig. 3K,L). The shortened mandible is a consequence of deformations in Meckel's cartilage (Fig. 3M). These abnormalities indicate that *Ctgf*-deficient cartilage has inferior mechanical properties, rendering it susceptible to deformation during development. This hypothesis provides an explanation for the enlargement of mutant vertebrae, suggesting that they become distended as a result of their inability to resist the forces of the expanding neural tube.

We performed a histological analysis to investigate these defects in more detail. At E12.5, when *Ctgf* mRNA is localized to the perichondrium, the sizes and morphologies of the cartilaginous condensations in *Ctgf* mutants and wild-type littermates are indistinguishable (Fig. 4A, and data not shown). By E14.5, chondrocytes in the midshaft regions of long bones from wild-type mice are undergoing differentiation into prehypertrophic and hypertrophic cells (Fig. 4B). No histological differences were detected in the proliferative zones of wild-type and mutant littermates at this stage. However, in *Ctgf* mutants, long bones are already bent near the junction between hypertrophic and nonhypertrophic cells (Fig. 4B). The hypertrophic zones did not differ in length in *Ctgf* mutants and wild-type littermates at this stage (Fig. 4B, and data not shown). At E16.5, when *Ctgf* is most highly expressed in hypertrophic chondrocytes, endochondral ossification has commenced in long

bones from wild-type and mutant littermates (Fig. 4C). An enlarged and disorganized hypertrophic zone is seen in mutants, and this persists at birth (Fig. 4D).

Thus, loss of *Ctgf* leads to distorted cartilages. The presence of these defects prior to ossification, along with high levels of *Ctgf* expression seen in differentiating chondrocytes, indicate a primary role for *Ctgf* in cartilage. Moreover, although *Ctgf* is expressed in perichondrium beginning at E12.5, histological differences are not apparent until E14.5, coincident with the expression of *Ctgf* in maturing chondrocytes. These results suggest that *Ctgf* is involved in late stages of chondrocyte proliferation and/or differentiation.

### Deficient cell proliferation in *Ctgf*<sup>-/-</sup> chondrocytes

CTGF promotes chondrocyte proliferation in vitro (Nakanishi et al., 2000), and *Ctgf*<sup>-/-</sup> mice exhibit morphological and histological features consistent with proliferative defects. Therefore, proliferation was examined by staining for proliferative cell nuclear antigen (PCNA). These analyses revealed a proliferative defect in neonatal *Ctgf*<sup>-/-</sup> growth plates (Fig. 4E). No differences were noted at E12.5. However, by E14.5, when *Ctgf* is highly expressed in prehypertrophic chondrocytes (Fig. 1K,L), the percentage of proliferating chondrocytes was decreased in mutants. This proliferative defect was more pronounced at E16.5 (Fig. 4E). Therefore, *Ctgf* appears does not regulate cell proliferation at early stages of chondrogenesis, but appears to be required at later stages.

TUNEL staining was performed to determine whether altered rates of apoptosis might contribute to the cartilage deformations and/or expansion of the hypertrophic zone in mutants. In both wild-type and mutant growth plates, apoptosis is confined to terminal chondrocytes (data not shown). Therefore, apoptosis does not appear to contribute to the defective mechanical properties of *Ctgf* mutant cartilage, and the expansion of the hypertrophic zone in mutants cannot be attributed to an inability of mutant chondrocytes to undergo apoptosis.

*Ctgf* is highly expressed in prehypertrophic chondrocytes at E14.5 and promotes chondrocyte proliferation at this stage (Fig. 1K,L, Fig. 4E). *Ihh*, which coordinates the progression of chondrocytes to hypertrophy and promotes cell proliferation (Long et al., 2001; St-Jacques et al., 1999), is expressed in a pattern overlapping that of *Ctgf*. Therefore, to determine whether *Ctgf* might affect chondrocyte proliferation by regulating *Ihh* levels, we examined *Ihh* expression by in situ hybridization and semi-quantitative RT-PCR. We also examined the expression of *ColX* because CTGF promotes *ColX* expression in chondrocytes in vitro (Nakanishi et al., 2000). Semi-quantitative RT-PCR analysis of growth plates at E14.5 revealed no apparent differences in levels of *Ihh* or *ColX* expression between *Ctgf* mutants and wild-type littermates (data not shown). In situ hybridization studies also indicated that the expression of these markers is not altered in *Ctgf* mutants. In wild-type mice at E14.5, *Ihh* is expressed in prehypertrophic and hypertrophic chondrocytes, while *ColX* expression is restricted to hypertrophic chondrocytes (Fig. 5A-C). At this stage, *Ctgf* expression overlaps with that of *Ihh* and *ColX*, indicating that *Ctgf* is expressed primarily in hypertrophic chondrocytes (Fig. 5A-C). In E14.5 *Ctgf* mutant littermates, *Ihh* and *ColX* are similarly expressed in prehypertrophic and hypertrophic chondrocytes (Fig. 5D,E). At E16.5 in wild-type mice, the pattern of *Ctgf* expression overlaps extensively with that of *ColX* in hypertrophic chondrocytes; *Ctgf* transcripts persist in chondrocytes adjacent to the perichondrium in the prehypertrophic zone (Fig. 5F-H). By E16.5, the hypertrophic zone is expanded in *Ctgf* mutants. At this stage, *Ihh* and *ColX* were appropriately expressed in the prehypertrophic and hypertrophic regions, respectively (Fig. 5I,J). Owing to the distorted shapes of the *Ctgf* mutant skeletal elements, we were unable to determine unequivocally whether the zones of expression of these markers were expanded in mutants. However, RT-PCR analysis revealed no differences in levels of *ColX* expression (data not shown).

Therefore, although the hypertrophic zone in *Ctgf* mutants is enlarged by E16.5, this is not accompanied by obvious expansions of the domains of *Ihh* or *ColX* expression (Fig. 5G,I). The basis for these observations is unknown at present. It is possible that the subtle expansions in the domains of *Ihh* and/or *ColX* expression collectively lead to the expanded growth plates seen in *Ctgf* mutants.

In summary, *Ctgf* is required for normal rates of chondrocyte proliferation in vivo. Proliferative defects were detected beginning at E14.5, coincident with the upregulation of *Ctgf* in prehypertrophic and hypertrophic chondrocytes. However, the decreased rate of cell proliferation in *Ctgf* mutants does not appear to be due to a decrease in *Ihh* mRNA levels, indicating that *Ctgf* acts downstream of *Ihh*, and/or by an independent mechanism. Finally, although CTGF promotes chondrocyte differentiation in vitro (Nakanishi et al., 2000), cleared skeletal preparations, histological examination, and analysis of *Ihh* and *ColX* expression revealed no apparent alterations in chondrocyte progression to hypertrophy in *Ctgf* mutants.

