Published in final edited form as:

Crit Rev Eukaryot Gene Expr. 2011 ; 21(1): 43–69.

The Role of Connective Tissue Growth Factor (CTGF/CCN2) in Skeletogenesis

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Abstract

Connective tissue growth factor (CTGF) is a 38kDa, cysteine rich, extracellular matrix protein composed of four domains or modules. CTGF has been shown to regulate a diverse array of cellular functions and has been implicated in more complex biological processes such as angiogenesis, chondrogenesis, and osteogenesis. A role for CTGF in the development and maintenance of skeletal tissues first came to light in studies demonstrating its expression in cartilage and bone cells which was dramatically increased during skeletal repair or regeneration. The physiological significance of CTGF in skeletogenesis was confirmed in CTGF-null mice, which exhibited multiple skeletal dysmorphisms as a result of impaired growth plate chondrogenesis, angiogenesis, and bone formation/mineralization. Given the emerging importance of CTGF in osteogenesis and chondrogenesis, this review will focus on its expression in skeletal tissues, its effects on osteoblast and chondrocyte differentiation and function, and the skeletal implications of ablation or over-expression of CTGF in knockout or transgenic mouse models, respectively. In addition, this review will examine the role of integrin-mediated signaling and the regulation of CTGF expression as it relates to skeletogenesis. We will emphasize CTGF studies in bone or bone cells, and will identify opportunities for future investigations concerning CTGF and chondrogenesis/osteogenesis.

Keywords

bone; cartilage; osteoblast; chondrocyte; integrin; TGF-beta

CTGF and the CCN Family of Proteins

Connective tissue growth factor (CTGF) is a 38kDa, cysteine rich, extracellular matrix protein that belongs to the CCN family of proteins. This family was named after the three original members including Cysteine-rich 61 (Cyr61/CCN1), CTGF/CCN2, Nephroblastoma overexpressed (Nov/CCN3).¹ More recent members include the Wnt-induced secreted

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Conflict of Interest Statement: All authors declare that there are no conflicts of interest.

proteins-1 (WISP-1/CCN4), -2 (WISP-2/CCN5) and -3 (WISP-3/CCN6).² To date, a total of six distinct members have been identified and a nomenclature committee named them CCN1-6 according to the order in which they were discovered.³ Members of the CCN family are multi-modular proteins containing four conserved modules (with the exception of CCN5 which lacks the C-terminal or fourth module) that are also present in other unrelated extracellular proteins.⁴⁻⁶ The CTGF gene consists of 5 exons, the first coding for a signal peptide (for secretion) and exons 2-5 coding for each of the four different modules. Module 1 is an insulin-like growth factor (IGF)-binding domain, module 2 is a von Willebrand type C (VWC) domain, module 3 is a thrombospondin-1 (TSP-1) domain, and module 4 is a C-terminal (CT) domain containing a putative cysteine knot (Fig. 1A).^{2,7,8}

CTGF has been shown to regulate a diverse array of cellular functions including proliferation, migration, adhesion, survival, differentiation and synthesis of extracellular matrix (ECM) proteins in various cell types.^{2,6-8} CTGF has also been implicated in more complex biological processes such as angiogenesis, chondrogenesis, osteogenesis, wound healing, fibrosis and tumorigenesis (Fig. 1B).⁶⁻⁹ The diverse biological activities of CTGF are consistent with its modular structure. Recent studies have shown that individual domains can regulate different cellular functions,¹⁰ certain modules can act interdependently,² and the presence or absence of other growth factors can influence the biological response of the target cell.⁹⁻¹¹

CTGF was first identified in the conditioned media of human umbilical vein endothelial cells.¹² The direct stimulation of CTGF by transforming growth factor beta (TGF- β), the most potent inducer of CTGF expression, was first demonstrated in human skin fibroblasts, and during wound repair *in vivo* where there is a coordinated up-regulation of TGF- β followed by CTGF expression.¹³ In addition to TGF- β , CTGF is also induced by other growth factors and secreted into the ECM, where it associates with cell surface proteins and extracellular matrix components.⁹ Studies have demonstrated that CTGF binds to specific cell surface integrins via its C-terminal module in a cell type-dependent manner.¹⁴⁻¹⁶ This interaction with cell surface integrins can account for some of its functions such as cell adhesion, migration and extracellular matrix protein deposition, events that are mediated by the activation of focal adhesion kinase (FAK) and other intracellular signaling molecules.¹⁷⁻¹⁹ It has also been shown that CTGF can interact with other growth factors, and therefore, positively or negatively modulate growth factor signaling.⁹ For example, it has been shown that CTGF can bind to TGF- β 1 and enhance TGF- β receptor signaling while inhibiting bone morphogenetic protein (BMP) receptor signaling through its interaction with BMP-4.²⁰

A role for CTGF in the skeletal tissues began to emerge in studies demonstrating its expression in developing cartilage, bone and teeth.²¹⁻²⁴ Studies have also shown that CTGF expression is markedly increased during the repair or regeneration of skeletal tissues.²⁴⁻²⁷ Culture studies using chondrocytes, osteoblasts or surrogate cell lines demonstrated that treatment with recombinant forms of CTGF stimulates the proliferation and differentiation of these cells.^{24,28-31} The importance of CTGF in skeletogenesis was confirmed in CTGF-null (KO) mice that exhibited multiple skeletal dysmorphisms as a result of impaired growth plate chondrogenesis, angiogenesis, and bone formation/mineralization.²¹ Previous reviews of CTGF have focused on either its effects on non-skeletal cell and tissue types or its effects on chondrocyte differentiation and function.^{2,8,32,33} It has been implied that the effects of CTGF on osteogenesis are largely secondary to its chondrogenic effects, such as those during endochondral ossification.^{32,33} However, there is an ever increasing number of studies that demonstrate a direct and important role for CTGF in regulating bone cell development and function that is independent of its chondrogenic effects. In fact, the levels of CTGF expressed in and produced by osteoblasts and their progenitors at active bone

forming sites *in vivo*, as well as in culture, are equivalent to or greater than in cartilage.^{23,24,27,34,35} Given the importance of CTGF in osteogenesis and chondrogenesis, this review will focus on its expression in skeletal tissues, its effects on osteoblast and chondrocyte differentiation and function, and the skeletal implications of ablation or over-expression of CTGF in knockout or transgenic mouse models, respectively. In addition, this review will examine the role of integrin-mediated signaling and the regulation of CTGF expression as it relates to skeletogenesis. We will emphasize CTGF studies in bone or bone cells, and will identify opportunities for future investigations concerning CTGF and chondrogenesis/ osteogenesis.

Expression of CTGF During Skeletal Development and Repair

The skeletal system is primarily composed on bone and cartilage, mesodermal tissues formed by osteoblasts and chondrocytes, respectively. The osteochondral progenitor cell is a common mesenchymal cell that gives rise to both osteoblasts and chondrocytes. Skeletal development begins with condensation of these mesenchymal progenitor cells, which aggregate at future skeletal sites. Mesenchymal progenitors from the neural crest give rise to the craniofacial skeleton, those from the sclerotome of the somites give rise to the axial skeleton, and those from the lateral plate mesoderm give rise to the appendicular (limb) skeleton.³⁶ There are two processes by which bone is formed; endochondral and intramembranous ossification. Endochondral ossification involves the differentiation of osteochondral progenitors into chondrocytes that form a cartilage template for the eventual bone, and most axial and appendicular bones (e.g. long bones) are formed via this process. Intramembranous ossification involves the direct differentiation of osteochondral progenitors into bone-forming osteoblasts and this is the process whereby the flat skull bones and the clavicle are formed.

