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Connective Tissue Growth Factor (CTGF/CCN2) Negatively Regulates BMP-2 Induced Osteoblast Differentiation and Signaling

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Abstract

Connective tissue growth factor (CTGF/CCN2) and bone morphogenetic protein (BMP)-2 are both produced and secreted by osteoblasts. Both proteins have been shown to have independent effects in regulating osteoblast proliferation, maturation and mineralization. However, how these two proteins interact during osteoblast differentiation remains unknown. In this study, we utilized two cell culture model systems, osteoblasts derived from CTGF knockout (KO) mice and osteoblasts infected with an adenovirus which over-expresses CTGF (Ad-CTGF), to investigate the effects of CTGF and BMP-2 on osteoblast development and function *in vitro*. Contrary to a previously published report, osteoblast maturation and mineralization were similar in osteogenic cultures derived from KO and WT calvaria in the absence of BMP-2 stimulation. Interestingly, in KO and WT osteoblast cultures stimulated with BMP-2, the KO osteoblasts exhibited enhanced osteoblast differentiation. This increase in osteoblast differentiation was accompanied by increased protein levels of phosphorylated Smad 1/5/8 and mRNA expression levels of bone morphogenetic protein receptor Ib. We also examined osteoblast differentiation in cultures that were infected with an adenoviral-CTGF vector (Ad-CTGF) and in controls. Continuous over-expression of CTGF resulted in decreased osteoblast maturation and mineralization in both unstimulated and BMP-2 stimulated cultures. Impaired osteoblast differentiation in cultures over-expressing CTGF was accompanied by decreased protein levels of phosphorylated Smad 1/5/8. Collectively, the data from these studies demonstrate that CTGF acts to negatively regulate BMP-2 induced signaling and osteoblast differentiation, and warrant additional studies to determine the precise mechanism(s) responsible for this effect.

Keywords

CTGF/CCN2; BMP-2; BMP signaling pathway; Osteoblast maturation and mineralization

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Introduction

Osteoblast differentiation is a complex process, which involves the commitment of mesenchymal cells to osteoblasts followed by synthesis and deposition of bone matrix proteins. The differentiation of pre-osteoblasts to matrix-secreting osteoblasts encompasses three phases: 1) proliferation, 2) maturation, and 3) mineralization (Neve et al., 2011). These phases require the appropriate expression of several osteoblast markers, including runt-related transcription factor 2 (*Runx2*), osterix, alkaline phosphatase (*Alp*), collagen type I alpha 1 (*Col1a1*) and osteocalcin (*Oc*) to ensure proper osteoblast development and function. This process is highly regulated by local factors such as bone morphogenetic proteins (BMPs). BMP-2 has been widely studied and is known to induce bone formation *in vivo* and promote osteoblast differentiation *in vitro* (Harris et al., 1995; Wang et al., 1990; Yamaguchi et al., 1991). The mechanism by which BMP-2 promotes osteogenesis is well understood. BMP-2 exerts its effects through the canonical Smad pathway involving Smads 1, 5, and 8, which are phosphorylated by serine/threonine kinase receptors composed of type I and II components. Once activated, Smads 1, 5, and 8 form a complex with Smad 4, which translocates to the nucleus and induces the transcription of target genes, such as *Runx2* (Cao and Chen, 2005; Phimpilai et al., 2006).

In addition to BMP-2, connective tissue growth factor (CTGF) has been shown to regulate osteogenesis. CTGF is expressed and secreted by osteoblasts during bone formation and fracture healing (Safadi et al., 2003). CTGF knockout (KO) mice exhibit skeletal dysmorphisms due to defects in chondrogenesis and extracellular matrix production (Ivkovic et al., 2003). In a study in which osteoblasts derived from these KO mice exhibited delayed osteoblast maturation and mineralization in culture, suggesting that CTGF is essential for normal osteoblast differentiation (Kawaki et al., 2008). Our lab conducted an in-depth characterization of CTGF KO mice and found these mice to exhibit numerous site-specific defects in the axial, appendicular, and craniofacial skeleton (Lambi et al., 2012). Interestingly, we found that mRNA expression of *Runx2*, *Alp*, and *Oc* differed depending on the skeletal site (Lambi et al., 2012). In addition to the CTGF KO model, others have generated transgenic models in which CTGF is over-expressed under the control of different promoters (Nakanishi et al., 2001; Smerdel-Ramoya et al., 2008). *In vivo* over-expression of CTGF resulted in osteopenic mice, and osteoblasts derived from these mice exhibited decreased *Alp* and *Oc* gene expression (Smerdel-Ramoya et al., 2008). On the contrary, *in vitro* studies have shown that the addition of recombinant CTGF to osteoblast cultures promotes the proliferation and differentiation of osteoblasts (Kawaki et al., 2011; Kawaki et al., 2008; Nishida et al., 2000; Safadi et al., 2003). Collectively, these studies support a role for CTGF as a regulator of osteoblast development and function, but the precise mechanisms whereby CTGF regulates osteoblast differentiation remain unknown.