### Defective extracellular matrix production in *Ctgf* mutants

Cartilage ECM components are the primary determinants of its elastic and tensile properties. The deformed cartilages seen in *Ctgf* mutants suggested that CTGF is required for synthesis of normal levels of cartilage ECM components. Therefore we examined whether abnormalities in ECM content might contribute to the defective properties of *Ctgf* mutant cartilage. As previously discussed, no clear differences were seen in *ColX* mRNA levels in midgestation *Ctgf* mutants and wild-type littermates (Fig. 5, and data not shown). Similar results were obtained when the expression of type II collagen (*ColII*), the most abundant collagen present in cartilage, was examined by semi-quantitative RT-PCR and in situ hybridization from E12.5-17.5 (data not shown). Examination of neonates also revealed no obvious differences in the amount or distribution of collagen types II and X in cartilage matrix in *Ctgf* mutants (Fig. 6A,B). Therefore, although CTGF is a major regulator of type I collagen production during fibrotic responses, and induces the transcription of types II and X collagens in vitro (Nakanishi et al., 2000), CTGF does not appear to be a major regulator of their expression in vivo.

We also examined proteoglycan levels, as CTGF induces proteoglycan synthesis in vitro (Nakanishi et al., 2000). Safranin-o staining, a measure of overall proteoglycan content, revealed no apparent differences between wild-type and mutant littermates in the reserve and proliferative zones, but confirmed that the hypertrophic zone, which does not stain intensely with safranin-o, is expanded in mutants (Fig. 6C). However, levels of aggrecan, the main cartilage proteoglycan (Fig. 6D), and link protein, which stabilizes aggregates of aggrecan and hyaluronin (Fig. 6E), are reduced in *Ctgf*<sup>-/-</sup> growth plates, confirming that *Ctgf*-deficient growth plate cartilage exhibits defects in ECM content. Therefore, *Ctgf* is required for the expression of wild-type levels of specific cartilage ECM components in vivo, and the inferior mechanical properties of *Ctgf* mutant cartilage can be attributed to the reduced expression of these components.

### Defective growth plate angiogenesis and osteopenia in *Ctgf* mutants

Histological examination revealed a number of defects in neonatal growth plates of *Ctgf* mutants. Consistent with the proliferative defects detected by PCNA staining, longitudinal columns are disorganized within the hypertrophic zones in mutants (Fig. 7A). Staining by the von Kossa method revealed apparently normal mineralization of the hypertrophic cartilage matrix (Fig. 7A). Mineralized bone collars, which normally form in the perichondrium adjacent to prehypertrophic and hypertrophic chondrocytes, are lengthened in *Ctgf* mutants, consistent with the expansion of the hypertrophic zone (Fig. 7B).



As impairment of angiogenesis leads to an enlarged zone of hypertrophy (Gerber et al., 1999; Haigh et al., 2000; Vu et al., 1998), and *Ctgf* mutants exhibit expanded hypertrophic zones, we examined whether growth plate angiogenesis is defective. In wild-type neonates, abundant capillaries are seen invading the mineralized hypertrophic cartilage. However, few intact capillaries are visible in growth plates of mutants (Fig. 7A). Immunostaining for PECAM confirmed that growth plate angiogenesis is defective in *Ctgf* mutants; the fine network of capillaries, well developed in the ossification zones of wild-type neonates, is less extensive in *Ctgf* mutants, although blood vessels are present within intertrabecular spaces (Fig. 8A).

Defective growth plate angiogenesis is associated with decreased trabecular bone density (Gerber et al., 1999). Consistent with a defect in growth plate angiogenesis, the bone collar appears thinner, and less trabecular bone is present in *Ctgf* mutants (Fig. 7B). A primary role for CTGF in osteoblast function is also possible as CTGF is expressed in osteoblasts and promotes their proliferation and differentiation in vitro (Nishida et al., 2000; Xu et al., 2000a). Additional studies will be required to discriminate between direct and indirect roles for CTGF in osteoblasts.

### CTGF regulates the availability of local factors required for growth plate angiogenesis

Angiogenesis at the growth plate requires localized proteolytic modification of the ECM to permit invasion by endothelial cells, and MMPs play essential roles in this process (Vu et al., 1998; Zhou et al., 2000). MMP9 is required for growth plate angiogenesis, and is expressed in osteoclasts/chondroclasts (Reponen et al., 1994). MMP9 immunostaining in wild-type neonates is most intense at the hypertrophic cartilage-bone junction. By contrast, MMP9 immunostaining at this junction is diminished in growth plates of *Ctgf* mutants (Fig. 8B). Semi-quantitative RT-PCR analysis confirmed that *Mmp9* mRNA levels are reduced in the growth plates of mutants (Fig. 8C). To determine whether the decrease in MMP9 levels in mutant growth plates reflects a generalized loss of osteoclasts, staining for tartrate-resistant acid phosphatase (TRAP) activity, a marker for cells of the osteoclast lineage, was performed. In wild-type neonates, TRAP-positive cells are distributed throughout the bone marrow, and co-localize with MMP9-expressing cells at the cartilage-bone junction (Fig. 8D). By contrast, in *Ctgf* mutants, although TRAP-positive cells are found at normal levels in bone marrow (data not shown), few are seen at the cartilage-bone junction (Fig. 8D). Therefore, CTGF is important for efficient infiltration of the calcified cartilage matrix by MMP9-expressing osteoclasts/chondroclasts.

VEGF produced by hypertrophic cartilage promotes angiogenesis, is activated by MMP-mediated degradation of the cartilage matrix and is chemotactic for osteoclasts (Gerber et al., 1999; Haigh et al., 2000). VEGF protein is expressed at low levels in maturing chondrocytes at E14.5, and at high levels in hypertrophic chondrocytes of the neonatal wild-type growth plate (Carlevaro et al., 2000; Engsig et al., 2000). By contrast, VEGF immunostaining per cell is diminished in the expanded hypertrophic zone in newborn *Ctgf* mutants (Fig. 9A). This decrease in VEGF expression is seen only in the hypertrophic cartilage; expression in osteoblasts (Horner et al., 2001) is at normal levels (data not shown). We used semi-quantitative RT-PCR to examine whether the reduced VEGF immunostaining in *Ctgf* mutants is due to decreased VEGF mRNA levels (Fig. 9B,C). At E14.5, when *Vegf* is expressed at low levels in perichondrium and maturing chondrocytes (Zelzer et al., 2002), no differences in levels of *Vegf* expression can be detected in long bones of *Ctgf* mutants and wild-type littermates. However, by birth, when *Vegf* mRNA is highly expressed in hypertrophic chondrocytes, levels of *Vegf* mRNA are reduced in growth plates of *Ctgf* mutants, despite the enlargement of the hypertrophic zone (Fig. 9B,C).

Expression of VEGF in hypertrophic chondrocytes is dependent on *Cbfa1/Runx2* (Zelzer et al., 2001). However, no differences in levels of *Cbfa1/Runx2* expression were observed in mutant growth plates, suggesting that CTGF acts downstream of *Cbfa1/Runx2*, or in an independent pathway to induce and/or maintain *Vegf* mRNA expression in hypertrophic chondrocytes (Fig. 9B,C). In summary, VEGF is a target of CTGF action in hypertrophic cartilage. Reduced expression of this factor can account for the expanded zone of hypertrophy, reduced numbers of MMP9-expressing cells at the growth plate and diminished angiogenesis observed in *Ctgf* mutants.