During embryogenesis, CTGF is expressed at different times and places in the developing skeleton. It is initially expressed during condensation of mesenchymal cells that will form the cartilaginous anlage of the endochondral bones,^{21,23,37} and in developing Meckel's cartilage.³⁸ The mesenchymal progenitor cells must proliferate and migrate to the sites of future endochondral bones. Cell motility occurs via the activation of the FAK/Src signaling pathway, which is the first step in cell condensation.³⁹ Once these motile cells aggregate into pre-cartilage condensations, FAK is down-regulated and cell-cell contacts are highly favored. Condensation has been shown to be directed by TGF- β , a signaling protein that regulates this process through its simultaneous regulation of fibronectin and several cell adhesion molecules, such as N-CAM, necessary for cell-cell communication.^{40,41} Recent studies have shown that CTGF is also essential for the formation of pre-cartilage condensations. Studies have demonstrated that CTGF stimulates mesenchymal cell proliferation, migration and aggregation (condensation), while knockdown of CTGF or application of CTGF neutralizing antibodies prevented these effects.^{37,38} Silencing of CTGF using siRNA and antisense oligonucleotide approaches demonstrated that TGF- β -induced condensation of C3H10T1/2 mesenchymal cells was inhibited in micromass cultures.³⁷ Cell proliferation and migration, two crucial events for condensation, were also significantly reduced in these cells. In another study, mesenchymal cells isolated from E10 first branchial arches formed increased numbers of cellular aggregates (nodules) in micromass cultures treated with recombinant CTGF (rCTGF) and this effect was blocked in the presence of CTGF neutralizing antibodies.³⁸ In a study using E13.5 CTGF-null mesenchymal cells obtained from CTGF KO mice, when plated at high density the cells failed to undergo condensation and exhibited a decrease in proteoglycan synthesis (marker of the initiation of cell differentiation) compared to mesenchymal cells from wild-type mice.⁴² Additionally, the loss of FAK induced the expression of a few chondrogenesis markers, including L-SOX-5, Col2a1 and CTGF.⁴² The data from these studies reveal that proper timing and

regulation of CTGF expression is important for cell condensation and subsequent chondrocyte differentiation during chondrogenesis.^{37,38,42}

Immunofluorescent localization of CTGF, TGF- β 1 and SOX-9 during development of vertebral bodies in mice demonstrated that both CTGF and TGF- β 1 were highly expressed and co-localized in mesenchymal cell condensations at E10.5.³⁷ On the contrary, the expression of SOX-9 and CTGF were inversely related, with SOX-9 being highly expressed in chondrocytes of developing cartilage at E12.5 while CTGF expression was low in chondrocytes but high in the surrounding perichondrium.³⁷ Although CTGF appears to play an important role in mesenchymal cell condensation, the absence of its expression in differentiating chondrocytes suggests that its down-regulation is required for chondrocyte differentiation to proceed. Future studies could examine whether sustained CTGF expression in mesenchymal cells blocks subsequent chondrocyte differentiation following condensation.

In addition to its role during cell condensation and subsequent cartilage differentiation, CTGF is also expressed in the growth plate, in the periosteum, and in osteoblasts of developing endochondral bones.^{21,23,34} In the growth plate, it is expressed at moderate levels in the proliferative zone and at high levels in the hypertrophic zone.^{21,43} Corresponding to its expression in hypertrophic chondrocytes, absence of CTGF results in inhibition of terminal chondrocyte differentiation.^{21,43}

A role for CTGF in osteogenesis was first appreciated when it was discovered by virtue of its dramatic over-expression in osteopetrotic bone.³⁴ Since then, several studies have demonstrated increased CTGF expression accompanying active bone formation. During postnatal bone growth, CTGF is highly expressed in active osteoblasts lining osteogenic surfaces.²⁴ CTGF is expressed at moderate levels in osteoblasts and osteocytes in alveolar bone adjacent to the periodontal ligament as a result of bone forming activity associated with physiological tooth movement.⁴⁴ The mechanical stimulation of bone caused by experimental tooth movement dramatically increased the expression of CTGF in osteoblasts and osteocytes around the periodontal ligament.²⁷ In a model for distraction osteogenesis, CTGF expression was increased in mesenchymal cells, hypertrophic chondrocytes and osteoblasts in the area of new bone formation, suggesting that CTGF plays an anabolic role in endochondral and intramembranous bone forming processes.^{45,46} CTGF expression was dramatically increased in active osteoblasts during formation of the hard (bony) callus in studies of fracture healing.^{24,46}

Role of CTGF in Osteoblast Differentiation and Function

CTGF is produced and secreted by osteoblasts in culture.^{24,47} Analysis of the temporal pattern of CTGF expression in primary osteoblast cultures revealed that CTGF levels were high during the proliferative phase, abated in confluent cultures, and increased again to maximal levels during matrix production and maturation, remaining at high levels during mineralization.²⁴ Different groups have also demonstrated that CTGF stimulates osteoblast proliferation, matrix production and differentiation in cultures of osteoblasts.^{24,28,34,35,48} Treatment of primary osteoblasts or osteoblastic cell lines (Saos-2 or MC3T3-E1) with rCTGF promotes proliferation, up-regulates the expression of various markers of osteoblast differentiation, including type I collagen (Col-I), alkaline phosphatase (ALP), osteopontin (Op) and osteocalcin (Oc), and stimulates matrix mineralization and mineralized nodule formation.^{24,28} On the contrary, calvarial osteoblasts and stromal cells isolated from CTGF transgenic mice displayed decreased ALP and Oc mRNA levels (see section on CTGF and Animal Models).⁴⁹

CTGF levels in osteoblasts are stimulated by TGF- β 1 and BMP-2,^{35,47,50} a finding that is consistent with a role for CTGF in the effects of these proteins on osteoblast growth and differentiation. Mesenchymal cells stimulated with Wnt-3A and BMP-9 identified CTGF as a target during osteoblast lineage-specific differentiation.⁵¹ At the early stages of osteoblast differentiation, CTGF was up-regulated by Wnt-3A and BMP-9, and CTGF silencing blocked BMP-9-induced osteogenic differentiation. This up-regulation of CTGF was not evident at later stages of osteoblast differentiation. Furthermore, constitutive expression of CTGF had the opposite effect, inhibiting both Wnt-3A and BMP-9-induced osteoblast differentiation. These results suggest that CTGF plays a role in early events of osteogenic differentiation, such as proliferation and recruitment of osteoprogenitors.⁵¹ The tight regulation of CTGF expression and the precise temporal interactions between CTGF and other osteogenic growth factors appear to be critical for normal osteoblast differentiation and additional studies are needed to elucidate the role of CTGF in this process.

Considering the apparent beneficial effects of CTGF on osteoblast differentiation and bone formation, recent studies have tested applications of its use in bone regeneration to promote bone healing or new bone formation. Injection of rCTGF into the marrow cavity of rat femurs elicited an osteoinductive response in the form of osteoblast differentiation and active bone formation.^{24,34} When a hydroxyapatite carrier loaded with CTGF was implanted into bone defects within a rabbit mandible, CTGF significantly enhanced the proliferation and migration of human bone marrow stromal cells, induced cell invasion and enhanced bone formation compared with the carrier alone.⁵² Using the intractable bone defect model, treatment with rCTGF induced the osteoblast mineralization markers and enhanced the bone regeneration.⁵³ The majority of *in vivo* and *in vitro* studies support an anabolic role for CTGF on bone formation, and therefore, this factor is a candidate for the development of novel clinical therapeutic approaches to stimulate bone formation. Information concerning possible molecular mechanisms for CTGF actions and its regulation in bone are reviewed in subsequent sections.

Role of CTGF in Tooth Development

CTGF has been shown to have a specific temporal and spatial expression in developing tooth germs.²² It is initially expressed in the dental lamina, invaginating epithelium and condensing mesenchyme at the tooth bud stage. It is subsequently expressed in the enamel knot and in preameloblasts. CTGF expression in the dental epithelium required the interaction with mesenchyme and could be maintained by treating the epithelium with TGF- β 1 or BMP-2. Using neutralizing antibodies, this group showed that blocking endogenous CTGF production in explants of tooth germs resulted in inhibition of proliferation of the epithelium and mesenchyme and delayed the cytodifferentiation of ameloblasts and odontoblasts. Treatment of cultured epithelial and mesenchymal cells with rCTGF stimulated their proliferation.²² This study clearly established the spatial and temporal expression of CTGF during odontogenesis and its role in the proliferation and differentiation of both the ameloblast and odontoblast cell lineages.