CTGF is a matricellular protein that interacts with several growth factors, including BMPs. Studies revealed that CTGF interacts with BMP-2 and BMP-4 through its von-Willebrand type C and C-terminal domains (Abreu et al., 2002; Maeda et al., 2009). More specifically, when CTGF interacts with BMP-4, CTGF sequesters BMP-4 thereby preventing the ligand from binding to its cognate receptor, BMPR-1a, thus inhibiting BMP signaling (Abreu et al., 2002). Osteoblasts that over-express CTGF have decreased protein levels of phosphorylated Smad (p-Smad) 1/5/8, suggesting that CTGF regulates the BMP signaling pathway (Smerdel-Ramoya et al., 2008). However, the functional significance of the interaction between CTGF and BMP-2 during osteoblast differentiation *in vitro* has not been studied. Based on the previous literature, we hypothesize that CTGF acts as a negative regulator for BMP signaling during osteoblast differentiation *in vitro*. In this study, we investigated and characterized the interaction between CTGF and BMP-2 during osteoblast differentiation using two cell culture models; one in which endogenous CTGF expression is absent

(osteoblasts derived from CTGF KO mice) and the other in which CTGF is over-expressed (osteoblasts infected with an adenovirus-CTGF vector). Our findings support a mechanism in which CTGF negatively regulates BMP-2 signaling and subsequent osteoblast differentiation.

Material and Methods

Source of Animals

CTGF heterozygous mice (CTGF^{+/LacZ}) were used as breeders to obtain CTGF KO (CTGF^{LacZ/LacZ}) mice as previously described (Crawford et al., 2009). Genotype was determined as previously described (Crawford et al., 2009). All animals were maintained and used according to the principles in the NIH Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, Publ. No. 86-23, 1985) and guidelines established by the IACUC of Temple University.

Primary Osteoblast Cell Culture

Embryonic day 18.5 embryos were isolated and individually genotyped to identify CTGF wild-type (WT) and knockout (KO) embryos. Primary cells were isolated from parietal calvaria of WT and KO embryos from which the cranial sutures were removed to reduce non-osteoblast cell contamination. Calvaria pieces were placed in digestion media consisting of 0.1% Collagenase (Sigma)/2.5% trypsin and subjected to a series of digestions of 5, 15, 30, 20, 15, and 15 minutes at 37°C. The purpose of the first digestion is to remove non-osteoblastic cells and this digestion was discarded. The osteoblast cell population was obtained from the remaining digestions. Cells were plated in 100 mm dishes (Corning) at 5×10^5 cells/plate in Alpha Minimal Essential Medium (α -MEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; HyClone). The cells were incubated at 37°C with 5% CO₂ with a change of media every three days until they reached 80% confluence. For experiments, cells were cultured in osteogenic media containing 50 μ g/ml ascorbic acid (Sigma) and 10 mM β -glycerophosphate (Sigma) in addition to α -MEM/10% FBS to stimulate osteoblast differentiation. The purity of the WT and KO cultures was confirmed by Western blot and quantitative PCR (qPCR) to ensure that there was no cross contamination between WT and KO cells.

Adenoviral CTGF infection of primary osteoblasts

Primary cells were isolated from parietal calvaria of postnatal day 3 rats as described in the preceding paragraph. For experiments, cells were infected with a recombinant CTGF adenovirus tagged with a green fluorescent protein (GFP) reporter dye (Ad-CTGF) at Day 1 after plating and Day 3 at the start of treatment with 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate in α -MEM/2% FBS to stimulate osteoblast differentiation. The CTGF adenovirus was generated and provided by Dr. Tong-Chuan He (He et al., 1998). Controls included uninfected cells and cells infected with adenovirus tagged with GFP only.

BMP-2 Stimulation

Recombinant BMP-2 (R&D Systems) was reconstituted to a concentration of 10 μ g/ml in sterile 4 mM HCl containing 0.1% BSA and stored at -20°C. Cells were treated with a standard concentration of 100 ng/ml of BMP-2 every three days during osteoblast differentiation. For evaluating levels of phosphorylated Smad 1/5/8, WT and KO cells or Control and Ad-CTGF infected osteoblasts were serum starved for 24 hours then stimulated with BMP-2 for 0-60 minutes. For evaluating BMP receptor levels, WT and KO cells were stimulated with BMP-2 for 0 and 8 hours.

