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The role of connective tissue growth factor in skeletal growth and development

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Summary

Connective tissue growth factor (CTGF) is a secreted, extracellular matrix-associated protein that regulates diverse cellular functions in different cell types. CTGF gene belongs to a larger CCN gene family that also includes Cyr61 and NOV. It modulates many cellular functions, including proliferation, migration, adhesion, and extracellular matrix production, and it is involved in many biological and pathological processes. CTGF has special importance in skeletal development. During Meckel's cartilage development, CTGF acts as a down-stream molecule of TGFβ to stimulate cell-cell interactions and the expression of condensation-associated genes. CTGF promotes endochondral ossification and articular cartilage regeneration. During the healing of experimental bone fracture, CTGF was expressed in periosteal cells and hypertrophic chondrocytes. It promotes the proliferation of chondrocytes and osteoblasts. CTGF is a down-stream mediator for prostaglandin E2 (PGE2) in osteoblast-induced proliferation. It also regulates signaling through the Wnt pathway, in accord with its ability to bind to the Wnt co-receptor LDL receptor-related protein 6 (LRP6). Constitutive expression of CTGF was shown to inhibit both BMP-9- and Wnt3A-induced osteogenic differentiation.

key words/ abbreviations:

CCN family – Cyr-61, CTGF, NOV family; **CTGF** – connective tissue growth factor; **Cyr61/CCN1** – cysteine-rich 61; **NOV/CCN3** – nephroblastoma overexpressed; **fisp12** – fibroblast inducible secreted protein; **TGF**β – transforming growth factor-beta; **HUVEC** – human umbilical vein endothelial cell; **VEGF** – vascular endothelial growth factor; **ECM** – extracellular matrix production; **IB domain** – insulin growth factor-binding protein; **VWC domain** – Von Willebrand factor type C; **TSP_1 domain** – thrombospondin type I; **HMCs** – human mesangial cells; **HFL-1** – human lung fibroblast line; **human chondrosarcoma-derived chondrocytic cell line HCS-2/8, HSPG** – heparan sulfated proteoglycans; **LPR** – low-density lipoprotein receptor-related protein; **P44/42 MAPK/ ERK** – P44/42 mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase; **BMP2** – bone morphogenetic protein 2; **LDL** – platelet-derived growth factor; **LRP6** – receptor-related protein 6

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CTGF Gene and Protein

The CCN family comprises cysteine-rich 61 (Cyr61/CCN1), connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (NOV/CCN3), Elm-1/Wisp1/CCN4, Wisp2/rCop/CCN5, and Wisp3/CCN6 [1]. A proposal was put forth to unify the nomenclature of the CCN family proteins in the order of their description in the literature, i.e. CCN1 (CYR61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3) [2].

CTGF was discovered in 1991 while screening a human umbilical vein endothelial cell (HUVEC) cDNA expression library with anti-PDGF antibody [3]. It was called connective tissue growth factor due to its mitogenic and chemotactic properties in fibroblasts. CTGF is produced by multiple cell types, such as fibroblasts [4], endothelial cells 5], vascular smooth muscle cells [6], mesangial cells [7], osteoblasts [8], and chondrocytes [9]. The human CTGF (hCTGF) gene has been cloned and sequenced. CTGF gene maps to human chromosome 6, it spans approximately 4.5 kb, and is organized into five exons and four introns (Figure 1). CTGF encodes 349 amino acids and is 91% homologous to the fisp-12 gene, which maps to murine chromosome 10. CTGF protein consists of four domains, one of which is an insulin growth factor-binding protein (IB domain), which contains the motif that binds to insulin growth factors (IGF). Another is a Von Willebrand factor type C (VWC domain). Many proteins that contain a VWC domain participate in oligomerization that might be preceded by dimerization. CTGF's thrombospondin type I (TSP_1) domain might be a cell attachment motif that binds to sulfated glycoconjugates, and the last domain is a cysteine-knot domain (Figure 2) [10].

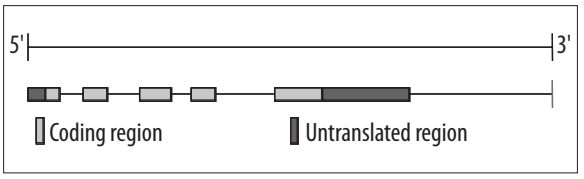

Figure 1. Schematic diagram of the CTGF genomic structure. CTGF has 5 coding regions (exons) that encode 4 different protein domains and 4 untranslated regions (introns).