Role of CTGF in Chondrocyte Differentiation and Function

In 1997, differential display PCR studies revealed that the gene, Hcs24, was expressed in human chondrosarcoma-derived chondrocytes (HCS-2/8 cell line) and that this gene is identical to CTGF.⁵⁰ It was subsequently shown that when treated with rCTGF, HCS-2/8 chondrocytic cells and primary rib growth plate chondrocytes (RGCs) demonstrated increases in both cell proliferation and proteoglycan synthesis.²⁹ Furthermore, it was shown that CTGF promotes chondrocyte maturation hypertrophy, exhibited by an increase in ALP activity in treated RGCs.²⁹ A closer look at the synthesis, processing and secretion of CTGF

in HCS-2/8 chondrocytic cells, revealed differences depending on the stage of maturation of the cells.⁵⁴ In proliferating chondrocytes, CTGF was immediately released upon synthesis, and most of the secreted CTGF was free in the culture supernatant. After the cells reached confluence, secretion slowed as the cells matured, and most of the secreted CTGF accumulated in the ECM surrounding the cells.⁵⁴ These data demonstrated differential regulation of CTGF secretion and processing depending on the status of cell growth and differentiation.

To elucidate a mechanism through which CTGF regulates chondrocyte proliferation and differentiation, investigators have examined its interaction with BMPs) as well as mitogen-activated protein kinase (MAPK) signaling pathways, as both have been shown to play a significant role in skeletal development.⁵⁵ Biochemical studies have shown that CTGF directly interacts with BMP-4 via its cysteine-rich domain (domain 4) and prevents BMP-4 from binding to its receptor, thereby inhibiting BMP signaling.²⁰ A more recent study demonstrated that CTGF also interacts with BMP-2 via its fourth domain.⁵⁶ When examining the combined effect of CTGF and BMP-2 on chondrocyte proliferation and differentiation using HCS-2/8 chondrocytic cells, there was no synergistic effect on proliferation compared to separate treatment with each growth factor.⁵⁶ However, combined treatment did enhance proteoglycan synthesis, indicative of chondrocyte differentiation. This was further demonstrated in primary mouse chondrocytes where treatment with both growth factors simultaneously increased cartilage differentiation markers, such as type II collagen, aggrecan, type X collagen, and Runx2/Cbfa1.⁵⁶

In addition to BMP signaling, CTGF has also been shown to regulate the proliferation and differentiation of chondrocytes via Protein Kinase and MAPK signaling pathways.^{30,31} Involvement of protein kinase C (PKC) in CTGF-mediated signaling in chondrocytes was demonstrated by inhibition of PKC in rCTGF-treated HCS-2/8 chondrocytic cells. These cells demonstrated significantly reduced proliferation and differentiation (Fig. 2). Furthermore, PKC is an important upstream protein in CTGF-mediated signaling to multiple kinase signaling pathways; these include MEK/ERK, p38, and protein kinase B (PKB).^{30,31} Using specific inhibitors, it was shown that CTGF-mediated activation of MEK/ERK, p38, or PKB causes selective stimulation of chondrocyte proliferation, maturation, or terminal differentiation, respectively (Fig. 2). CTGF also activates JNK, another member of the MAPK family, and JNK activation is involved in the proliferation and maturation of RGCs and HCS-2/8 chondrocytic cells (Fig. 2). Lastly, a role for phosphatidylinositol 3-kinase (PI3K), an upstream kinase to PKB, was also identified. Treatment of RGCs with CTGF and specific inhibitors of PI3K and PKC inhibited ALP activity, suggesting that both PKC and PI3K are involved in terminal differentiation of chondrocytes (Fig. 2). These studies demonstrate that CTGF is an important regulator of chondrocyte proliferation and differentiation.

CTGF and Animal Models: An *In* and *Ex Vivo* Look

Elucidating the *in vivo* importance of CTGF in skeletogenesis has required a multifaceted approach utilizing overexpressing transgenic as well as knockout mice. The action of many growth factors requires an appropriate balance in physiologic levels, where either too much or too little can be deleterious. Therefore, an essential approach to characterizing the effects of CTGF on skeletal formation requires the generation of genetically engineered animal models where the physiologic scale is tipped in either direction. Here we provide an overview of the studies and findings to date using animal models to study the role of CTGF in skeletogenesis; these include global and conditional knockout models, as well as transgenic models. We will discuss both *in vivo* findings, as well as *ex vivo* studies that utilized skeletal cells derived from these genetically engineered animal models.

A role for CTGF in skeletal development has been illustrated in studies using one of two global knockout models, both of which appear to produce a similar skeletal phenotype (as described below).^{21,57} The first model described in 2003 in the context of growth plate chondrogenesis and endochondral bone formation, was generated by replacing a fragment containing exon 1 (signal peptide), the TATA box and the transcription start site with the neomycin-resistance gene. Due to the design of the construct, Southern blot analysis could be used to genotype resulting litters when crossing heterozygous parents, and therefore discern wild-type (WT, +/+)¹, heterozygous (+/-) and null (-/-) offspring.²¹ The second model, described in 2008 in the context of pancreatic islet development, was generated by replacing the coding sequence of CTGF within exons 3 through 5 with a transmembrane domain-lacZ/Neomycin phosphotransferase cassette.⁵⁷ While a single RT-PCR amplification is not possible to discern the three genotypic possibilities from crossing two heterozygous parents (CTGF +/+, +/-, -/-), a clear advantage of this model is the ability to utilize an antibody to β -gal and/or X-gal staining to determine the location and distribution of the CTGF- β -gal fusion protein in heterozygous and null offspring. To date, much of the *ex vivo* work done with cells isolated from global CTGF knockout mice has utilized the former model; the details of which will be discussed subsequently.^{43,57-65}

The importance of CTGF in skeletogenesis was first demonstrated by Lyons and colleagues in CTGF null mice, which showed defects in growth plate chondrogenesis, angiogenesis, bone formation/mineralization, and ECM production. While skeletal defects were region specific, they reproducibly included kinked ribs, tibiae, radii and ulnae, as well as craniofacial abnormalities.²¹ Ultimately, global ablation of CTGF results in neonatal lethality caused by respiratory failure secondary to misshapen ribs and pulmonary hypoplasia.^{21,62} The neonatal lethality of these knockout mice represents a challenge in studying the full role of CTGF in skeletogenesis, namely the inability to study the effects of CTGF ablation during postnatal skeletal development, maintenance, and aging.

Further *in vivo* analysis of skeletogenesis in CTGF KO mice has focused on embryonic development at E15.5, 16.5 and 18.5. At E15.5 only the periosteal bone collar is visible microscopically in extremity long bones, and at E16.5 trabecular bone is just beginning to form in the primary centers of ossification.^{64,66} Therefore, studies at these times have provided information that is perhaps more applicable to the role of CTGF in bone formed by intramembranous ossification. Using these time points, it was demonstrated that CTGF KO mice have delays in craniofacial ossification, collagen deposition, osteoblast proliferation, and mineralization, as well as decreased tibial trabecular collagen deposition (also at E18.5), ALP activity, periosteal collar mineralization and osteoblast proliferation.⁶⁴ *Ex vivo* studies of CTGF KO and WT primary osteoblasts also showed less Col1a1, ALP and Oc expression levels, as well as decreased ALP activity and osteoblast proliferation. Furthermore, it was shown that rCTGF could rescue defects in osteoblast proliferation, mineralization and ALP activity in the CTGF-null osteoblasts.⁶⁴ Lastly, it has been shown that the ossified area in CTGF KO tibiae (E18.5) was reduced in length when compared to WT littermates, while total bone length did not appear different.⁴³ Despite the initial 2003 findings of striking skeletal defects, a more in depth, quantitative analysis of the effect of CTGF ablation on bone formation is warranted.