Cell Proliferation

Cell number was determined using the CyQUANT[®] NF Cell Proliferation Assay Kit (Molecular Probes) according to the manufacturer's protocol. Briefly, CTGF WT and KO osteoblasts were plated at 2.5×10^3 cells/well in a 96 well plate (Falcon) in α -MEM/10% FBS. On Days 3 and 7 media was aspirated and replaced with DNA binding dye solution. Cells were incubated at 37°C for 1 hour and samples were measured using a Wallac 1420 fluorometer. Cell number was calculated based on a standard curve generated for primary osteoblasts. For Control and Ad-CTGF infected osteoblasts, cell number was determined using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's protocol. Briefly, Control and Ad-CTGF infected osteoblasts were plated at 4×10^3 cells/well in a 96 well plate in α -MEM/10% FBS. On Day 3 20 μ l/well of CellTiter 96[®] AQueous One Solution Reagent was added to the media. After 1 hour at 37°C, the absorbance at 490nm was recorded using a microplate reader (Bio-rad).

Alkaline Phosphatase Staining and Activity

CTGF WT and KO cells and Control and Ad-CTGF cells were plated in a 48-well plate (Corning) at 1.1×10^4 cells/well and 24-well plate (Corning) at 1×10^4 cells/well, respectively in osteogenic media, which was changed every three days. The osteoblast cultures were stopped at Days 7 and 14 to evaluate the production of alkaline phosphatase. Alkaline phosphatase staining was performed using the Leukocyte Alkaline Phosphatase Kit (Sigma). Cells were washed with 1X HBSS and fixed in a citrate buffer containing acetone and formaldehyde. Following fixation, cells were washed with ddH₂O and incubated with a staining solution consisting of equal parts of sodium nitrite solution, FRV-alkaline solution and naphthol AS-BI alkaline solution for 25 minutes at room temperature. Cells were washed with ddH₂O and allowed to air dry. Wells were observed and images taken using the Nikon Eclipse TE300 inverted microscope. Quantification of alkaline phosphatase production was determined using Quantichrom[™] Alkaline Phosphatase Assay Kit (BioAssays Systems). Cells were lysed in ddH₂O water containing 0.2% Triton X-100 for 20 minutes at room temperature. Cell lysates were collected and the assay was carried out according to the manufacturer's protocol. Alkaline phosphatase activity was normalized to total protein content. Total protein content was determined using BCA protein assay kit (Pierce) on identical cultures.

Alizarin Red Staining

CTGF WT and KO and Control and Ad-CTGF osteoblast cultures were stopped at Day 21 to evaluate mineralization. For alizarin red staining, cells were washed in 1X HBSS and fixed in 10% paraformaldehyde for 15 minutes at room temperature. Cells were washed with 1X PBS and stained with 40 mM Alizarin Red S (Sigma) for 15 minutes at room temperature. Cells were washed with ddH₂O and allowed to air dry. Wells were observed and images taken using the Nikon Eclipse TE300 inverted microscope.

RNA Isolation and Quantitative PCR

Total RNA was isolated from CTGF WT and KO osteoblasts and Control and Ad-CTGF infected osteoblasts at Days 7, 14, and 21. To evaluate changes in BMP receptor levels, CTGF WT and KO osteoblasts were serum starved for 24 hours, stimulated with BMP-2 (100 ng/ml), and RNA was collected at 0 and 8 hours. RNA was isolated from cell cultures using Trizol reagent (Invitrogen) according to the manufacturer's directions. RNA was purified using the RNeasy Mini Kit (Qiagen) and treated with DNase using the RNase-Free DNase Kit (Qiagen). RNA quality and quantity was determined using spectrophotometry and the integrity of all samples was confirmed using 1% formaldehyde-agarose gel. For cDNA synthesis, 1 μ g of cDNA was transcribed from total RNA using SuperScript[®] First-

Strand Synthesis System (Invitrogen). Gene expression for connective tissue growth factor (*CTGF*), runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (*Alp*), collagen type I alpha 1 (*Col1a1*), osteocalcin (*Oc*), bone morphogenetic protein receptor Ia (*BMPRIa*), and bone morphogenetic protein receptor Ib (*BMPRIb*) was determined by qPCR, as described previously (Lambi et al., 2012). The primer information for the abovementioned genes has been previously published (Lambi et al., 2012; Pache et al., 2006).

Protein Isolation and Western Blotting

CTGF WT and KO cell monolayers were washed in cold 1X PBS and lysed in 1X RIPA (Millipore) for 1 hour at 4°C. Cell lysates were centrifuged and the resultant supernatants were used for determination of protein concentration using BCA Protein Assay Kit (Pierce). Western blot was performed as previously described (Safadi et al., 2003). The membrane was blocked with 5% BSA/1X PBS-Tween20 for 1 hour and incubated with the following primary antibodies: anti-goat CTGF (1:100; Santa Cruz), anti-rabbit phospho-Smad (p-Smad) 1/5/8 (1:1000; Cell Signaling), anti-rabbit Smad 1/5/8 (1:250; Santa Cruz Biotechnology) and anti-rabbit actin (1:1000; Sigma) overnight at 4°C. The membrane was washed with 1X PBS-Tween 20 and incubated with horseradish-peroxidase conjugated donkey anti-rabbit (1:10,000; Jackson ImmunoResearch) for 1 hour at room temperature. The membrane was washed again, incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and exposed to film. Quantification of the bands was done using Image J software.