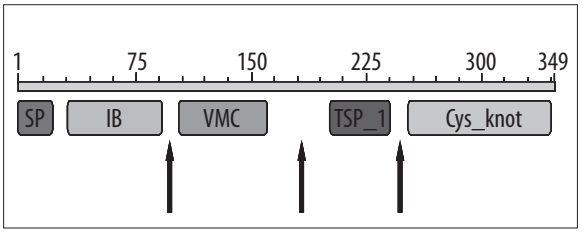

Figure 2. Schematic diagram of CTGF protein, indicating the arrangement of the different motifs. Each motif is encoded by a separate exon. SP – signal peptide (1-22 aa); IB – insulin-like growth factor binding protein domain (31..> 93 aa); VWC – von Willebrand factor type C repeat (103..> 162 aa); TSP_1 – thrombospondin type I repeat (202..> 242 aa); Cys_knot – C-terminal cysteine knot domain (261..> 330 aa). The proteolytic cleavage sites are indicated with the black arrows.

Figure 1. Schematic diagram of the CTGF genomic structure. CTGF has 5 coding regions (exons) that encode 4 different protein domains and 4 untranslated regions (introns).

Transforming growth factor β1 (TGFβ1) and CTGF

CTGF is transcriptionaly activated by (TGFβ1) and appears to mediate some of the extracellular matrix (ECM) inducing properties that have been previously attributed to TGFβ1. In fibrotic liver, CTGF mRNA and protein are produced by hepatic stellate cells (HSCs), a production that is primarily regulated by TGFβ [11]. Moreover, in a human lung fibroblast line (HFL-1), TGFβ1 enhanced CTGF mRNA levels in a time-and concentration-dependent manner [12]. Nuclear run-on assays in primary cultures of murine osteoblasts revealed that TGFβ1, bone morphogenetic protein (BMP2), and cortisol enhanced CYR61 and CTGF transcription [13]. High glucose stimulated a striking increase in gremlin (BMP antagonist) mRNA levels in parallel with increases in mRNA for the growth factors vascular endothelial growth factor (VEGF), TGFβ1, and CTGF in cultured bovine retinal pericytes [14]. CTGF and TGFβ stimulated connective tissue cell proliferation and ECM synthesis *in vitro* and exhibited shared fibrogenic and angiogenic properties *in vivo*; in human mesangial cells (HMCs), TGFβ induced CTGF as a downstream mediator, which in turn stimulated fibronectin synthesis [15].

CTGF is also a down-stream effector molecule, mediating TGFβ1 up-regulation of integrins and cellular adhesion. In human mesangial cells (HMCs), changes induced by TGFβ on $\alpha_5\beta_1$ expression were mediated through TGFβ induction of CTGF. Treatment of cells with TGFβ and anti-sense CTGF oligonucleotides significantly reduced the TGFβ-induced increase in $\alpha_5\beta_1$ levels [15]. Moreover, CTGF mediated TGFβ-induced increase in cell adhesion to fibronectin, the main substrate for $\alpha_5\beta_1$, since CTGF anti-sense oligonucleotides significantly reduced the number of adherent cells to fibronectin from TGFβ-stimulated cultures [15]. CTGF also acts as down-stream mediator of TGFβ-induced fibroblast proliferation [16] and it mediates TGFβ anchorage independent growth of NIH3T3 cells [17]. CTGF is also involved in extracellular matrix production. *In vitro* studies with normal rat kidney fibroblasts demonstrated that CTGF induces collagen synthesis, and transfection with CTGF antisense oligonucleotide blocked TGFβ-stimulated collagen synthesis [18].

In a recent study, CTGF was shown to directly bind TGFβ1. CTGF binding to TGFβ1 enhances the ability of this cytokine to bind to its receptor. Thus, CTGF activates TGFβ1 signaling by direct binding [19]. Collectively, data in the literature show that CTGF is both a regulator for and regulated by TGFβ.

CTGF's Role in Cellular Functions and Biological, and Pathological Processes

CTGF modulates many cellular functions. The mitogenic activities of CTGF have been reported and it was found to promote the proliferation of chondrocytes and osteoblasts [8,20]. CTGF also acts as a chemotactic protein. Using an

anti-sense oligonucleotide and anti-sense RNA, the role of CTGF in the migration of vascular endothelial cells was investigated in a Boyden chamber assay. Pre-treatment with anti-sense oligonucleotide markedly inhibited the migration of bovine aorta endothelial cells. Thus, CTGF appears to play a role in angiogenesis [21]. In addition, CTGF has role in pathological processes such as wound healing, where it stimulates cell proliferation, adhesion, chemotaxis, angiogenesis, and ECM production [1]. The divergence of CTGF actions on connective tissue cell types was revealed by the finding that the N-terminal domain of CTGF mediates myofibroblast differentiation and collagen synthesis, whereas the C-terminal domain of CTGF mediates fibroblast proliferation [22]. CTGF may also be involved in the cyclical proliferation of the uterine gland epithelium and in the early stages of follicular maturation, as well as in the neuropeptide regulation in the gut and cardiovascular and renal systems [23].