A critical role for CTGF in growth plate chondrogenesis has been more extensively examined in global ablation studies where CTGF-null mice display chondrodysplasia in limb and rib cartilage, as well as deformations in Meckel's cartilage.²¹ It had previously been shown that rCTGF stimulated chondrocyte proliferation *in vitro*.²⁹ This was confirmed in CTGF-null chondrocytes, which had an impaired ability to proliferate *in vivo* beginning

¹Unless otherwise specified, CTGF 'WT' mice includes both CTGF +/+ and +/- genotypes.

at E14.5, the time at which CTGF is upregulated in prehypertrophic and hypertrophic chondrocytes, and this resulted in fewer chondrocytes by E18.5.^{21,43} *Ex vivo* studies using CTGF-null primary chondrocytes have confirmed decreased chondrocyte proliferation, as well as shown decreased $\alpha 5$ integrin expression.⁵⁹ The $\alpha 5$ integrin is important in prehypertrophic chondrocyte differentiation and its expression overlaps that of CTGF in cartilage.^{59,67} Additional studies revealed a potential role for Nov/CCN3 in the CTGF-null developing cartilage, as CCN3 mRNA is elevated in null chondrocytes, despite decreased levels of CCN6, SOX-9, aggrecan, and Col2a1 mRNA.⁴³

The apparent *in vivo* defects of chondrocyte proliferation at E14.5 also coincided with an increased expansion in the hypertrophic zone of certain bones (e.g. humeri) in CTGF-null mice; this expansion continued until birth.²¹ The difference in the length of the zone, while apparent and qualitatively described in preceding studies,^{21,43} has not been previously quantified. We performed measurements in proximal tibial metaphyses from CTGF KO mice and WT littermates (Fig. 3A-D) and demonstrated a statistically significant increase in the length of the hypertrophic zone in CTGF KO tibiae (Fig. 3E).

The noticeable expansion of the hypertrophic zone in CTGF KO mice warranted a closer look as to whether defects in angiogenesis were present in these growth plates. It had previously been shown that defects in vascular endothelial factor (VEGF)- and matrix metalloproteinase 9 (MMP-9)-mediated angiogenesis resulted in lengthened hypertrophic zones.⁶⁸⁻⁷⁰ In CTGF KO mice, immunostaining demonstrated that while blood vessels were present in the metaphyseal intertrabecular spaces, the overall network of capillaries in the ossification zone adjacent the hypertrophic zone, was less extensive in the mutant bones (radii) compared to WT littermates.²¹ Further analysis revealed decreased MMP-9 and VEGF expression levels.²¹ Taking into account previous findings that impaired angiogenesis was concomitant with decreased trabecular bone density,⁶⁹ as well as the appearance of decreased trabecular bone and thinner bone collars in CTGF KO mice, it was concluded that growth plate angiogenesis was indeed defective in these mice.²¹ However, using metatarsals isolated from CTGF WT (+/+), heterozygous (+/-), and KO (-/-) embryos, it was later demonstrated that *in vitro* angiogenesis was not significantly different between these groups.⁶⁰ Further studies are necessary to clarify the extent to which angiogenesis is impaired in the CTGF-null growth plates.

Despite the inability to study CTGF-null mice postnatally, some knowledge on the effects of CTGF ablation has been garnered using CTGF heterozygous mice. It has been shown that CTGF haploinsufficiency results in roughly 50% normal protein levels.⁶⁰ When compared to CTGF wild-type (+/+) littermates, heterozygous (+/-) mice are phenotypically normal, lacking any of the aforementioned skeletal defects seen in the null (-/-) mice (Lambi, Popoff, unpublished data).⁷¹ Furthermore, CTGF heterozygous mice demonstrated reduced trabecular number and resultant transient osteopenia at 1 month of age, which was not observed in 4- and 6-month old CTGF heterozygous mice.⁷¹

Additional information on CTGF ablation has been obtained through generation of two conditional CTGF knockout models. The first of which was constructed using the 2.4-kb paired-related homeobox gene 1 (Prx1) enhancer, previously shown to drive expression beginning in E10.5 limb buds and later in E15.5 tendons and periosteum of fore- and hindlimbs.^{72,73} Conditional CTGF-null mice under this model demonstrated an osteopenic phenotype at 1 month of age only in male mice.⁷¹ Bone parameters were normal at the other ages examined in male mice and at all ages examined in female mice. The second model was constructed using the 3.9-kb human Oc promoter capable of driving expression in terminally differentiated (mineralizing) osteoblasts.⁷⁴ Conditional CTGF-null mice under the control of the Oc promoter demonstrated an osteopenic phenotype at 6 months of age,

but again only in male mice.⁷¹ These results suggest that CTGF has a more important role in prenatal skeletal development. Since the phenotype in conditional knockout mice can vary depending on the promoter being used to target specific cell populations, additional studies utilizing other established skeletal cell-specific promoters are warranted.

While the majority of the research involving CTGF and skeletogenesis has relied on CTGF ablation, several studies have utilized overexpressing transgenic lines to provide more information of CTGF's role in bone formation. A model of CTGF overexpression under the control of the type XI collagen promoter (ColXIa1), localized to chondrocytes,⁷⁵ resulted in decreased size of transgenic mice compared to WT littermates, as well as decreased bone density in the hindlimbs of transgenic mice.⁷⁶ A subsequent study generated bone-specific CTGF overexpression under the control of a 3.8-kb Oc promoter.⁴⁹ These transgenic mice displayed decreased bone mineralization and trabecular bone volume, as well as decreased rates of bone formation and mineral apposition. *Ex vivo* studies of calvarial osteoblasts and stromal cells isolated from these animals demonstrated decreased ALP and Oc mRNA levels in cells from transgenic mice, as well as varying effects on BMP/Smad, Wnt/ β -catenin, and (insulin-like growth factor 1) IGF-I/Akt signaling.⁴⁹ These results are contrary to previously reported *in vitro* studies where rCTGF increased the expression of ALP, Oc, and other markers of osteoblast differentiation in MC3T3-E1 osteoblastic cells and primary calvarial osteoblasts.^{24,28} Perhaps the conflicting results from these experiments are caused by differences in the magnitude of CTGF over-expression and/or from differences between intermittent (short-term) exposure to exogenously added rCTGF versus continuous (long-term) exposure from endogenous, over-expression. Such discrepancies highlight yet another area where future studies are necessary, not only utilizing various skeletal cell-specific promoters, but also inducible constructs.

From the studies to date concerning the effects of CTGF on skeletogenesis, one broad conclusion can be drawn: appropriate physiological levels of CTGF are necessary for normal skeletal development, as animal models with either ablation or overexpression of CTGF have demonstrated phenotypes related to cartilage and/or bone development.

CTGF and Integrin-Mediated Signaling and Adhesion

The development of the skeletal system requires proper synthesis and remodeling of the ECM, which provides the cells a milieu rich in nutrients, growth factors, and other components necessary for cell function. Basic components of the ECM include structural proteins, such as collagens and proteoglycans, as well as adhesion proteins, such as fibronectin (FN) and laminin.⁷⁷ For cell adhesion and communication with these extracellular proteins, cells use integrins, a family of $\alpha\beta$ -heterodimeric cell surface receptors. Associated with cytoskeletal actin filaments, these integrin receptors transduce extracellular information across the plasma membrane, leading to activation of various intracellular signaling cascades that regulate cell growth and differentiation.⁷⁸ The various subtypes of α and β integrin subunits, and their resultant combinations, confer unique binding specificities and signaling properties; these impact cell function as well as cell-to-cell or cell-matrix interactions.⁷⁹ Furthermore, binding to a specific ECM-associated (matricellular) protein can occur via different integrin heterodimers in different cell types.

