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Molecular Requirements for Induction of CTGF Expression by TGF- β 1 in Primary Osteoblasts

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Abstract

Connective tissue growth factor (CTGF/CCN2) is a cysteine rich, extracellular matrix protein that acts as an anabolic growth factor to regulate osteoblast differentiation and function. In osteoblasts, CTGF is induced by TGF- β 1 where it acts as a downstream mediator of TGF- β 1 induced matrix production. The molecular mechanisms that control CTGF induction by TGF- β 1 in osteoblasts are not known. To assess the role of individual Smads in mediating the induction of CTGF by TGF- β 1, we used specific Smad siRNAs to block Smad expression. These studies demonstrated that Smads 3 and 4, but not Smad 2, are required for TGF- β 1 induced CTGF promoter activity and expression in osteoblasts. Since the activation of MAPKs (Erk, Jnk and p38) by TGF- β 1 is cell type specific, we were interested in determining the role of individual MAPKs in TGF- β 1 induction of CTGF promoter activity and expression. Using dominant negative (DN) mutants for Erk, Jnk and p38, we demonstrated that the expression of DN-Erk caused a significant inhibition of TGF- β 1 induced CTGF promoter activity. In contrast, the expression of DN-p38 or DN-Jnk failed to inhibit activation of CTGF promoter activity. To confirm the vital role of Erk, we used the Erk inhibitor (PD98059) to block its activation, demonstrating that it prevented TGF- β 1 activation of the CTGF promoter and up-regulation of CTGF expression in osteoblasts. Since Src can also act as a downstream signaling effector for TGF- β in some cell types, we determined its role in TGF- β 1 induction of CTGF in osteoblasts. Treatment of osteoblasts with a Src family kinase inhibitor, PP2, or the expression of two independent kinase-dead Src mutant constructs caused significant inhibition of TGF- β 1 induced CTGF promoter activity and expression. Additionally, blocking Src activation prevented Erk activation by TGF- β 1 demonstrating a role for Src as an upstream mediator of Erk in regulating CTGF expression in osteoblasts. To investigate the involvement of the TGF- β 1 response element (TRE) and the SMAD binding element (SBE) in CTGF induction, we cloned the rat CTGF proximal promoter (−787 to +1) containing the TRE and SBE motifs into a pGL3-Luciferase reporter construct. Using a combination of CTGF promoter deletion constructs and site-directed mutants, we demonstrated the unique requirement of both the TRE and SBE for CTGF induction by TGF- β 1 in osteoblasts. Electro-mobility shift assays using specific probes containing the TRE, SBE or both

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showed TGF- β 1 inducible complexes that can be ablated by mutation of the respective motif, confirming their requirement for TGF- β 1 induced CTGF promoter activity. In conclusion, these studies demonstrate that CTGF induction by TGF- β 1 in osteoblasts involves Smads 3 and 4, the Erk and Src signaling pathways, and requires both the TRE and SBE motifs in the CTGF proximal promoter.

Keywords

Osteoblast; CTGF; TGF- β 1; SMADs; MAPKs; Src

Introduction

Connective tissue growth factor (CTGF/CCN2) is a 38kDa, cysteine rich, extracellular matrix protein that belongs to the CCN family (CTGF, Cyr61, nov, WISP1, WISP2 and WISP3) of immediate early growth-responsive genes [1]. CTGF has been shown to regulate a diverse array of cellular functions including proliferation, migration, adhesion, survival, differentiation and synthesis of ECM proteins in various cell types [1–5]. CTGF has also been implicated as a key regulatory factor in complex biological and pathological processes [6–13]. Studies in several connective tissue cell types have shown that TGF- β 1 is a potent inducer of CTGF expression [14] and that CTGF acts as a downstream mediator of some of the effects of TGF- β 1 on cell proliferation, migration, adhesion and matrix production [15–26].