CTGF RECEPTORS

CTGF is a ligand that induces its effects by binding receptors on the cell surface. A cell surface receptor of the 620-kDa low-density lipoprotein receptor-related protein (LPR) was identified as a receptor for CTGF in several cell lines, including MG63 (human osteoblast cell line) [24]. In chondrocytes, a study revealed the formation of an $^{(125)}$I-rCTGF-receptor complex with an apparent molecular weight of 280-kDa protein [25], suggesting an interaction between CTGF and an integrin receptor. CTGF was found to induce chondrocyte proliferation, through P44/42 mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase (P44/42 MAPK/ERK), and the authors postulated that this process is either initiated by CTGF binding to receptor tyrosine kinase (RTK) or to an integrin receptor [26]. Many studies have identified integrins as receptors for CTGF, namely $\alpha_v\beta_3$ on endothelial cells, $\alpha_{11b}\beta_3$ on platelets, $\alpha_6\beta_1$ integrin on fibroblasts, and $\alpha_m\beta_2$ in peripheral blood monocytes [21,27–29].

CTGF AND CHONDROCYTES

CTGF plays important roles in both the growth and differentiation of chondrocytes. CTGF efficiently promotes endochondral ossification and articular cartilage regeneration [30]. It was found to regulate chondrocyte function, particularly in the hypertrophic zone. The stability of chicken CTGF mRNA is regulated in a differentiation stage-dependent manner in chondrocytes. Stimulation by BMP2, platelet-derived growth factor, and CTGF stabilized CTGF mRNA in proliferating chondrocytes, but it destabilized the mRNA in prehypertrophic-hypertrophic chondrocytes. Therefore, gene expression of CTGF mRNA during endochondral ossification is properly regulated, at least in part, by changing the stability of the mRNA, which arises from the interaction between the RNA cis-element located within the area between 100 and 150 bases from the polyadenylation tail and a putative 40-kDa trans-factor in the nuclei and cytoplasm [31].

CTGF may play a role in Meckel's cartilage development by acting down-stream of TGF-β and stimulating cell-cell interactions and expression of condensation-associated genes. CTGF was strongly expressed in anterior, central, and posterior regions of embryonic day (E) 12 condensing Meckel's mesenchyme. Expression decreased in E15 newly differentiated chondrocytes, but surged again in E18 hypertrophic chondrocytes located in the anterior region and the most-rostral half of central region. rCTGF treatment of micromass cultures stimulated the expressions of both condensation-associated macromolecules (fibronectin and tenascin-C) and chondrocyte differentiation [32].

In long-bone anlagen *in vivo* and hypertrophic chondrocyte, interference with retinoid signaling blocked the expression of CTGF and other posthypertrophic markers cultures, whereas all-trans-retinoic acid (RA) boosted CTGF expression and even induced it in immature proliferating cultures. Exogenous recombinant CTGF stimulated chondrocyte maturation, but failed to do so in the presence of retinoid antagonists. Retinoid signaling regulates CTGF expression in hypertrophic chondrocytes with differential involvement of MAP kinases [33].

CTGF AND OSTEOBLASTS

CTGF mRNA and protein were expressed *in vivo* in rows of plump, cuboidal osteoblasts lining bony trabeculae [8]. CTGF was also expressed *in vitro* in primary osteoblast cultures, in which treatment of primary osteoblast cultures with PGE2 resulted in increased CTGF expression (PGE2 is known for its roles in increasing osteoblast proliferation). This finding suggests that CTGF is a down-stream mediator for prostaglandin E2 (PGE2) in osteoblasts-induced proliferation [34]. Also, CTGF promoted MC3T3-E1 osteoblast-like cell line cell proliferation in a dose- and time-dependent manner [35]. Another study showed that CTGF-mediated MC3T3-E1 osteoblast adhesion, to the αvβ5 integrin, resulted in activation of distinct signaling pathways. These pathways regulate the activation of intracellular kinases, the formation of focal adhesions, and the reorganization of actin cytoskeleton, which suggests a role for CTGF in regulating osteoblast cell morphology, viability, migration, and proliferation [36]. CTGF was identified as a potential target of Wnt and BMP signaling since it was up-regulated at the early stage of BMP-9 and Wnt3A stimulations of mesenchymal stem cells. The constitutive expression of CTGF was shown to inhibit both BMP-9- and Wnt3A-induced osteogenic differentiation. Wnt3A regulated CTGF expression in a beta-catenin-dependent manner [37].