## DISCUSSION

A large body of evidence links CCN proteins to many diseases, including fibrosis and tumorigenesis (Ayer-Lelievre et al., 2001; Denton and Abraham, 2001; Xu et al., 2000b). However, the roles of CCN proteins in normal developmental processes have received little attention. This study demonstrates that CTGF is important for multiple aspects of chondrogenesis. CTGF regulates chondrocyte proliferation, ECM synthesis and angiogenesis.

CTGF stimulates DNA synthesis in chondrocytes in vitro (Nakanishi et al., 2000), and chondrocyte proliferation is impaired in *Ctgf*<sup>-/-</sup> mice (Fig. 4E, Fig. 7A). Interestingly, in spite of high levels of expression in perichondrium at E12.5, no differences in rates of proliferation can be detected at this stage. Differences are first detected at E14.5, when *Ctgf* expression occurs at the highest levels in prehypertrophic and hypertrophic chondrocytes. This suggests that *Ctgf* acts in a paracrine manner to control chondrocyte proliferation.

Defects in ECM content in *Ctgf* mutants confer defective mechanical properties on mutant cartilage. CTGF induces collagen and proteoglycan synthesis in chondrocytes in vitro (Nakanishi et al., 2000). Interestingly, no differences in types II and X collagen expression were seen in mutants. Therefore, although CTGF is a potent inducer of collagen synthesis in chondrocytes in vitro (Nakanishi et al., 2000), it is not required for collagen synthesis in vivo. Compensatory pathways may restore types II and X collagen levels in *Ctgf* mutants. The related CCN family member *Cyr61 (Ccn1)* is of particular interest in this regard. *Cyr61* is expressed in chondrocytes and induces the synthesis of collagen and other ECM components in vitro (Wong et al., 1997). Therefore, *Ctgf* and *Cyr61* may have overlapping roles in cartilage.

That CTGF is required in vivo for ECM production is demonstrated by the severely reduced levels of aggrecan and link protein in the growth plates of *Ctgf* mutants (Fig. 6D,E). Parallels between the phenotypes of *Ctgf* mutants and mice deficient in link protein (*Crt11*) highlight the essential role played by CTGF as a regulator of ECM content in cartilage. Link protein is an ECM component, and is essential for the acquisition of tensile strength in cartilage (Morgelin et al., 1994). *Crt11* and *Ctgf* mutants have similar constellations of defects: shortened mandibles, enlarged vertebrae, and bends and kinks in the same subset of long bones. Finally, chondrocyte columns are disorganized and endochondral ossification is delayed in both strains (Watanabe and Yamada, 1999). However, there are important differences between *Ctgf* and *Crt11* mutants. *Crt11*<sup>-/-</sup> mice exhibit more severe reductions in proteoglycan levels, and a greater disorganization of the growth plate. Moreover, *Ctgf* mutants exhibit defects in cell proliferation and enlarged hypertrophic zones not seen in *Crt11* mutant mice. Therefore, some, but not all, of the defects in *Ctgf* mutant cartilage are caused by decreased proteoglycan content.

Our results show that CTGF is important for efficient recruitment of MMP9-expressing cells to the growth plate (Fig. 8B-D). The crucial role that MMPs play in ECM remodeling in

skeletal tissues is illustrated by the skeletal phenotypes of MMP-deficient mice (Vu et al., 1998; Zhou et al., 2000). Recruitment of MMP9-expressing chondroclasts/osteoclasts to the growth plate is dependent on VEGF (Engsig et al., 2000). The paucity of these cells at the growth plates of *Ctgf* mutants is probably due, at least in part, to the decreased expression of VEGF in *Ctgf*<sup>-/-</sup> hypertrophic cartilage.

There are several mechanisms through which the reduced levels of MMP9 seen in *Ctgf* mutants can lead to growth plate defects. MMP9 degrades collagens and proteoglycans expressed in cartilage and is thus required for ECM remodeling (D'Angelo et al., 2001; Sternlicht and Werb, 2001). Therefore, loss of MMP expression would impair cartilage ECM turnover. This is consistent with the suspected role of CTGF as a key mediator of fibrotic responses, where matrix degradation and synthesis must occur simultaneously (Martin, 1997). In addition, MMPs control angiogenesis, cell migration and differentiation by cleaving cell surface molecules, growth factors and their binding proteins (Sternlicht and Werb, 2001). For example, MMP9 can activate latent TGF $\beta$  (D'Angelo et al., 2001; Yu and Stamenkovic, 2000). The reduced levels of MMP9 in growth plates of *Ctgf* mutants is thus expected to lead to changes in the distribution and activities of growth factors such as TGF $\beta$ .

CTGF may control MMP9 expression in several ways. MMP expression can be induced by integrin-mediated interactions. CTGF, by altering ECM composition, may alter integrin-induced MMP9 expression. CTGF can bind directly to integrins to induce MMP transcription (Chen et al., 2001a). CTGF could also affect levels of MMP9 post-translationally by altering its retention and/or degradation. This is especially interesting given that direct associations occur between CTGF and LRP (low density lipoprotein receptor-related protein), and between MMP9 and LRP (Hahn-Dantona et al., 2001; Segarini et al., 2001), raising the possibility that CTGF controls clearance of MMPs by altering their degradation via LRP-mediated endocytosis.

We show that CTGF acts as a cartilage matrix-associated molecule that couples hypertrophy to growth plate angiogenesis and trabecular bone formation (Figs 7-9). CTGF promotes neovascularization through integrin-mediated signaling (Babic et al., 1999), and direct engagement of integrins is therefore one mechanism through which CTGF may act in the growth plate. CTGF can also regulate angiogenesis by modulating MMP expression, as MMPs directly activate integrins on endothelial cells to induce angiogenic responses (Sternlicht and Werb, 2001).

Our results also show that CTGF plays an important role in VEGF localization in hypertrophic chondrocytes (Fig. 9). VEGF is required for growth plate angiogenesis (Gerber et al., 1999; Haigh et al., 2000). The mechanism by which VEGF expression in the growth plate is controlled is not well understood. The transcription factor CBFA1/RUNX2 is required for VEGF expression in hypertrophic cartilage (Zelzer et al., 2001). The observation that CBFA1/RUNX2 levels are not altered in *Ctgf* mutants suggests that CTGF acts downstream of CBFA1/RUNX2, or in an independent pathway. The transcription factor hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) is expressed by hypertrophic chondrocytes and is required, but not sufficient, for VEGF expression (Schipani et al., 2001). HIF1 $\alpha$ -independent pathways are also essential, and one of these may involve TGF $\beta$ , as HIF1 $\alpha$  and SMAD3 form a complex that synergistically induces VEGF expression (Sanchez-Elsner et al., 2001). CTGF may interact with TGF $\beta$  to induce VEGF expression, since CTGF binds directly to TGF $\beta$ , and enhances the ability of TGF $\beta$  to interact with its receptors (Abreu et al., 2002). CTGF may also act independently of TGF $\beta$  to induce VEGF expression. For example, CTGF induces adhesive signaling in fibroblasts through integrins, leading to activation of p42/44 MAPKs (Chen et al., 2001a), and the p42/44 MAPK pathway has been shown to be required for VEGF expression in fibroblasts (Milanini et al., 1998). These

results raise the possibility that CTGF induces VEGF expression via activation of p42/44. Whether a similar pathway controls VEGF expression in hypertrophic chondrocytes is not yet known.