CTGF functions as a matricellular protein that can serve as both an adhesive substrate for cells and as a molecular bridge between other ECM proteins, such as FN.^{15,58,80} However, to date, no one has identified a specific high-affinity signaling receptor for CTGF. Instead, studies have shown that integrins serve as functional signaling receptors for CTGF,^{14-16,18,19,58,81-87} and do so in a non-RGD-dependent fashion.⁵ It has also been demonstrated that heparan sulfate proteoglycans (HSPGs) serve as co-receptors in these

CTGF-integrin interactions.^{16,17,58,81,87,88} In this context, some of the biological activities of CTGF reside in the unique interactions with specific integrin subtypes expressed by a given target cell. These interactions initiate intracellular signaling cascades that ultimately regulate gene transcription and cell function.²

Taking into account the multi-modular structure of CTGF, numerous studies have revealed that the structural interaction of CTGF with integrins (and HSPGs) occurs via its fourth (cysteine knot) module. Further complexity arises as a given cell type may bind multiple CTGF modules through different integrin heterodimers. Such is the case with the hepatic stellate cells, where adhesion to the CTGF CT domain occurs via the $\alpha_v\beta_3$ integrin (and HSPGs),¹⁴ while adhesion to the TSP-1 domain occurs via the $\alpha_6\beta_1$ integrin.⁸⁹ As a result, different integrins on the surface of the same cell type can bind to different CTGF modules, thereby activating independent signaling pathways and mediating different cell functions.

Most of our current knowledge of CTGF-integrin interactions comes from studies using non-skeletal cells. In short, CTGF has been shown to bind to $\alpha_v\beta_3$ on endothelial cells and hepatic stellate cells,^{14,81} $\alpha_6\beta_1$ on fibroblasts,¹⁷ $\alpha_M\beta_2$ on peripheral blood monocytes,⁹⁰ $\alpha_{IIb}\beta_3$ on blood platelets,⁸⁴ and $\alpha_v\beta_1$ on pancreatic stellate cells.¹⁶ However, to fully understand the role of CTGF in skeletogenesis, it is necessary to understand the integrin complexes required for CTGF signaling in skeletal cells, how these specific interactions regulate cell growth and differentiation, and how they can play a role in disease processes such as tumorigenesis and metastasis to bone. We have summarized the findings to date regarding CTGF-integrin interactions in skeletal and non-skeletal cells in Table 1. For the purposes of this section, we will focus on CTGF-integrin interactions in skeletal cells.

CTGF-Integrin Interactions in Skeletal Cells

Cell attachment is a crucial step in the development, maintenance and regeneration of skeletal tissues. CTGF was shown to enhance human bone marrow stromal cell (hBMSC) adhesion in a dose-dependent manner.¹⁸ This multipotent stem cell population was enriched in mesenchymal stem cells (MSCs; most cells were STRO-1 positive) and had the ability to differentiate into osteoblasts, chondrocytes or adipocytes depending on culture conditions. This effect was shown to reside in the CT domain of CTGF and was mediated by the integrin $\alpha_v\beta_3$. This CTGF-integrin interaction resulted in the phosphorylation of p38 MAPK while activation of ERK and JNK was unaffected. Furthermore, CTGF also promoted the attachment of hBMSCs to hydroxyapatite (Hap) plates, suggesting its utility in bone regeneration when combined with Hap scaffolds to repair large bone defects.¹⁸

Little is known about the CTGF interaction with chondrocytes or about the receptors that transduce CTGF-derived signals and activities in chondrocytes. An early study using radiolabeled (¹²⁵I) recombinant CTGF demonstrated two classes (high and low affinity) of CTGF receptors on HCS-2/8 chondrocytic cells with an apparent molecular weight (the ¹²⁵I-CTGF-receptor complex) of 280kDa.⁹¹ However, there have been no follow up studies to identify the structure or function of these CTGF-specific receptors. In a more recent study, it was demonstrated that FN is a major binding partner of CTGF in the HCS-2/8 chondrocytes.¹⁵ This cell line is often used in chondrocyte studies since it has a differentiated phenotype that is similar to normal chondrocytes in terms of their ECM production and cell surface integrin profiles. Both full length CTGF and the CT domain bound directly to FN and enhanced adhesion of HCS-2/8 chondrocytes via the integrin receptor $\alpha_5\beta_1$ in a concentration-dependent manner.¹⁵ The signaling pathways activated as a result of this integrin-CTGF-FN interaction have not yet been explored and further studies would help determine its functional significance in chondrocytes.

In addition to the physiological importance of CTGF-integrin interactions in chondrocytes, studies have also shown that CTGF-integrin interactions can regulate cell migration and invasion in cancer cells.^{19,83} Chondrosarcoma is a malignant primary tumor of bone with a poor prognosis due to its high invasiveness and metastatic potential.⁹² CTGF has been shown to promote the migration and invasive behavior of human chondrosarcoma cells (JJ012 cell line) by enhancing the expression of MMP-13. MMP-13 degrades type II collagen (the major collagen in cartilage matrix),⁹³ and plays an important role in ECM turnover and tumor metastasis.⁹⁴ CTGF-enhanced migration and MMP-13 expression in chondrosarcoma cells was mediated by binding to cell surface integrin $\alpha_v\beta_3$ receptors and subsequent activation of the FAK, ERK, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signal transduction pathway.¹⁹ Since CTGF levels are elevated in advanced stages of breast cancer,⁹⁵ a separate study demonstrated that CTGF modulates cytoskeletal reorganization and enhances migration in human breast cancer (MCF-7) cells.⁸³ The mechanism of action for this effect involved the cell surface integrin $\alpha_v\beta_3$, ERK activation and up-regulation of the pro-metastatic gene *S100A4*. Together these studies suggest that CTGF-integrin interactions can play a critical role in regulating tumor invasiveness and metastatic potential in skeletal tissues, representing yet another opportunity ripe with investigative potential. Based on the perceived importance of CTGF in tumor invasion and metastasis, the therapeutic potential of blocking CTGF in specific cancers is currently being pursued.

In bone cells, the importance of integrin-mediated signaling in regulating cell differentiation and function are well documented. However, there is little information concerning CTGF-integrin interactions in bone cells and how this may regulate the effects of CTGF on cell growth, differentiation and function. Several integrins are involved in osteoclast binding to bone matrix, including $\alpha_v\beta_3$ (osteopontin, vitronectin, bone sialoprotein), $\alpha_v\beta_5$ (fibronectin), and $\alpha_5\beta_1$ (collagen).^{96,97} Among these integrins, $\alpha_v\beta_3$ is the predominant integrin on osteoclasts, and failure to express the complex results in a dramatic change in the osteoclast cytoskeleton, including failure to form actin rings and ruffled border membranes *in vivo*.⁹⁸ Osteoclasts lacking the β_3 integrin subunit are incapable of resorbing bone, resulting in osteopetrosis *in vivo*.⁹⁹ There is only one publication, which indirectly links the $\alpha_v\beta_3$ integrin on osteoclasts to CTGF in rheumatoid arthritis (RA).⁸⁶ Serum levels of CTGF were found to be significantly elevated in patients with RA and the production of CTGF in synovial fibroblasts was up-regulated by tumor necrosis factor-alpha (TNF- α) stimulation.⁸⁶ Furthermore, the presence of CTGF enhanced macrophage colony-stimulating factor / receptor activator of NF- κ B ligand (M-CSF/RANKL)-mediated osteoclastogenesis and bone resorption *in vitro*. CTGF also activated FAK and ERK in osteoclasts, and the phosphorylated ERK was recruited by the integrin $\alpha_v\beta_3$, suggesting that this integrin serves as a receptor for CTGF on osteoclasts. Although a direct interaction between CTGF and the $\alpha_v\beta_3$ integrin was not demonstrated, they concluded that CTGF produced by synovial fibroblasts in RA contribute to increased osteoclastogenesis and function through integrin $\alpha_v\beta_3$ signaling.⁸⁶ Therefore, it is proposed that CTGF plays a role in the cartilage and bone destruction associated with RA.