Control and Ad-CTGF cell monolayers were prepared for Western blot as above with the following changes. The membrane was blocked using Odyssey blocking buffer (LI-COR) for 1 hour and incubated with the following primary antibodies: anti-goat CTGF (1:100; Santa Cruz), anti-rabbit phospho-Smad (p-Smad) 1/5/8 (1:1000; Cell Signaling), anti-rabbit Smad 1 (1:1000; Cell Signaling), and anti-rabbit actin (1:1000; Sigma) overnight at 4°C. After washing, the membrane was incubated with donkey anti-goat or anti-rabbit IRDye® 800CW (1:5000; LI-COR). After washing, the membrane was scanned using the LI-COR Odyssey Infrared Imaging System. Quantification of the bands was done using Image J software.

Statistical Analysis

Unpaired Student's t test was used to determine whether the absence or over-expression of CTGF had any effect on quantitative parameters related to osteoblast proliferation, maturation, and mineralization compared to WT and Control cultures. Data are expressed as mean $\pm$ SEM. A P-value < 0.05 was considered statistically significant.

Results

CTGF KO osteoblasts display decreased cell proliferation but normal maturation and mineralization in vitro

To confirm the purity of the osteoblasts cultures, we assessed CTGF protein and mRNA expression levels from CTGF WT and KO osteoblast cultures. As shown in Figure 1A, CTGF expression was undetectable at the protein and mRNA level in the CTGF KO osteoblast cultures. To determine whether the absence of CTGF affects osteoblast proliferation, we examined the proliferative capacity of WT and KO osteoblasts at Days 3 and 7. Three days after plating, the number of KO osteoblasts was significantly less compared to WT osteoblasts. However, by Day 7 there was no difference in cell number between WT and KO osteoblasts (Fig. 1B), suggesting that with time the KO osteoblasts reach confluence. To investigate the role of CTGF during osteoblast differentiation, we cultured primary WT and KO osteoblasts for a period of 21 days. Alkaline phosphatase

(ALP) is present on the surface of osteoblasts and is an early marker for osteoblast differentiation. When we evaluated osteoblast maturation at Day 14 by ALP staining and activity, there was no difference between WT and KO cultures (Fig. 1C). Next, we examined osteoblast mineralization at Day 21 by alizarin red staining. In both WT and KO cultures, osteoblasts started to aggregate, which was demonstrated by an increase in alizarin red staining at sites of the cell aggregations. There was no delay in osteoblast mineralization in the KO cultures, which were similar in appearance to the WT cultures (Fig. 1D).

Runx2 is an essential transcription factor for osteoblast differentiation and is known to up-regulate other critical genes important for later stages of osteoblast differentiation, such as *Colla1* and *Oc* (Ducy et al., 1997). Therefore, we measured *Runx2* mRNA expression in WT and KO cultures at Day 7. Although the KO cultures showed an increase in *Runx2*, it was not significant. *Colla1* and *Oc* are important for the maturation and mineralization phases, respectively, with *Colla1* gene expression peaking at Day 14 and *Oc* peaking at Day 21. In the KO osteoblast cultures, *Colla1* and *Oc* mRNA expression was similar when compared to WT cultures (Fig. 1E). Contrary to previous reports, these data demonstrate that osteoblasts differentiate under normal osteogenic culture conditions in the absence of endogenous CTGF production (KO osteoblasts).

CTGF KO osteoblasts exhibit enhanced differentiation in response to BMP-2

BMP-2 is a well-known osteoinductive agent both *in vivo* and *in vitro* (Harris et al., 1995; Wang et al., 1990; Yamaguchi et al., 1991). CTGF interacts with BMPs (Abreu et al., 2002; Maeda et al., 2009), but it is unknown how CTGF and BMP-2 interact during osteoblast differentiation. To examine this interaction, we treated osteoblast cultures with recombinant BMP-2 for 21 days. Interestingly, ALP staining at Day 14 revealed the presence of aggregates or nodules in the KO cultures, which were not seen in the WT cultures, indicative of enhanced osteoblast maturation. The staining was accompanied with an increase in ALP production (Fig. 2A). Alizarin red staining at Day 21 showed the presence of small, mineralized nodules in WT cultures, whereas the KO cultures displayed a greater number of nodules, which were fused with one another to form larger mineralized nodules (Fig. 2B). In addition, *Runx2*, *Colla1* and *Oc* mRNA expression levels were significantly up-regulated in CTGF KO cultures (Fig. 2C). These data demonstrate that osteoblast differentiation is accelerated and enhanced in response to exogenous BMP-2 in KO compared to WT cultures.