Secreted proteins controlling VEGF expression in the growth plate have not been previously identified. In endothelial cells, VEGF induces CTGF expression (Suzuma et al., 2000). Taken together with our results, CTGF and VEGF may therefore participate in a positive-feedback loop in hypertrophic chondrocytes. In addition to this transcriptional control, CTGF appears to act post-translationally by binding to VEGF, and impairing VEGF-induced angiogenesis (Inoki et al., 2002). These findings suggest that, in addition to its role as an inducer of VEGF transcription, CTGF plays a role in regulating VEGF activity. CTGF may sequester VEGF in an inactive form in the hypertrophic ECM through direct physical association, and may regulate the release of active VEGF to induce maximal angiogenic activity.

In summary, CTGF is important for chondrocyte proliferation, acquisition of tensile strength by cartilage, ECM remodeling and growth plate angiogenesis. A role for multiple members of the CCN family in angiogenesis and chondrogenesis is likely. For example, both CTGF and *Cyr61* promote neovascularization and chondrogenesis in vitro (e.g., Chen et al., 2001a; Kireeva et al., 1997). Mice that lack *Cyr61* die in midgestation because of defects in nonsprouting angiogenesis within the placenta (Mo et al., 2002). Thus, *Cyr61* and CTGF have similar activities in vitro and are co-expressed, but regulate distinct aspects of angiogenesis in vivo.

The related family member WISP3/CCN6 may also share overlapping functions with CTGF. Although the sites of WISP3 expression and its in vitro activities are unknown, loss of WISP3 in humans causes progressive pseudorheumatoid dysplasia, a disease characterized by degeneration of articular cartilage (Hurvitz et al., 1999). Therefore, multiple members of the CCN family may be required for angiogenesis and the formation and maintenance of cartilage. Analysis of double mutants will provide insights into the roles of these genes in other developmental processes.

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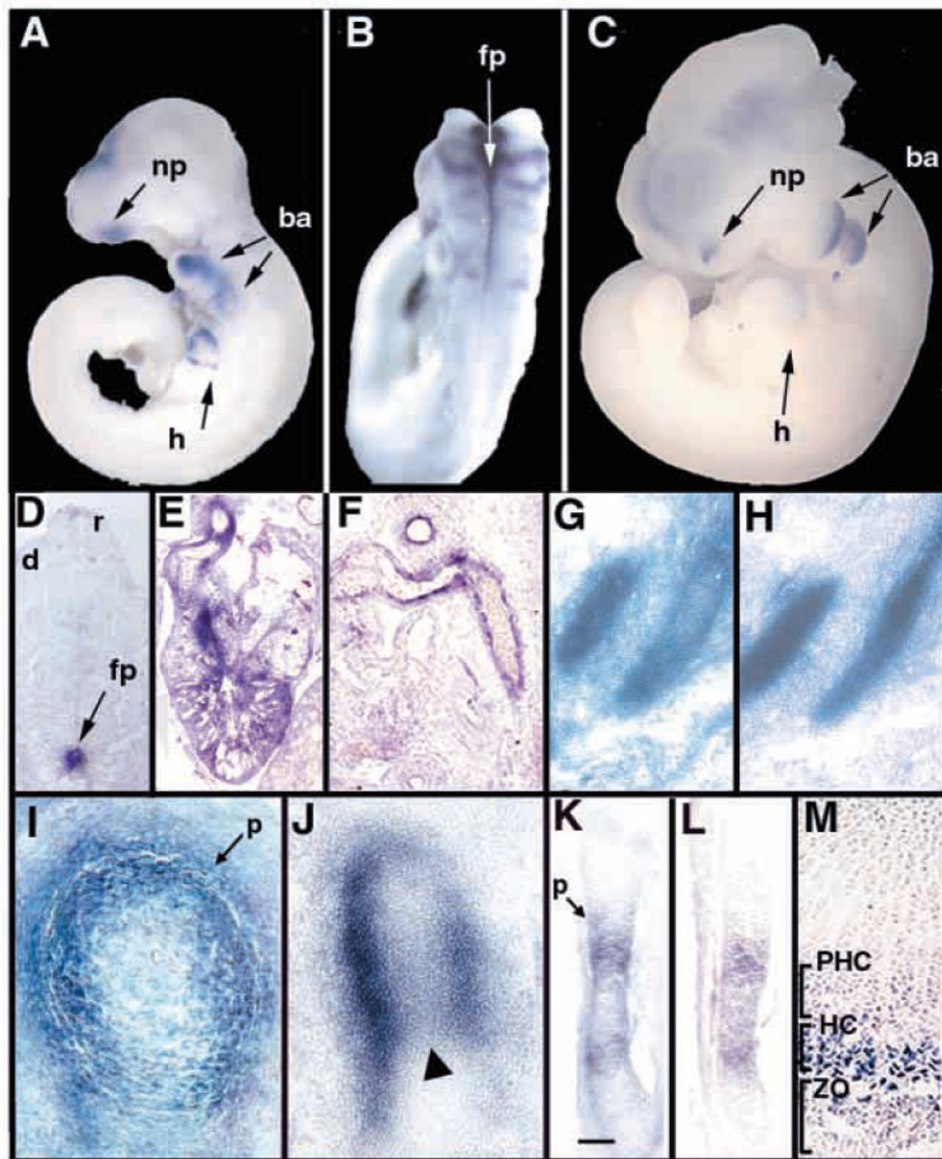
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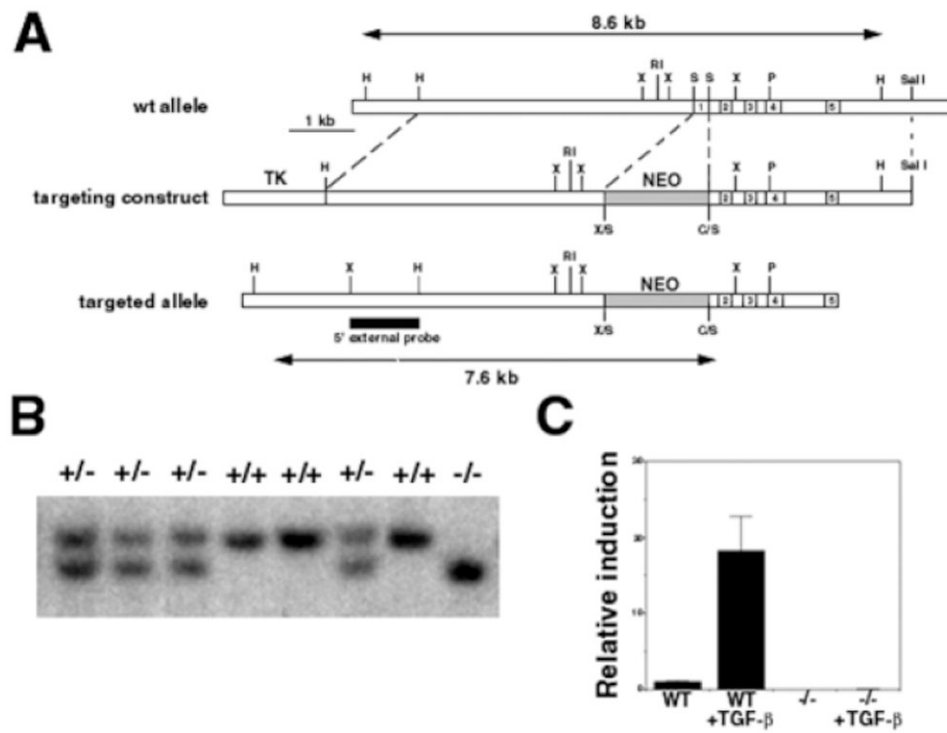
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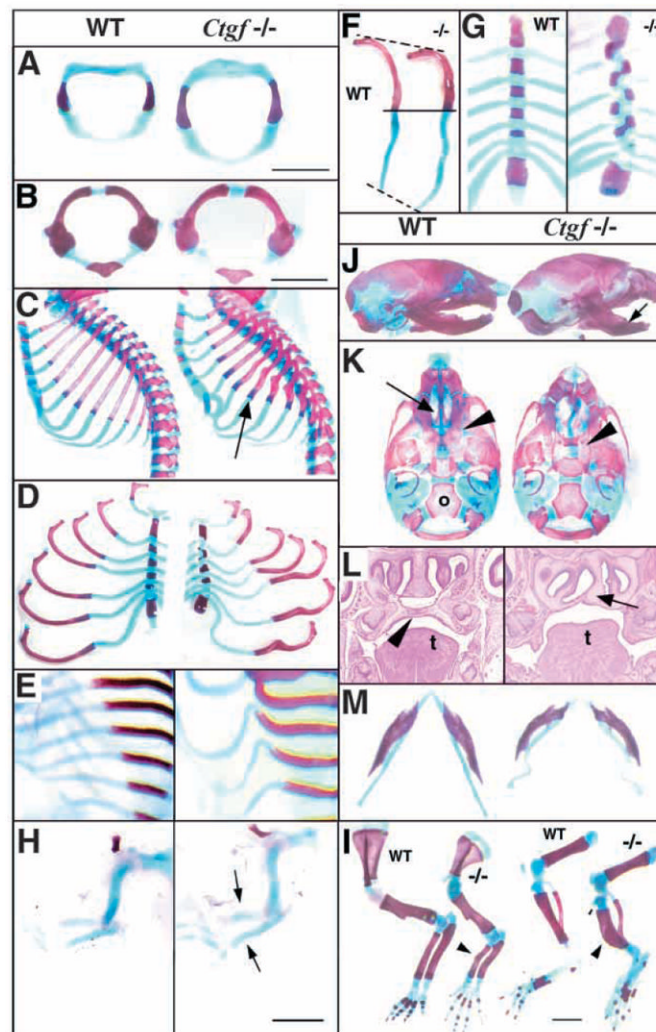
**Fig. 1.** Expression of *Ctgf* in midgestation embryos. Whole-mount in situ hybridization of E9.5 (A,B) and an E10.5 (C) embryo treated with antisense *Ctgf*, demonstrating expression in heart, branchial arches, neuronal tissues and nasal process. (D) Frontal section through an E11.5 embryo showing high levels of *Ctgf* expression in the floorplate, and lower levels in the roofplate and dermomyotome. (E) *Ctgf* expression in the heart at E13.5. Transcripts are present in ventricular myofibroblasts and in fusing endocardial cushion tissue at the midline of the outflow tract. (F) *Ctgf* expression in the endothelium and smooth muscle layers of major blood vessels at E13.5. (G,H) Adjacent parasagittal sections through E12.5 ribs showing *Ctgf* expression in perichondrium (G) and collagen type II (*ColIII*) (H) expression in proliferating chondrocytes. (I) Transverse section through an E13.5 femur showing *Ctgf* expression in perichondrium and in adjacent chondrocytes. (J) Sagittal section through the femur of an E13.5 embryo showing strong *Ctgf* expression in the perichondrium, and lower levels in chondrocytes at the center of the diaphysis (arrowhead). (K-M) Adjacent sections through an E14.5 femur showing expression of *Ctgf* (K), and *Ihh* (L). Scale bar in K: 100  $\mu$ m