Osteoblasts express various integrin receptors that interact with matricellular proteins and regulate their growth and differentiation. Osteoblasts can utilize the β_1 integrin for adherence to FN and type I collagen, or the β_3 integrin for adhesion to vitronectin, bone sialoprotein, or osteopontin.¹⁰⁰⁻¹⁰³ The importance of these integrin-ECM interactions in osteoblasts has been illustrated in studies demonstrating that disruption of these interactions, specifically those involving FN and type I collagen, can block osteoblast differentiation.¹⁰⁴ It has also been shown that adhesion to ECM via the integrins $\alpha_v\beta_3$ and $\alpha_2\beta_1$ play a critical role in regulating osteoblast mineralization.¹⁰⁵

Despite the importance of integrin-ECM interactions in osteoblasts, there are no published reports that establish CTGF as an adhesive signaling protein in this cell type. We have examined osteoblast adhesion to MC3T3-E1 osteoblastic cells (Kanaan, Popoff, unpublished observations). MC3T3-E1 osteoblasts adhered to immobilized rCTGF in a concentration-dependent manner and this interaction was mediated by the integrin $\alpha_v\beta_5$. Osteoblast adhesion to CTGF resulted in cytoskeletal reorganization, activation of FAK and ERK, and promoted osteoblast differentiation (as evidenced by increased ALP activity) when compared to cells cultured on control (BSA or Poly-L lysine-coated) substrates (Kanaan, Popoff, unpublished observations). Expression of different integrins on osteoblasts may be restricted to specific stages of their differentiation, and specific integrin-matrical protein interactions may represent important regulatory signals for osteoblast differentiation. Additional studies are warranted to examine the molecular mechanisms associated with osteoblast adhesion to CTGF and better understand the role that CTGF plays in the osteoblast function and ultimately the development, maintenance and remodeling of bone.

LRP as a CTGF Receptor

The low density lipoprotein receptor-related protein (LRP) was identified as a receptor for CTGF in bone marrow stromal cells, hepatic stellate cells and fibroblasts, yet no direct signaling by this receptor in response to CTGF binding was identified.¹⁰⁶⁻¹⁰⁸ Using a renal fibroblast cell line, it was demonstrated that CTGF induced phosphorylation of the cytoplasmic domain of LRP. Despite this effect, CTGF was unable to stimulate differentiation of these cells unless co-stimulated with TGF- β , upon which it increased TGF- β -induced myofibroblast differentiation. It has also been suggested that CTGF binding to LRP may merely represent a clearing mechanism via internalization and endosomal degradation.^{106,107,109} Lastly, it has been shown that in *Xenopus* embryos, CTGF binds the Wnt co-receptor LRP-6 and inhibits Wnt signaling.¹¹⁰ Given 1) the importance of LRP and Wnt signaling in skeletogenesis, 2) the aberrations in CTGF-null mice during skeletal development (see above), and 3) the established binding of CTGF to LRP in certain cell types, studies examining whether LRP acts as a receptor for CTGF on bone cells and the potential effects of this LRP/CTGF interaction on Wnt signaling should be pursued.

Regulation of CTGF Expression in Osteoblasts and Chondrocytes

CTGF is an early response gene and its expression is principally regulated at the level of transcription,¹¹¹ however, post-transcriptional controls that effect RNA stability have also been identified.¹¹²⁻¹¹⁶ The induction and regulation of CTGF expression is context dependent in that the signaling mechanisms that control CTGF induction are specific to certain disease states and/or cell types.¹¹⁷ CTGF gene expression can be regulated in numerous cell types by a number of different factors that include: various environmental conditions including hypoxia and biomechanical stress; soluble mediators including high glucose, cortisol, bioactive lipids, retinoids, certain amino acids, lipoproteins and glucocorticoids; drugs such as Iloprost and statins, as well as, specific growth factors.¹¹⁸⁻¹³³ Concerning skeletal cells, the major inducers of CTGF in both chondrocytes and osteoblasts include BMP-2 and TGF- β 1. In addition, glucocorticoids, retinoids and taurine are reported to mediate CTGF induction in chondrocytes, while endothelin and cortisol mediate CTGF in osteoblasts.^{122,130,132-135} Among all of these CTGF inductive agents, TGF- β 1 is one of the most potent regardless of cell type.^{117,121,136,137} Beyond its potency, TGF- β 1 is by far the most studied CTGF inducer and the majority of data regarding the control of CTGF induction in skeletal cells comes from studies of its regulation by TGF- β 1. In this section of the review, we will discuss the regulation of CTGF expression in the context of skeletal cells, with special emphasis given to CTGF regulation by TGF- β 1.

In general, TGF- β 1 signals through a generic Smad-mediated pathway involving Smads 2, 3 and 4 (Fig. 4).¹³⁸ Smads 2 and 3 are phosphorylated by active transmembrane serine/threonine TGF- β 1 receptors and form a trimeric complex with Smad 4, which subsequently translocates to the nucleus, and binds to Smad binding elements (SBE) in promoters of TGF- β 1-responsive genes.¹³⁸⁻¹⁴⁰ Using specific knockdown studies in osteoblasts, we found that Smads 3 and 4 were essential, while Smad 2 dispensable, for CTGF induction by TGF- β 1. This observation is consistent with the finding that Smad 3 tends to be required for CTGF induction by TGF- β 1 regardless of cell type, while Smad 2 is also dispensable in other connective tissue cell types.¹⁴¹⁻¹⁴³ However an important exception to this is found in hepatocytes, where Smad2 and not Smad3 is required for CTGF induction by intracellular TGF- β .¹⁴⁴

Smad signaling is also dependent on other signaling molecules, including transcriptional activator and/or repressor proteins, which act as co-factors to achieve nuclear translocation and DNA binding.¹³⁹ While data using specific knockdown studies is not available regarding the role of Smads for CTGF induction in chondrocytes, studies in the chondrocytic cell lines HCS-2/8 and ATDC5, demonstrate that the SBE cooperates with other cis-regulatory elements in the CTGF promoter to control transcription, implicating the involvement of Smad signaling for CTGF induction in these cells (we will discuss in greater detail some cis-regulatory elements important for CTGF induction later in this review).^{145,146} Thus, Smad signaling is often required, but in most cases not sufficient alone to achieve CTGF induction by TGF- β 1.

CTGF induction is also mediated by MAPK signaling in a cell type-dependent fashion. Several studies examining the interactions between MAPKs and Smad signaling demonstrated that TGF- β 1-induced Smad signaling is regulated by MAPKs in osteoblasts (Fig. 4).¹⁴⁷⁻¹⁵¹ While ERK mediates CTGF promoter transactivation by a TGF- β 1/ endothelin-1 synergism in osteoblast cell lines and in primary osteoblasts,^{152,153} we found that although TGF- β 1 activated all three MAPKs (ERK1/2, p38 and JNK), only ERK was required for TGF- β 1 induction of CTGF expression and promoter activity.¹⁵⁴ In chondrocytes, the role MAPKs play in CTGF induction remains less defined. In one study assessing retinoid induction of CTGF in chondrocytes, ERK was found to be necessary for retinoid stimulated CTGF, whereas p38 inhibited this stimulation.¹³⁴ Future studies are necessary to assess the potential role that MAPKs play in CTGF regulation in chondrocytes.