CTGF AND SKELETAL DEVELOPMENT

Several findings concerning the role of CTGF in bone have been recently elucidated. An initial study showed that CTGF is over-expressed (8- to 10-fold) in osteopetrotic bone versus normal littermates, suggesting an increase in the number of mesenchymal cells committed to osteoblast lineages or an increase in pre-osteoblast cell proliferation and recruitment to the bone surfaces where active osteogenesis takes place [26].

Subsequent studies were performed to examine CTGF's effects on bone formation *in vivo*. A study using a local delivery system in which 1 μg rCTGF was administrated via a single injection into the marrow cavity of the femur revealed that rCTGF-injected femurs had islands of newly formed woven bone within the marrow cavity [8]. These results established

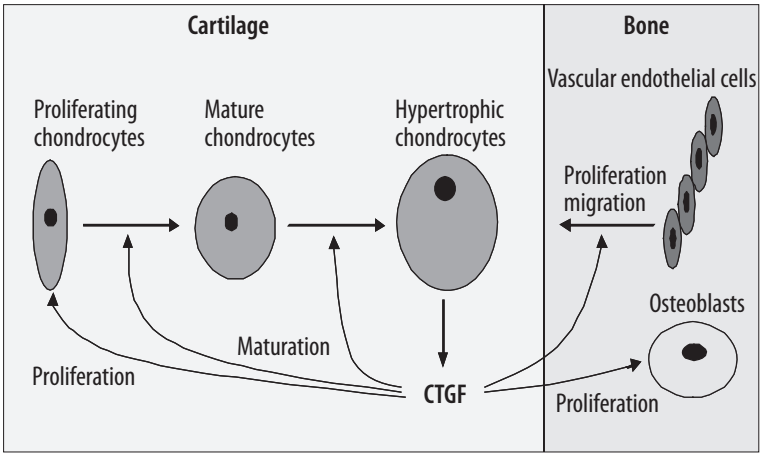

Figure 3. Schematic diagram of CTGF's role in skeletal development. CTGF is maximally produced by hypertrophic chondrocytes and promotes the entire process of endochondral ossification by acting on chondrocytes, osteoblasts, and endothelial cells as a paracrine factor.

CTGF as an osteo-inductive agent *in vivo*. Additional studies to further characterize CTGF's effects were performed on adult Sprague-Dawley rat femurs which were subjected to a controlled mechanical force to induce fracture. Femur sections from fracture callus where osteoblast cell activity (osteogenesis) is at its peak were subjected to immunofluorescent microscopy examination for CTGF expression. This examination revealed high signals in active osteoblasts lining surfaces of newly formed bone within the callus [8]. This is consistent with the finding that CTGF is expressed in periosteal cells and hypertrophic chondrocytes during the healing of experimental bone fracture [38]. It is also consistent with the finding that CTGF mRNA is expressed in osteoblasts and osteocytes localized around periodontal ligaments under control conditions. This expression was found to be more intense following experimental tooth movement, indicating that CTGF could play a role in regulating bone cell function during mechanical stimulation [36].

CTGF deficiency *in vivo* in a mouse knockout model resulted in skeletal dysmorphisms, perhaps due to impaired chondrocyte proliferation and extracellular matrix deposition within the hypertrophic zone. Decreased expression of specific extracellular matrix components (collagen fibers) and matrix metalloproteinases (MMP9) suggested that matrix remodeling within the hypertrophic zones in CTGF mutants was defective [37]. 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 results demonstrate that CTGF is important for cell proliferation and matrix remodeling during chondrogenesis (Figure 3), and is a key regulator coupling extracellular matrix remodeling to angiogenesis at the growth plate [39]. The elevated pattern of CTGF/Hcs24 mRNA expression during distraction osteogenesis suggests that CTGF/Hcs24 may play some roles in the endochondral and intramembranous ossification processes that occur during distraction osteogenesis [40]. Detecting the expression of CTGF gene in mouse embryo at day 12 (E12) in the cartilage proper just condensing from mesenchym suggested a role for CTGF in mesenchymal cell condensation [41]. Condensation is a critical phase that precedes differentiation [42]. Recent *in vitro* studies showed CTGF is involved in this process [43]. CTGF also regulates signaling through the Wnt pathway, in accord with its ability to bind to the Wnt co-receptor LDL receptor-related protein 6 (LRP6). This interaction is likely to occur through the C-terminal (CT) domain of CTGF, which is distinct from the BMP- and TGFβ-interacting domain [44]. All the above findings, in addition to the well-known role of CTGF in angiogenesis, suggest that CTGF promotes the process of skeletal development and repair by acting on mesenchymal cells, chondrocytes, osteoblasts, and endothelial cells (Figure 3).

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