for K,L. *Ctgf* is expressed in the domain of *Ihh* expression, as well as in prehypertrophic chondrocytes adjacent to the perichondrium. (M) Section through the proximal growth plate of a P0 femur, showing *Ctgf* expression in hypertrophic chondrocytes. ba, branchial arch; d, dermomyotome; fp, floorplate; h, heart; HC, hypertrophic chondrocytes; m, meninges; np, nasal process; p, perichondrium; PHC, prehypertrophic chondrocytes; r, roofplate; sc, spinal cord; ZO, zone of ossification.



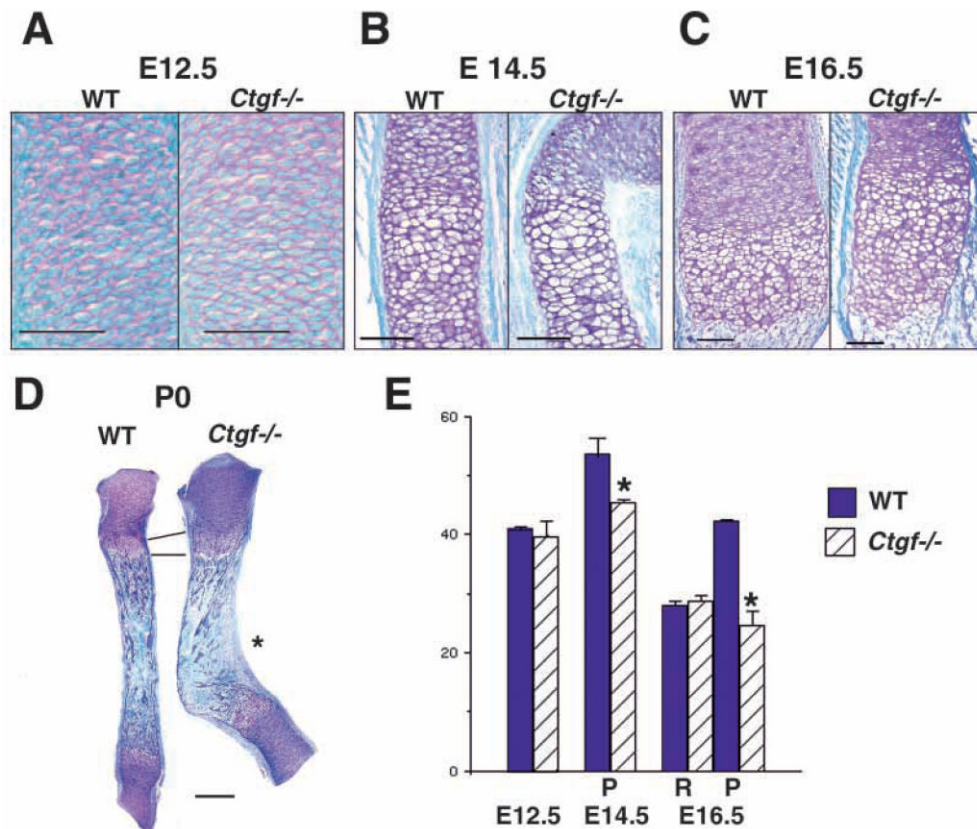


**Fig. 2.** Generation of *Ctgf*<sup>-/-</sup> mice. (A) Map of the *Ctgf* locus (wild-type allele), targeting vector and mutant allele produced by homologous recombination. The *Hind*III fragments expected for the wild-type (8.6 kb) and mutant (7.6 kb) alleles are indicated by double-headed arrows. A, *Apa*I; B, *Bam*HI; C, *Cl*aI; H, *Hind*III; N, *Not*I; P, *Ps*aI; R, *Eco*RI; S, *Sma*I; X, *Xba*I. (B) Southern blot analysis of genomic DNA from a heterozygote intercross. (C) Verification that the targeted locus encodes a null allele. RT-PCR analysis of fibroblasts from wild-type and mutant embryos treated with TGF $\beta$ 1 (5 ng/ml) to induce *Ctgf* expression. No *Ctgf* transcripts can be detected in mutants, even after TGF $\beta$  treatment.