MAPKs, such as ERK, can modulate the TGF- β 1/Smad pathway directly through effects on the phosphorylation of Smads or indirectly through activation/inactivation of required nuclear co-activators/co-repressors that mediate Smad DNA binding.¹⁵⁵ In osteoblasts, inhibiting ERK does not prevent activation (phosphorylation) of Smad 3 and 4 by TGF- β 1 treatment. However, using electro-mobility shift assays, we showed that pharmacological inhibition of ERK activation impaired transcriptional complex formation on the SBE of the CTGF promoter, demonstrating that ERK activation was necessary to facilitate SBE transactivation in osteoblasts.^{154,156} Recently, other intracellular proteins have also emerged that are important for CTGF regulation via the TGF- β 1 Smad or MAPK pathway. In osteoblasts, we have shown that Src is important for TGF- β 1 induced CTGF promoter activity and expression,^{154,156} and that Src is an essential upstream signaling transducer of ERK and Smad signaling with respect to TGF- β 1 induction of CTGF.¹⁵⁶ New evidence also suggests that ETS-1, a known ERK transcription factor essential for CTGF induction by TGF- β 1 in fibroblasts, also appears to be required for CTGF expression in osteoblasts (Arnett et al., unpublished data). In addition, the transcription factor FOXO, a known Smad co-factor that facilitates Smad DNA binding, also appears to regulate CTGF expression, however its potential relevance for skeletal cells needs to be evaluated further.¹⁵⁷

The CTGF gene is primarily driven by a core promoter containing a TATA box,¹⁵⁸ in proximity to several functional and putative cis-elements that constitute its proximal promoter.³³ Numerous studies have identified specific motifs within this proximal promoter that regulate CTGF expression in response to various stimuli in different cell types.^{111,118-129} The two predominant cis-regulatory elements that are required for TGF- β 1 induction of CTGF expression in osteoblasts and chondrocytes include the TGF- β 1 response element (TRE) (aka: basal control element) and/or the SBE, and numerous studies in different cell types have demonstrated divergent roles for each of these elements in either basal or TGF- β 1 induced regulation of CTGF.^{151,159}

The TRE was originally described by Grotendorst et al. and is unique to the CTGF promoter. The TRE is important for TGF- β 1 mediated induction of CTGF in scleroderma fibroblasts, pancreatic cancer cells, the chondrocytic cell line HCS-2/8 and also mediates CTGF induction in fibroblasts by endothelin-1.^{152,158,160,161} However, in normal skin fibroblasts and mesangial cells, the TRE only plays a role in basal CTGF expression, and the SBE alone mediates CTGF induction by TGF- β 1.¹⁶⁰ Interestingly in osteoblasts, using a combination of promoter deletion and site-directed mutant constructs we demonstrated that both the TRE and SBE are required for basal and TGF- β 1 induced CTGF expression.¹⁵⁴ This was the first study in which both elements together were recognized as being necessary for both induction of CTGF expression by TGF- β 1 and basal control in the same cell type. Despite this unique cooperation of both elements in CTGF regulation in osteoblasts, it is not uncommon for multiple transcriptional elements to be involved in the context of Smad mediated signaling involving the SBE. Since Smads themselves have an intrinsically weak DNA binding affinity they are reliant on formation of large nucleoprotein complexes with transcription factors that bind to one or more transcriptional DNA sequences (cognate motifs) adjacent to the SBE to achieve target gene activation.¹⁶² For example, in fibroblasts, the SBE element was reported to act in concert with a tandem repeat of an ETS element,^{160,163} and ETS-1 binding to this motif was required for CTGF induction by TGF- β 1 in these cells.¹¹¹ Using electromobility shift experiments, we found that the TRE enhances the affinity of the SBE for transcriptional complexes in osteoblasts,¹⁵⁴ demonstrating that synergistic function between the TRE and SBE is necessary to facilitate transcription factor binding necessary for CTGF induction in osteoblasts.

In the HCS-2/8 chondrocytes, known for their high basal expression of CTGF, the TRE, and not the SBE, is predominantly responsible for CTGF expression, however an additional upstream element designated transcriptional enhancer dominant in chondrocytes (TRENDIC) is also required to maintain high levels of basal CTGF expression.^{145,164,165} Although the TRENDIC element was also found to enhance CTGF expression in some other non-bone cell lines (MDA-231 breast adenocarcinoma cells), its involvement in CTGF expression in other cell types appears less significant with no effect in the osteoblastic cell line Saos-2.¹⁶⁵ Recently, a nuclear MMP3 was shown to bind the TRENDIC motif and regulate CTGF expression in chondrocytes,¹⁶⁶ however no functional studies in these cells regarding the significance of CTGF induction by MMP3 have been conducted. More recently, several T-cell factor/lymphoid enhancer factor (TCF/LEF) elements in the CTGF proximal promoter have also been identified that regulate CTGF expression in chondrocytes.¹⁶⁷ These TCF/LEF elements differentially bind either SOX-9 or β -catenin to regulate CTGF expression at different stages of chondrocyte differentiation and appear to mediate the timing of CTGF expression in these cells. However the potential contribution of these elements for CTGF regulation in osteoblasts needs to be evaluated moving forward.

In addition to promoter mediated regulation, CTGF transcription can also be regulated by non-promoter controls. In 2000, Takigawa et al. identified a post-transcriptional element in the 3-untranslated region (3-UTR) of CTGF, designated as a cis-acting element of structure-

anchored repression (CAESAR) that suppresses CTGF expression in chondrocytes.^{113,168} Additionally, no significant cis-regulatory elements have been found for a number of other CTGF inducers including: glucocorticoids, statins and mechanical strain suggesting that CTGF regulation may occur beyond the constraints of its proximal promoter, possibly through distant 5' or 3' enhancer sites.^{130,169-171} Given the importance of CTGF expression for both osteoblast and chondrocyte differentiation, future studies are necessary to develop a better understanding of CTGF regulation in skeletal cells. The identification of specific signaling cascades and perhaps even more importantly, cell specific molecules that control CTGF expression, may provide potential therapeutic targets for treating diseases of bone and cartilage in the future.

Concluding Remarks and Future Directions

This review has highlighted the studies to date establishing CTGF as an important regulator of skeletogenesis. Studies have shown that CTGF is important for the condensation of mesenchymal cells at sites of future bones and for regulating the proliferation and differentiation of chondrocytes and osteoblasts. Precise regulation of CTGF expression is essential for the normal progression of events during mesenchymal condensation, chondrogenesis and osteogenesis. The ability of CTGF to interact with and modulate the effects of other skeletal growth factors also plays a crucial role in its ability to regulate the development of the skeleton, and this is an area in which future investigations must be conducted to gain a better understanding of the interactions between CTGF and other skeletal growth factors. The physiological importance of CTGF during skeletogenesis was confirmed in CTGF null mice that demonstrated defects in the craniofacial, axial and appendicular skeleton. Although chondrogenesis and osteogenesis were negatively impacted by CTGF ablation, the neonatal lethality of these knockout mice prevented the use of this model to study the postnatal role of CTGF during skeletal modeling, remodeling and repair. To this end, conditional knockout mice using a promoter that targets osteoblasts at an early stage of their lineage should be established and evaluated. This same approach should also be utilized to create animal models with stage specific ablation of CTGF in chondrocytes or mesenchymal cells.

Transgenic mice with bone-specific CTGF overexpression under the control of the Oc promoter displayed decreased trabecular bone volume and bone formation. *Ex vivo* studies of calvarial osteoblasts and stromal cells isolated from these animals demonstrated decreased osteoblast differentiation and function. These results are contrary to *in vitro* studies where rCTGF increased osteoblast differentiation in MC-3T3-E1 osteoblastic cells and primary calvarial osteoblasts. Perhaps the conflicting results from these experiments are caused by differences in the relative levels of CTGF that the cells are exposed to and/or from differences between intermittent (short-term) exposure to exogenously added rCTGF versus continuous (long-term) exposure to endogenous, overexpression of CTGF. Such discrepancies highlight yet another area where future studies are necessary, utilizing various skeletal cell-specific promoters and inducible constructs to generate additional transgenic models for evaluation.

CTGF functions as a matricellular protein that can serve as both an adhesive substrate for cells and as a molecular bridge between other ECM proteins. Studies have shown that integrins serve as functional signaling receptors for CTGF and that some of the biological activities of CTGF reside in the unique interactions with specific integrin subtypes expressed by a given target cell. These interactions initiate intracellular signaling cascades that ultimately regulate gene transcription and cell function. Since most studies of integrin/CTGF interactions to date have been conducted using non-skeletal cells, relatively little is known about how these interactions regulate skeletal cell differentiation and function. Given

the importance of bone cell/ECM interactions in regulating cell differentiation and function, additional studies are needed to identify bone cell-specific integrin/CTGF interactions and to determine how these interactions affect the differentiation and function of osteoblasts and osteoclasts. Studies examining whether LRP acts as a receptor for CTGF on bone cells and the potential effects of this LRP/CTGF interaction on Wnt signaling should also be pursued.