Increased BMP signaling in CTGF KO osteoblasts

Since the BMP-2 ligand binds to the heteromeric complex composed of type II and I BMP receptors (Cao and Chen, 2005), we investigated the expression of these receptors. We stimulated WT and KO cultures with BMP-2 for 8 hours, and evaluated changes in mRNA expression of BMP receptors. In unstimulated conditions (time 0) there were no significant differences in *BMPR-Ia* and *BMPR-Ib* expression between WT and KO osteoblasts. Upon stimulation with BMP-2, *BMPR-Ia* levels decreased 0.5- and 0.75-fold in WT and KO osteoblasts, respectively (Fig. 3A). When WT and KO osteoblasts were treated with BMP-2 and assessed for *BMPR-Ib* expression levels, *BMPR-Ib* levels increased by 10- and 35-fold in WT and KO osteoblasts, respectively. The BMP-2 stimulated increase in *BMPR-Ib* was significantly greater in KO compared to WT osteoblasts (Fig. 3A). There were no significance differences in *BMPR-II* expression levels between WT and KO osteoblasts in either unstimulated or BMP-2 stimulated conditions (data not shown). Since Smads 1, 5, and 8 are important mediators of BMP signaling, we investigated whether the enhanced osteoblast differentiation seen in the BMP-2 stimulated CTGF KO cultures could be attributed to an increase in p-Smad 1/5/8 levels. We treated WT and KO osteoblasts with BMP-2 for various periods of time up to 60 minutes. The p-Smad 1/5/8 protein levels demonstrated a temporal increase with BMP-2 stimulation in both WT and KO osteoblasts,

yet p-SMAD 1/5/8 levels were significantly greater in KO compared to WT osteoblasts at each of the time points (Fig. 3B, C; top panel). Interestingly, total Smad 1/5/8 protein levels were also increased in KO osteoblasts (Fig. 3B, C; bottom panel). These data demonstrate that KO osteoblasts exhibit increased activation of Smad signaling in response to BMP-2, and suggest that BMPR-Ib is the prominent receptor required for BMP-induced signaling in osteoblast cultures.

Over-expression of CTGF results in impaired osteoblast maturation and mineralization *in vitro*

To determine the effect of over-expression of CTGF on osteoblast differentiation, we infected subconfluent rat primary calvarial osteoblasts with either Ad-GFP (control) or Ad-CTGF-GFP (Ad-CTGF). We also used uninfected cells as additional controls. Since there were no significant differences in osteoblast differentiation between the Ad-GFP infected or uninfected cells, we choose to use the data from the Ad-GFP control to compare with the adenovirus infected cells over-expressing CTGF (Ad-CTGF) and tagged with GFP as well. CTGF over-expression was verified throughout the stages of osteoblast differentiation by examining CTGF protein levels at specified time points (Fig. 4A, B). Additionally, the infection efficiency at Day 14 remained robust, thereby allowing us to examine the effects of CTGF over-expression during the entire process of osteoblast differentiation (Fig. 4C). To investigate the effect of CTGF over-expression on proliferation, we performed a MTT assay at Day 3 of culture. Ad-CTGF osteoblast cultures displayed an increase in cell number compared to control cultures, suggesting that CTGF over-expression enhances osteoblast proliferation (Fig. 5A). Next, we evaluated the effect of CTGF over-expression on osteoblast maturation at Days 7 and 14. In the control osteoblast cultures, the intensity of ALP staining and production increased from Day 7 to Day 14. However, Ad-CTGF osteoblasts exhibited significantly decreased ALP staining and activity (Fig. 5B), as well as a decrease in mRNA expression of *Runx2*, *Alp*, and *Col1a1* compared to control osteoblasts (Fig. 5C). Finally, we evaluated the effect of CTGF over-expression on osteoblast mineralization. The control osteoblast cultures displayed well-formed, packed nodules, which stained intensely with alizarin red, and the cells surrounding the nodules were densely packed (Fig. 6A). In contrast, the Ad-CTGF osteoblast cultures revealed an absence of nodules with non-specific staining in the matrix and decreased *Oc* mRNA expression (Fig. 6A, B). When we treated these cultures with BMP-2, the Ad-CTGF infected osteoblasts exhibited the same degree of inhibition of maturation and mineralization as observed in the non-BMP-2 treated Ad-CTGF osteoblast cultures (data not shown). These results demonstrate that the continuous over-expression of CTGF adversely affects osteoblast differentiation *in vitro*, and it completely blocked the stimulation of osteoblast differentiation normally observed upon addition of exogenous BMP-2 to the cultures.

Over-expression of CTGF decreases BMP Signaling in osteoblasts

Since the CTGF KO osteoblasts displayed increased levels of p-Smad 1/5/8 in response to BMP-2, we wanted to investigate how the over-expression of CTGF in osteoblasts affects p-Smad 1/5/8 protein levels when stimulated with BMP-2. When we stimulated the control and Ad-CTGF osteoblasts with BMP-2 for 0 to 60 minutes, both the control and Ad-CTGF osteoblasts showed a time-dependent increase in p-Smad 1/5/8 levels (Fig. 7A, B). However, at each time point, the p-Smad 1/5/8 levels in the Ad-CTGF osteoblasts were significantly lower compared to control osteoblasts. Unlike the KO osteoblasts, there were no significant differences in total Smad 1 protein levels between the control and Ad-CTGF osteoblasts. These data confirm the effect of CTGF as a negative regulator of BMP-2 induced signaling and osteoblast differentiation.