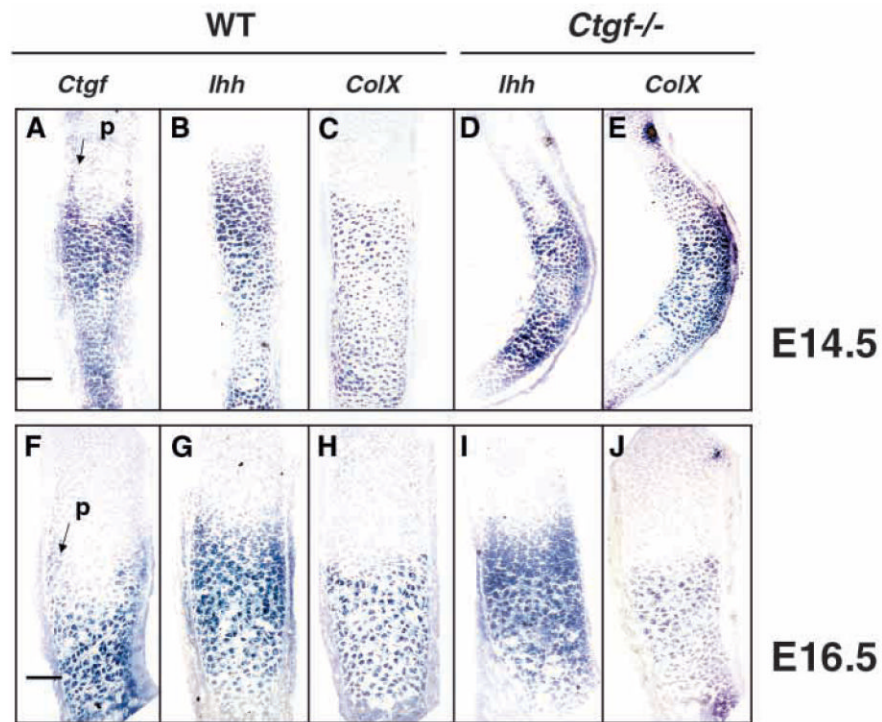


**Fig. 3.** *Ctgf*<sup>-/-</sup> mice exhibit multiple skeletal defects. In all panels, wild-type is towards the left and *Ctgf*<sup>-/-</sup> is to the right. Atlases from E14.5 (A) and P0 (B) mutants are broader than those from wild-type littermates. (C) Sagittal views of neonatal rib cages showing deformation of cartilage, and kinks in bone in *Ctgf*<sup>-/-</sup> mice (arrow). (D) Flat mounts of rib cages show that in *Ctgf* mutants, ribs are kinked and the sternum is shortened. (E) E14.5 rib cages, showing that the kinks seen in neonatal mutant ribs are preceded by distorted rib cartilage. (F) Endochondral ossification is delayed in *Ctgf* mutants. Seventh ribs from neonatal wild-type and mutant littermates are shown. (G) Misalignment of the sternal bars is seen in ~10% of neonatal mutants. (H) Cleared skeletal preparations of E13.5 forelimbs, showing kinks in the radius and ulna (arrows) prior to ossification. (I) Cleared skeletal preparations of neonatal forelimbs and hindlimbs showing deformations (arrowheads) in the radius and ulna, and tibia and fibula. (J) Side views of neonatal skulls showing domed skull and shortened mandibles (arrow). (K) Ventral views of neonatal skulls, showing lack of elevation of the palatal shelves, leading to cleft palate (arrowhead), deformation of nasal cartilage, and consequent absence of the adjacent ethmoid bones (arrow in wild type), but no apparent defects in other membrane bones such as the occipital (o). (L) Frontal sections of E15.5 skulls, showing cleft palate (palate in wild type is indicated by arrowhead) and deformed

nasal cartilage (arrow in mutant) in mutants. t, tongue. (M) *Ctgf* mutant neonates exhibit deformation of Meckel's cartilage and shortened mandibles. Scale bars: 1 mm.