Concerning the regulation of CTGF expression in skeletal cells, the major inducers of CTGF in both chondrocytes and osteoblasts include BMP-2 and TGF- β 1. In addition, glucocorticoids, retinoids and taurine have been reported to mediate CTGF induction in chondrocytes, while endothelin and cortisol have been shown to regulate CTGF in osteoblasts. Among these CTGF inductive agents, TGF- β 1 is the most potent regardless of cell type, and the majority of data regarding the control of CTGF induction in skeletal cells comes from studies of its regulation by TGF- β 1. Studies have shown that osteoblasts are unique compared to other cell types studied thus far, in that both the Smad binding element (SBE) and the TGF- β -binding element (TBE) in the proximal CTGF promoter are required for TGF- β 1 induction of CTGF expression in osteoblasts. Furthermore, Erk and Src activation are also required for TGF- β 1 induction of CTGF in osteoblasts, and it appears that Src plays a central role in regulating both Smad and Erk signaling. Additional studies are needed to further elucidate signaling mechanisms involved in TGF- β 1-mediated regulation of CTGF expression in osteoblasts and to determine whether any of these mechanisms are unique to osteoblasts. In addition, the regulation of CTGF by other inductive agents in skeletal cells, especially BMP-2, awaits future investigation.

Acknowledgments

NIH/NIAMS R01 AR047432 (to SNP) and PA Department of Health (to AGL).

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Figure 1.

CTGF Modular Structure and Function

A) The CTGF transcript contains a signal peptide (SP) as well as four modules: module 1 is an insulin-like growth factor (IGF)-binding domain; module 2 is a von Willebrand type C domain; module 3 is a thrombospondin-1 domain; and module 4 is a C-terminal domain containing a putative cysteine knot. Modules II and III are separated by a variable hinge region susceptible to enzymatic cleavage. Also shown below each module are molecules known to interact with this region of the secreted CTGF protein.

B) The mosaic structure of these proteins allows for their involvement in many normal cellular events that contribute to key physiologic processes necessary for skeletogenesis.

Figure 2.

CTGF Signaling in Chondrocytes

CTGF interacts with (an) unknown receptor(s) on the surface of chondrocytes via binding of one or more of its four distinct modules. This interaction triggers various intracellular pathways. PKC plays an important upstream role in chondrocyte proliferation, maturation, and terminal differentiation via its activation of MEK, p38, and PKB, respectively. CTGF also activates the JNK pathway, which stimulates chondrocyte proliferation. CTGF-mediated activation of PI3K also stimulates chondrocyte terminal differentiation via PKB. (Note: This figure was adapted from Yosimichi, G., *et al.*, (2006). *Bone*, 38(6), 853-863).

Figure 3.

Increased hypertrophic zone in CTGF KO compared to WT mice.

A,C) Tibiae from newborn WT (**A,B**) and CTGF KO (**C,D**) mice were stained with von Kossa and counterstained with toluidine blue (scale bars are equal to 500μm. **B,D**) High magnification of the proximal region of endochondral ossification, with hypertrophic zones depicted by bracket on right side (scale bars are equal to 100μm). **E)** An increase in thickness of the hypertrophic zone was observed in CTGF KO metaphyses. Values below represent the mean ± SEM, where $n=4$.

Figure 4.

Regulation of CTGF Expression by TGF-β1 in Osteoblasts

TGF-β1 induction of CTGF in osteoblasts requires Src, Smad and ERK signaling. The canonical TGF-β1/Smad signaling pathway involves TGF-β1 activation of transmembrane serine/threonine TGF-β1 receptors (TβRI and TβRII) that in turn phosphorylate receptor Smads (Smad 2 and Smad 3). Following activation, Smad 2 and 3 form a trimeric complex with Smad 4, and this complex subsequently translocates to the nucleus, where it binds to Smad binding elements (SBE) in DNA through association with other transcriptional co-activators and/or co-repressors to regulate CTGF. Smad 2 is dispensable for CTGF induction by TGF-β1 in osteoblasts. Src plays a central role in TGF-β1 signaling in osteoblasts by regulating the activation of both Erk and Smad 2 and 3. ERK functions in a combinatorial manner with Smad signaling and facilitates SBE transactivation. In addition, the TRE and SBE are also required for basal and TGF-β1 induced CTGF expression. Future studies are needed to identify other CTGF regulatory mechanisms in osteoblasts.

Table 1

CTGF and Integrin-Mediated Signaling and Adhesion

Cell type	Integrin receptor	Form of CTGF Used	Cellular Events Stimulated	Reference
Endothelial Cell (HMVEC)	$\alpha_v\beta_3$	Mouse full length	Adhesion, migration, survival	81
Platelet	$\alpha_{IIb}\beta_3$	Mouse full length	Activation (ADP)-dependent adhesion	84
Fibroblast (HDF)	$\alpha_6\beta_1$	Mouse full length	Adhesion; formation of filopodia and lamellipodia; FAK, Paxillin, Rac, and ERK activation; increased expression of MMP-1 and MMP-3	17
Fibroblast (MEF)	$\alpha_4, \alpha_5, \beta_1$	Endogenous mouse	Adhesion to and spreading on FN; α -SMA stress fiber formation; ERK and FAK activation	58
Monocyte (PBMC, THP-1)	$\alpha_M\beta_2$	Mouse full length	Activation (ADP/PMA)-dependent adhesion	87
Hepatic Stellate Cell	$\alpha_v\beta_3$	Human full length and CT domain	Adhesion	14
	$\alpha_6\beta_1$	TSP-1 domain	Adhesion, ERK activation, increased expression of FN and pro-collagen type IV(α_5)	89
Pancreatic Stellate Cell	$\alpha_5\beta_1$	Human full length, and CT domain	Adhesion, migration	16
Bone Marrow Stromal Cell	$\alpha_v\beta_3$	Human full length, and CT domain	Adhesion, p38 activation	18
Mammary Epithelial Cell (HC11)	$\alpha_6\beta_1$	Human full length	Adhesion	85
Breast Carcinoma Cell (MCF-7)	$\alpha_v\beta_3$	NS	^b Migration, ERK activation, increased <i>S100A4</i> expression	83
Ovarian Carcinoma Cell (DOV13)	$\alpha_3\beta_1, \alpha_6\beta_1$	NS	Adhesion	82
Chondrosarcoma Cell (JJ012)	$\alpha_v\beta_3$	NS	^b Migration; FAK, ERK, p65 and IKK α/β activation; increased MMP-13 expression; increased κ B luciferase activity	19
Chondrocyte (HCS-2/8)	$\alpha_5\beta_1$	Human full length, and CT domain	Adhesion to FN	15
Osteoblast (MC3T3-E1)	$\alpha_v\beta_5$	Rat full length	Adhesion, FAK and ERK activation	^h
Osteoclast (CD14 ⁺ PBMC)	$\alpha_v\beta_3$	Human full length	^b FAK and ERK activation	86

The cellular effects provided in the table occurred upon direct interaction of the CTGF used and the listed integrin complex(es), unless otherwise specified. All forms of CTGF listed above were used as a substrate to coat plates for experiments.

^b Results obtained from indirect interactions of CTGF with the integrin complex identified.

^h Kanaan and Popoff, unpublished data.

Abbreviations: NS= forms of CTGF not specified in the original publication. HMVEC=primary human dermal microvascular endothelial cells; HDF=adult human dermal fibroblast; MEF=mouse embryonic fibroblast; PBMC=human peripheral blood mononuclear cell; THP-1=human acute monocytic leukemia cell line; HC11=mouse mammary epithelial cell line; MCF-7=human breast cancer cell line;MDA-231=human breast carcinoma cell line; DOV13=human ovarian carcinoma cell line; JJ012=human chondrosarcoma cell line; HCS-2/8=human chondrosarcoma chondrocytic cell line; MC3T3-E1=mouse osteoblastic cell line.