Discussion

CTGF interacts with numerous growth factors and matrix proteins to regulate various cellular processes such as migration, adhesion, proliferation, and differentiation (Arnott et al., 2011). These processes are important for proper osteoblast development and function. Previous studies have shown that CTGF interacts with BMP-4 and BMP-2. One group demonstrated that CTGF interacts with BMP-4 and prevents BMP-4 from binding to its receptor, BMPR-1a, thereby inhibiting the action of BMP-4 during embryonic patterning (Abreu et al., 2002). Another group demonstrated that CTGF interacts with BMP-2 and together, these proteins can modulate chondrocyte proliferation and differentiation (Maeda et al., 2009). Both proteins are produced and secreted by osteoblasts, and have independent effects in regulating osteoblast proliferation, maturation and mineralization. However, the functional impact of CTGF binding to BMP-2 during osteoblast differentiation has yet to be explored. In this study, we compared the differentiation of CTGF KO and WT osteoblasts, as well as Ad-CTGF infected and control osteoblasts to investigate the effects of CTGF and BMP-2 on osteoblast development and function *in vitro*.

During the first phase of osteoblast differentiation, the cells actively proliferate in culture and express cell cycle and cell growth regulated genes. When cells come into contact with each other, they undergo cell contact inhibition, which initiates arrest of cell growth and start of differentiation (Stein et al., 1990). Our data shows that WT osteoblasts proliferate more than KO osteoblasts at Day 3, yet by Day 7, there is no significant difference in cell number between WT and KO cultures (Fig. 1A). Additional studies investigating the expression levels of Ki67 or proliferating cell nuclear antigen (PCNA) will confirm that proliferation is indeed affected early on in the CTGF KO cultures. Although KO osteoblasts initially proliferate more slowly than WT, the KO cultures are able to reach confluence by Day 7 and thereafter differentiation proceeds normally.

The second and third phases of osteoblast differentiation are matrix production/maturation and mineralization, respectively. Following cell cycle arrest, the osteoblasts undergo a series of morphological changes and temporally express *Alp*, *Colla1*, and *Oc*, all of which are necessary for the synthesis of a mineralized bone matrix (Stein et al., 1990). We evaluated these two phases in osteoblast cultures derived from CTGF WT and KO embryos. Under normal osteogenic culture conditions that were not stimulated with exogenous BMP-2, there was no difference in ALP staining and activity, alizarin red staining, or the mRNA expression of osteoblast markers when comparing WT and KO osteoblast cultures (Fig. 1B-D). These results are contradictory to what has been previously published regarding the differentiation of osteoblasts derived from CTGF KO mice. This previous study showed a significant reduction in the differentiation of CTGF KO compared to WT osteoblasts (Kawaki et al., 2008). One possible explanation for the difference in our results from those previously published may be related to cell plating densities and the time points for assessing differentiation parameters in the two studies. The authors of the previous study used a significantly lower cell plating density than that was used in this study and they assessed differentiation parameters during a shorter culture period. Interestingly, when we plated WT and KO osteoblasts at half of our normal cell density which is still higher than that used in the Kawaki et al. study, the KO osteoblasts showed less ALP activity at Day 14 than WT, but by Day 21 the levels of ALP activity in WT and KO cultures were comparable (data not shown). Our results demonstrate that CTGF is not essential for osteoblast differentiation under normal osteogenic culture conditions.

However, when we treated WT and KO osteoblast cultures with BMP-2, the KO osteoblasts exhibited a markedly accelerated differentiation. It is well documented that Runx2 is required for osteoblast differentiation (Komori et al., 1997; Otto et al., 1997). This essential

transcription factor is expressed throughout osteoblast differentiation and it up-regulates several downstream genes necessary for later stages of osteoblast differentiation, such as *Colla1* and *Oc* (Ducy et al., 1997; Yang et al., 2003). Interestingly, studies have shown that BMPs up-regulate *Runx2* mRNA expression and inhibition of BMP signaling disrupts the ability of Runx2 to stimulate osteoblast differentiation *in vitro* (Phimphilai et al., 2006). Our data show that upon stimulation with BMP-2, *Runx2* mRNA expression levels were significantly up-regulated in KO osteoblast cultures, which resulted in enhanced osteoblast maturation and mineralization compared to WT osteoblast cultures (Fig. 2).