Recently CTGF was found to play an important role in osteoblast differentiation and osteogenesis [10,13,27–29]. CTGF is produced and secreted by osteoblasts where it acts in an autocrine fashion as an anabolic growth factor to regulate osteoblast differentiation and function [13,28]. We recently demonstrated that CTGF could be induced by TGF- β 1 in primary osteoblasts during their differentiation and that CTGF is a downstream effector of TGF- β 1 induced ECM synthesis, an essential step in this process [25]. However the molecular mechanisms that mediate TGF- β 1 induced synthesis of CTGF in osteoblasts have not been studied.

In general, TGF- β 1 exerts its cellular effects and induces gene expression through Smad and mitogen-activated protein kinase (MAPK) mediated signaling pathways [30]. TGF- β 1 activated Smad transcription factors exist in three subgroups based on their function: receptor activated Smads (Smad 2 and Smad 3), common Smads (Smad 4) and inhibitory Smads (Smad 7). Smads 2 and 3 are phosphorylated by active transmembrane serine/threonine TGF- β 1 receptors [30]. 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 TGF- β 1-responsive genes [31,32]. The inhibitory Smad, Smad 7, controls Smad signaling by preventing phosphorylation and/or nuclear translocation of R-Smads [33–35]. Thus in the context of TGF- β 1 signaling, Smads act as ligand-induced transcriptional regulators through translocation to the nucleus, where they interact with Smad binding elements (SBE) in the promoters of their target genes.

TGF- β 1 signaling is not limited to Smads alone, but can also be mediated by the MAPK pathway. TGF- β 1 can activate MAPKs including, extracellular signal regulated kinase (Erk 1/2), Jun-N-terminal kinase (Jnk 1/2) and p38 [22], and the specific MAPKs that become activated are cell type dependent [36]. Numerous studies have demonstrated evidence for crosstalk between the Smad and MAPK signaling cascades activated by TGF- β 1. MAPKs can potentiate, synergize or antagonize the TGF- β 1/Smad pathway [37,38], and Smads have been shown to mediate the activation of MAPKs [39,40]. Thus Smads and MAPKs cooperate to

achieve TGF- β 1 target gene activation, but this interaction varies in different cell types and has not been examined in primary osteoblasts.

TGF- β 1 induced CTGF gene activation has been attributed to the presence of two cisregulatory elements found in the CTGF promoter: the TGF- β 1 response element (TRE), which is unique to CTGF, and the Smad binding element (SBE) [40,41]. Both the TRE and the SBE have been identified in the CTGF promoter in various species, including human, mouse and rat [42]. The relative contributions of these two cis-regulatory elements in the up-regulation of CTGF expression induced by TGF- β 1 vary in a cell type dependent manner. Previous studies have demonstrated divergent roles for each of these elements in either basal or TGF- β 1 induced regulation of CTGF depending on cell type and the health status (normal versus diseased) of the cells being investigated [40,41]. However, in osteoblasts the role of the SBE and TRE in TGF- β 1 induction of CTGF has not been examined.

Recently, a number of different studies have shown that both the Smad and MAPK signaling pathways are involved in TGF- β induced CTGF promoter activation in some connective tissue cell types [14,43,44]. These studies have demonstrated that the identity of specific signaling components involved in regulating the induction of CTGF by TGF- β 1 varies depending on cell type [41,45]. Since we have previously shown that TGF- β 1 is a potent inducer of CTGF expression and that CTGF mediates the effects of TGF- β 1 on extracellular matrix production in osteoblasts, the purpose of this study is two-fold; 1) to examine the role that the Smad, MAPK and Src signaling pathways play in the induction of CTGF by TGF- β 1 in osteoblasts, and 2) to characterize the contributions of the TRE and SBE in TGF- β 1 induced CTGF promoter activity and expression in osteoblasts.