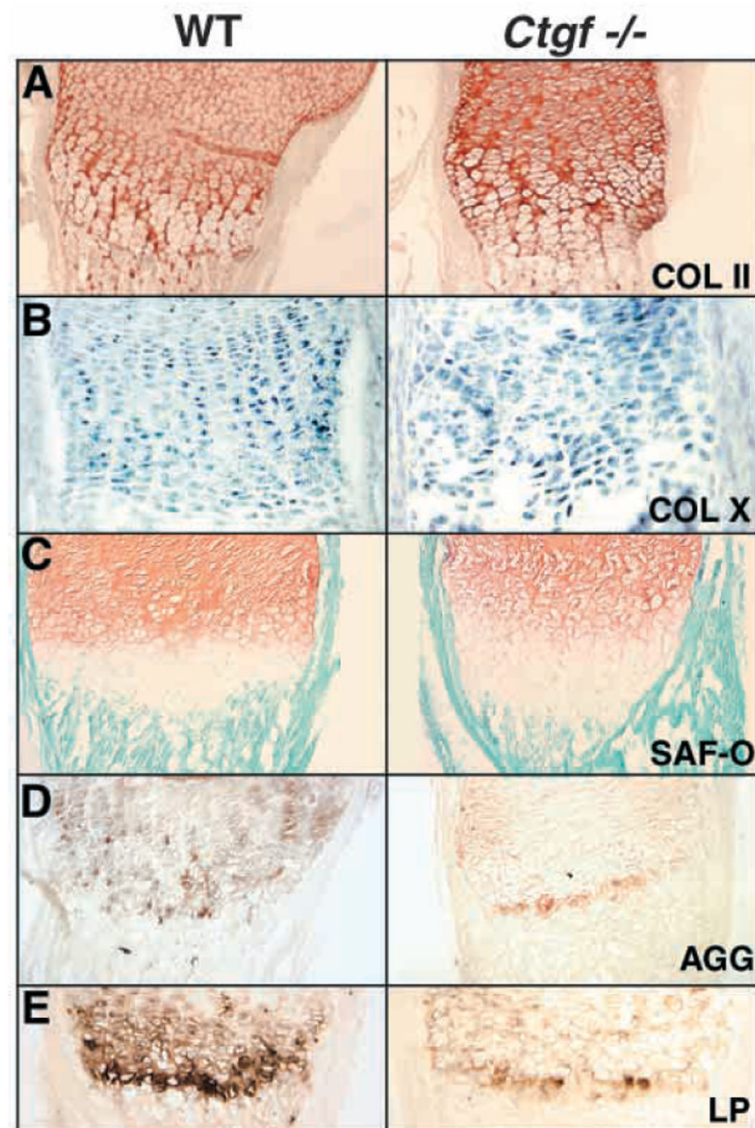


**Fig. 4.** Histological and proliferative defects in *Ctgf* mutant cartilage. (A) Sections through wild-type and *Ctgf* mutant humeri at E12.5, showing no apparent differences in size or morphology. Scale bar: 50  $\mu$ m. (B) Sections through wild-type and *Ctgf* mutant E14.5 radii at the metaphysis. Hypertrophic cells are present in wild-type and mutant littermates. Scale bar: 100  $\mu$ m. (C) Sections through growth plates of E16.5 wild-type and *Ctgf* mutant radii demonstrate that the growth plate is expanded in mutants. The junction between the zones occupied by prehypertrophic and hypertrophic chondrocytes is disorganized in mutants. Scale bar: 100  $\mu$ m. (D) Radii from newborn wild-type and *Ctgf* mutant littermates, demonstrating persistence of the enlarged hypertrophic zone. The epiphyses appear normal in mutants. The concave surface of the kink in mutants is a site of membrane bone formation (asterisk). Scale bar: 300  $\mu$ m. (E) Reduced rates of chondrocyte cell proliferation in *Ctgf* mutants. Quantification of PCNA labeling is shown in the graph, with values expressed as % labeled nuclei. Cells in five adjacent sections, each spanning 40  $\mu$ m, were scored by an observer blinded to the genotype. Statistical significance was assessed by Student's *t*-test. \* $P$ <0.01; P, proliferative zone; R, resting zone.

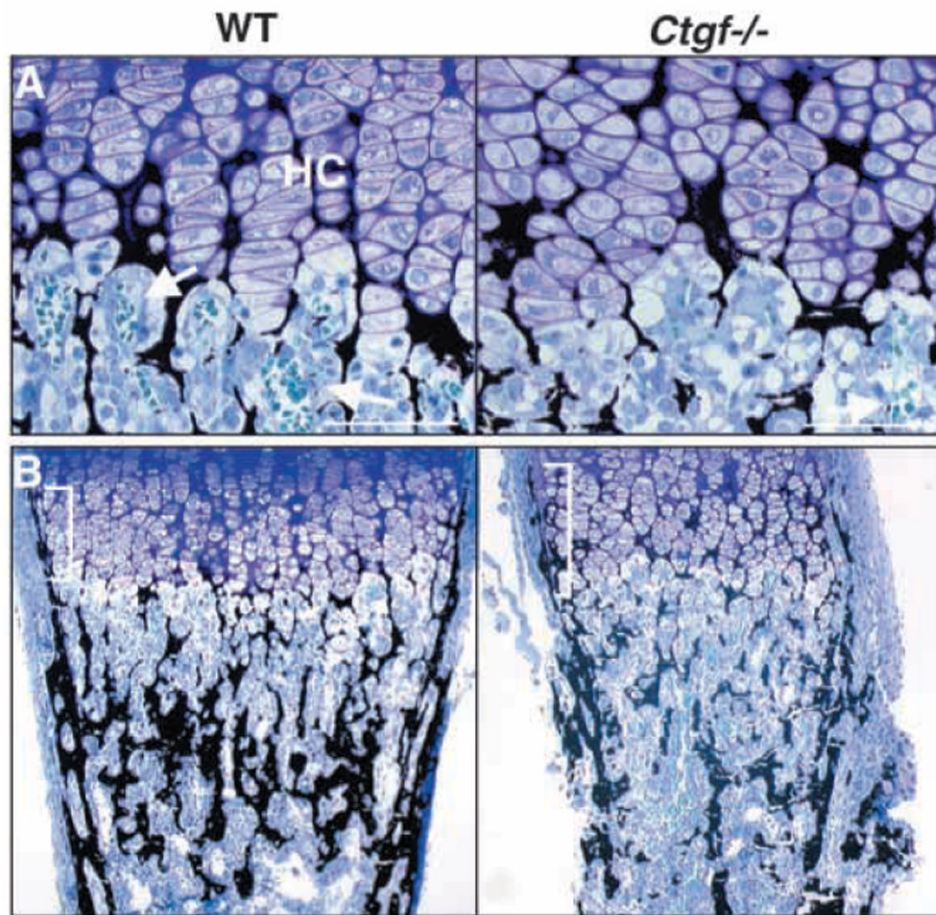


**Fig. 5.** Expression of *Ihh* and *ColX* in *Ctgf* mutants. (A-C) Expression of *Ctgf* (A), *Ihh* (B) and *ColX* (C) in the radius of an E14.5 wild-type embryo. The expression patterns of *Ctgf* and *Ihh* overlap in prehypertrophic and hypertrophic chondrocytes. *Ctgf* is co-expressed with *ColX* in hypertrophic chondrocytes at this stage and in prehypertrophic chondrocytes adjacent to the perichondrium (arrow in A). (D,E) Expression of *Ihh* (D) and *ColX* (E) in the radius of an E14.5 *Ctgf* mutant littermate. These markers are expressed in appropriate patterns in prehypertrophic and hypertrophic chondrocytes. Scale bar: 100  $\mu$ m for A-E (F-H) Expression of *Ctgf* (F), *Ihh* (G) and *ColX* (I) in the radius of an E16.5 wild-type embryo. *Ctgf* is expressed primarily in hypertrophic chondrocytes in a pattern overlapping that of *ColX*, but transcripts also persist in chondrocytes adjacent to the perichondrium in the prehypertrophic zone (arrow in F). (I,J) Expression of *Ihh* (I) and *ColX* (J) in the radius of an E16.5 *Ctgf* mutant littermate. The domains of expression of *Ihh* are indistinguishable in wild-type and mutant littermates, indicating that the expansion of the hypertrophic zone revealed by histological analysis is not accompanied by an expanded prehypertrophic zone. Scale bar: 50  $\mu$ m for F-J.