Since the BMP-2 ligand binds to a heteromeric complex composed of type I and II BMP receptors, we evaluated the mRNA expression of the receptors. Addition of BMP-2 into WT and KO osteoblast cultures decreased the expression of BMPR-1a, suggesting that BMPR-1a does not play an important role during BMP-2 induced osteoblast differentiation. This finding is also consistent with a previous study, in which BMP-2 treatment of 2T3 cells, an osteoblast cell line, resulted in decreased expression of BMPR-1a during osteoblast differentiation (Chen et al., 1998). On the contrary, previous studies have shown that BMPR-1b does play a critical role in osteoblast differentiation. Primary osteoblasts derived from transgenic mice over-expressing a truncated dominant negative BMPR-1b under the type I collagen promoter exhibited impaired osteoblast differentiation due to inhibition of BMP signaling (Chen et al., 1998; Zhao et al., 2002). We show that BMPR-1b is significantly up-regulated after treatment with BMP-2 in KO osteoblast cultures compared to WT osteoblast cultures (Fig. 3A). When we evaluated the levels of p-Smad 1/5/8 in WT and KO osteoblast cultures, the KO osteoblasts displayed increased levels of p-Smad 1/5/8, demonstrating increased BMP signaling (Fig. 3B, C). Taken together, these findings suggest that in the absence of endogenous CTGF there is an increase in BMP signaling that is mediated through BMPR-1b, which activates Smads 1,5, and 8, resulting in up-regulation of *Runx2* levels and enhanced or accelerated osteoblast differentiation.

To further confirm our hypothesis that CTGF negatively regulates BMP signaling during osteoblast differentiation, we over-expressed CTGF in primary osteoblasts using a recombinant CTGF adenovirus. Consistent with previous findings of Smerdel-Ramoya and colleagues (2008), our data demonstrate that a continuous exposure to increased CTGF has an inhibitory effect on osteoblast maturation and mineralization, an effect that is mediated by a decrease in BMP signaling (Fig. 6 and 7). In addition, when Ad-CTGF infected osteoblasts were treated with exogenous BMP-2, the elevated levels of CTGF prevented the stimulation of osteoblast differentiation that is normally induced by this treatment. The continuous over-expression of CTGF has an effect that appears to be contrary to that previously observed and published by our lab and others, namely that intermittent treatment of primary osteoblast cultures with CTGF has an anabolic effect on osteoblast differentiation (Nishida et al., 2000; Safadi et al., 2003). One explanation for this discrepancy is the duration and level of exposure to CTGF in these osteoblast cultures. Similar effects have been reported in osteoblast cultures treated with parathyroid hormone (PTH). Studies have shown that cells treated intermittently with PTH exhibited an increase in several osteoblastic bone parameters, such as ALP activity, calcium content, and numbers of bone nodules, whereas continuous treatment with PTH resulted in a significant decrease in these same parameters (Ishizuya et al., 1997; Schiller et al., 1999). Our data support the concept of differential effects of CTGF on osteoblast differentiation depending on the duration (continuous versus intermittent) and relative levels (pharmacologic versus physiologic) of exposure during the culture period. Nonetheless, the results of this study support a role for CTGF in regulating osteoblast differentiation through the BMP signaling pathway.

In conclusion, our findings clearly demonstrate, through functional studies, a novel function of CTGF as a negative regulator of BMP-2 induced osteoblast signaling and differentiation.

These findings are consistent with earlier studies suggesting that CTGF can act to inhibit BMP-2 and BMP-4 by preventing the ligands from binding to its cognate receptor. Based on the observations made in the current study, we propose a model (Fig. 8) in which CTGF sequesters BMP-2 and thereby limits the BMP-2 that is available to bind to and signal through its receptor, BMPR-1b, in osteoblasts. (A) Under normal conditions, endogenous CTGF binds and sequesters some of the bioavailable BMP-2 thus preventing it from binding to its receptor and stimulating osteoblast differentiation. (B) In CTGF KO osteoblasts, where this inhibitory effect is absent, all of the bioavailable BMP-2 binds to its receptor thereby increasing Smad signaling and enhancing osteoblast differentiation. (C) When CTGF is present in excess, such as in the Ad-CTGF infected osteoblasts, a much smaller pool of BMP-2 is available to bind to its receptor thereby decreasing Smad signaling and inhibiting osteoblast differentiation. Since the levels of BMPR-1b are also affected by CTGF, this model warrants further investigation. Future studies will address the following questions: 1) How does endogenous production of CTGF regulate BMP-2 bioavailability and/or receptor levels and 2) how does endogenous production of CTGF affect *Runx2* promoter activity? These additional studies will offer more comprehensive insights as to how CTGF negatively regulates BMP signaling during osteoblast differentiation.

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Figure 1. CTGF KO osteoblasts display decreased proliferation but normal differentiation *in vitro*

(A) Representative Western blot of CTGF protein levels and quantitative PCR of CTGF mRNA expression in CTGF WT and KO osteoblasts cultures. (B) Proliferation of CTGF WT and KO osteoblasts was assessed using Cyquant Proliferation assay on Days 3 and 7. Cell numbers were based on fluorescence at 520nm. (C) ALP staining of osteoblast cultures at Day 14. ALP activity quantified at Day 14 of culture. Activity was normalized to total protein content. (D) Alizarin Red S Staining of osteoblast cultures at Day 21. (E) mRNA gene expression of *Runx2* at Day 7, *Colla1* at Day 14, and *Oc* at Day 21. * $P < 0.05$; **** $P < 0.0001$. Scale bar: 50 μm . Abbreviations include alkaline phosphatase (ALP), runt-related transcription factor 2 (*Runx2*), collagen type I alpha 1 (*Colla1*) and osteocalcin (*Oc*). Experiments were repeated six times with similar results.

Figure 2. CTGF KO osteoblasts exhibit enhanced maturation and mineralization in the presence of BMP-2

(A) ALP staining of osteoblast cultures at Day 14. ALP activity quantified at Day 14 of culture. Activity was normalized to total protein content. (B) Alizarin Red S Staining of osteoblast cultures at Day 21. (C) mRNA gene expression of *Runx2* at Day 7, *Colla1* at Day 14 and *Oc* at Day 21. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Scale bar: 50 μm .

Abbreviations include alkaline phosphatase (ALP), runt-related transcription factor 2 (*Runx2*), collagen type I alpha 1 (*Colla1*) and osteocalcin (*Oc*). Experiments were repeated three times with similar results.

Figure 3. BMP signaling is increased in CTGF KO osteoblasts

(A) mRNA expression levels of *BMPR-Ia* and *BMPR-Ib* in the absence and presence of BMP-2. (B) Representative Western blot analysis of p-Smad 1/5/8 and total Smad 1/5/8 protein levels using 15 μ g total protein from WT and KO osteoblast cultures treated with BMP-2 for 0 to 60 minutes. (C) p-Smad 1/5/8 levels and total Smad 1/5/8 levels were normalized to actin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Abbreviations include bone morphogenetic protein receptor-Ia (*BMPR-Ia*) and bone morphogenetic protein receptor-Ib (*BMPR-Ib*). Experiments were repeated three times with similar results.

Figure 4. Over-expression of CTGF throughout osteoblast differentiation *in vitro*
(A) Representative Western blot analysis of CTGF protein levels at different time points during osteoblast differentiation for control and Ad-CTGF infected osteoblasts. **(B)** CTGF levels were normalized to actin. **(C)** Infection efficiency of osteoblasts over-expressing CTGF-GFP in culture at Day 14. Scale bar: 50 μ m.

Figure 5. Over-expression of CTGF increases osteoblast proliferation but inhibits maturation (A) Cell proliferation was assessed using the colorimetric MTT assay at Day 3 for control and Ad-CTGF infected osteoblasts. (B) ALP staining and ALP quantification of osteoblast cultures at Days 7 and 14. Activity was normalized to total protein content. (C) mRNA expression of *Runx2*, *Alp*, and *Colla1*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Scale bar: 50 μm . Abbreviations include alkaline phosphatase (ALP, *Alp*), runt-related transcription factor 2 (*Runx2*), collagen type I alpha 1 (*Colla1*). Experiments were repeated three times with similar results.

Figure 6. Over-expression of CTGF inhibits osteoblast mineralization

(A) Alizarin Red S Staining of control and Ad-CTGF infected osteoblast cultures at Day 21.

(B) mRNA expression of *Oc* at Day 21. * $P < 0.05$. Scale bar: 10 μ m. Osteocalcin (*Oc*).

Experiments were repeated three times with similar results.

Figure 7. Over-expression of CTGF decreases BMP signaling in osteoblasts

(A) Representative Western blot analysis of p-Smad 1/5/8 and total Smad 1 protein levels in control and Ad-CTGF infected osteoblasts treated with BMP-2 for 0 to 60 minutes. (B) p-Smad 1/5/8 protein levels were normalized to total Smad 1. Experiments were repeated three times with similar results.

Figure 8. Proposed model of CTGF regulating BMP signaling during osteoblast differentiation
(A) Normal levels of CTGF sequester some of the BMP-2, thereby limiting the amount of BMP-2 ligand that is available to bind to its cognate receptors, BMPRI and BMPRII. Once activated BMPRI activates Smads 1/5/8, which then forms a complex with Smad 4. This complex translocates into the nucleus, where it will bind to other DNA binding proteins, and bind to a region on the Runx2 promoter. This results in Runx2 activation and upregulation of genes critical for osteoblast differentiation. **(B)** In the absence of CTGF, all of the BMP-2 ligand is available to bind to its receptors. This leads to increased promoter activation and Runx2 expression, thus enhancing osteoblast differentiation. **(C)** Excess levels of CTGF sequesters a significantly greater amount of the available BMP-2 ligand, thus inhibiting ligand/receptor interaction and decreasing activation of Smad 1/5/8. Ultimately, this results in decreased osteoblast differentiation.