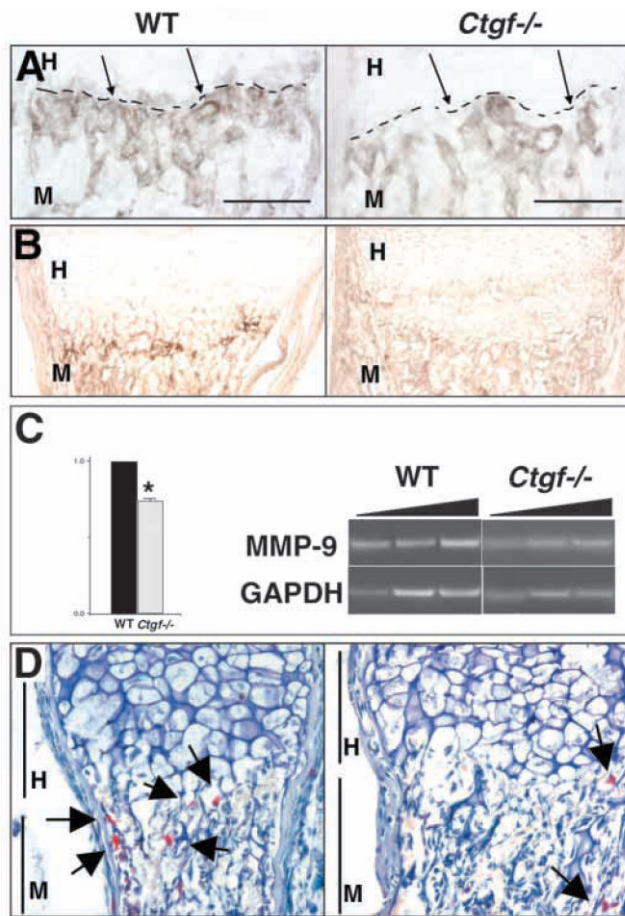




**Fig. 6.** Expression of ECM components is altered in *Ctgf* mutants. (A) Immunostaining for type II collagen protein is at comparable intensities in P0 growth plates of *Ctgf* mutants and wild-type littermates. (B) Levels of type X collagen mRNA are indistinguishable in wild-type and *Ctgf* mutant growth plates. (C) Safranin-o staining demonstrates that proteoglycan levels are normal in the resting and proliferative zones, and that the hypertrophic zone, which is not stained intensely by safranin-O, is expanded in mutants. (D,E) Expression of aggrecan (D) and link protein (E) is reduced in P0 growth plates of *Ctgf* mutants.

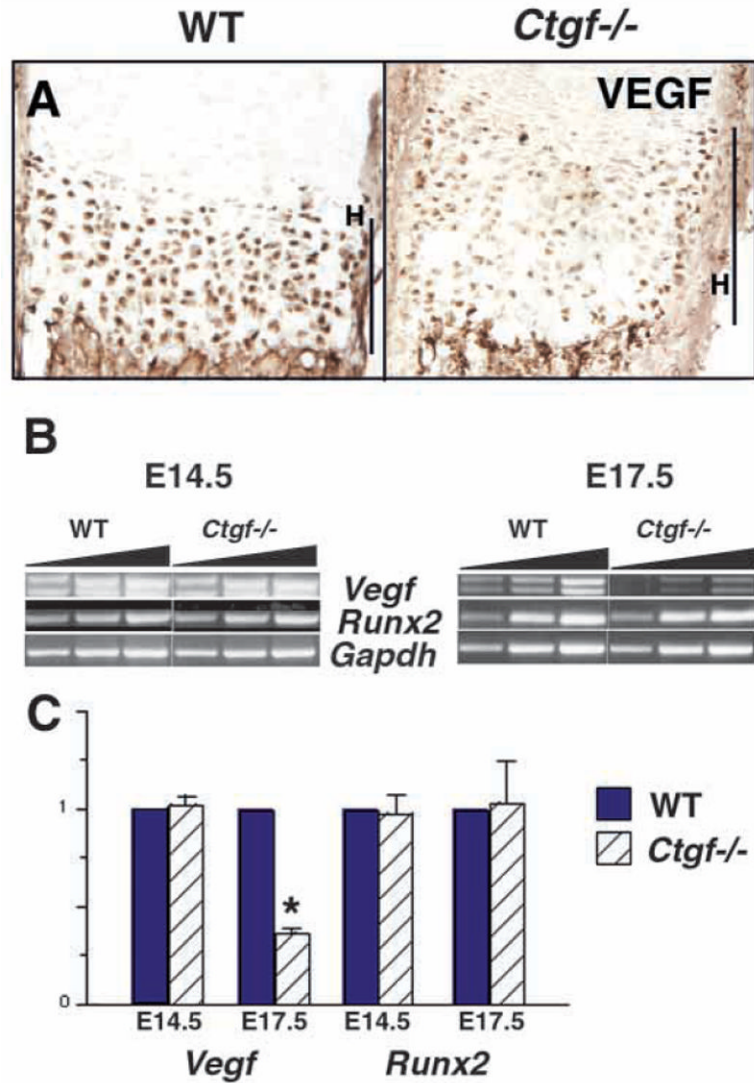


**Fig. 7.** Impaired angiogenesis and osteopenia in growth plates of *Ctgf* mutants. (A) Plastic sections through the growth plates of P0 femora stained by the method of von Kossa. The ECM of *Ctgf* mutants is mineralized (black stain), but hypertrophic chondrocyte columns (HC) are disorganized, and there are few capillaries (arrows) invading the cartilage matrix. A single capillary can be seen in the vicinity of the mutant growth plate. (B) von Kossa-stained plastic sections through neonatal femora from wild-type and *Ctgf*<sup>-/-</sup> mice demonstrate that mutants are osteopenic; the amount of mineralization (black stain) is greatly reduced in mutants. The bone collar (brackets) adjacent to the expanded hypertrophic zone is lengthened in mutants, but is thinner than in wild-type littermates. Scale bars: 40  $\mu$ m.



**Fig. 8.** Defective expression of angiogenic factors in *Ctgf* mutant growth plates. Growth plates of neonatal radii are shown in all panels. (A) PECAM immunostaining demonstrates the existence of blood vessels in the intertrabecular spaces of the metaphysis, but the fine network of capillaries seen at the ossification zone (broken line) in wild-type animals is not as extensive in *Ctgf* mutants. Scale bar: 100  $\mu$ m. (B) MMP9 immunostaining is reduced in neonatal mutants. (C) *Mmp9* RNA levels are reduced in neonatal mutants. The triangular bars above the photograph of the gel represent increasing numbers of amplification cycles (see Materials and Methods). Data are from three pairs on *Ctgf* mutants and wild-type littermates. Expression of *Mmp9* was normalized to *Gapdh* in each reaction. \* $P < 0.05$ . (D) TRAP-positive cells (arrows) are present in normal levels in the bone marrow of *Ctgf* mutants, but in reduced levels at the cartilage-bone junction, indicating a defect in recruitment of osteoclasts/chondroclasts to the hypertrophic region. MMP13 protein is present at diminished levels in hypertrophic chondrocytes in *Ctgf* mutants. H, hypertrophic zone; M, metaphysis.





**Fig. 9.** VEGF expression is reduced in the hypertrophic zones of *Ctgf* mutant growth plates. (A) VEGF immunostaining is reduced in the hypertrophic zones of newborn *Ctgf* mutants. (B) Semi-quantitative RT-PCR analysis of *Vegf* and *Runx2* expression. Representative data from a single *Ctgf* mutant and wild-type littermate at E14.5 and at E17.5 is shown. The triangular bars above the photograph of the gel represent increasing numbers of amplification cycles (see Materials and Methods). Expression of *Runx2* and *Vegf* was normalized to *Gapdh* in each reaction. (C) Expression of *Runx2* and *Vegf* in *Ctgf* mutants relative to wild-type littermates. Data are from five pairs of *Ctgf* mutants and wild-type littermates. Expression of *Runx2* is not altered in mutants at E14.5 or E17.5. *Vegf* transcripts are present at indistinguishable levels in *Ctgf* mutants and wild-type littermates at E14.5, but at reduced levels in mutants at E17.5; the upper and lower bands correspond to the 165 and 120 *Vegf* isoforms, respectively. \*  $P < 0.05$ . H, hypertrophic zone.