Materials and Methods

Source of Animals

Primary osteoblasts were derived from bone (calvaria) of neonatal Norway-Hooded rats that are maintained at Temple University School of Medicine (Philadelphia, PA). All animals were handled 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

Primary osteoblast cultures were obtained using neonatal rats as previously described [13,25, 28]. Primary cells were isolated from parietal calvaria from which the periosteum and cranial sutures were removed to reduce non-osteoblast cell contamination. Calvaria pieces were subject to five sequential digestions of 5, 15, 15, 25 and 25 min at 37° C in a shaking water-bath with % collagenase-P (Roche)/ 0.25% trypsin (Mediatec). The first two digestions are performed to remove non-osteoblast cells and are discarded. Osteoblast-enriched cell populations were obtained from the 3rd-5th digestions of the calvarial pieces. These cells were plated in 100mm dishes (Falcon) at 5×10^5 cells/plate in osteogenic media consisting of Earle's Minimal Essential Medium (EMEM; Mediatec) supplemented with 10% fetal calf serum (FCS; Mediatec), 50 μ g/ml ascorbic acid (Sigma) and 10mM β -glycerophosphate (Sigma). The cells were incubated at 37°C with 5% CO₂ with a change of media every three days until they reached 80% confluency. Cells were subcultured under identical conditions for utilization in experiments following the third passage. We have previously shown that these culture conditions result in an enhanced (>90%) population of cells committed to the osteoblast lineage using specific markers of osteoblast differentiation (e.g. Runx2, Osterix) [13,25,46,47].

DNA Constructs and Reagents

CTGF promoter deletion constructs: pGL3-T129, pGL3-S164 and pGL3-314 were created using PCR amplification of CTGF proximal promoter regions with primers flanked with NheI-HindIII and cloned directly into the NheI/HindIII sites of pGL3-Basic (Promega). Three 5' primers - all NheI flanked include: P314 5' TAGCTAGCCCTTGTTCATGTATTTCTAAGTTATATTTC TCAGG-3', S164 5' TAGCTAGCAATGTGGAGTGTCAAGGGGTCAGG-3', T129 5'-TAG CTAGCGTGAGTTGATGAGGCAGGAAGGTGG-3' and one common 3' primer - HindIII flanked: 5'-CTGFPCGAAGCTTGAGACTGGGCGGGTGG-3'. Inserts were confirmed by sequencing. The pGL3-W787 vector was constructed from a fragment of the CTGF proximal promoter containing nucleotides -787 to +1 from Rat CTGF cloned into the Sma I -Bgl II cloning site of the pGL3-Basic luciferase reporter vector (Promega). CTGF promoter mutation constructs were made using the Quick-Change site-directed mutagenesis kit (Stratagene). Multiple mutation constructs were created using previously characterized substitution of the SBE (Δ SBE), the TRE (Δ TRE) or both the SBE and TRE (Δ SBE/ Δ TRE) with a BamHI restriction site [41]. Point mutations were introduced using the following primers (Sigma Genosys): Δ SBE: 5'-GGACTGGGGAACCTGTGAGGTGTAAGGAGGCCTAGGTTTTTCGACCGTGTGAAATC GACC-3', 5'-GGTCGATTTTCACACGGTCGAAAACCTAGGCCTCCTTTTCACCTCACAGTTCCCCAG TCC-3'; Δ TRE: 5'-CAGACGGAGGAATGTGGAGGATCCAGGGGTCAGGATCAATCC-3', 5'-GGATTGATCCTGACCCCTGGATCCTCCACATTCCTCCGTCTG-3'; Δ SBE/ Δ TRE: 5'-ATCCGGAGGAATGTGGAGGATCCAGGGGTCAGGATCAATCC-3', 5'-GGATTGATCCTGACCCCTGGATCCTCCACATTCCTCCGGAT-3'. Mutations were confirmed with BamHI restriction digestion and sequencing (SeqRight). Dominant negative (DN) Jnk was kindly provided by Dr. Roger Davis (University of Massachusetts School of Medicine, Worcester, MA), DN-p38 was kindly provided by Dr. Ken Malcom (National Jewish Medical and Research Center, Denver, CO) and DN-Erk was kindly provided by Dr. Jacques Pouyssegur (Institute of Signaling, Developmental Biology and Cancer Research, CNRS, Nice, France). The kinase dead Src constructs, Src-KD [48] and Src-KDYF [49], were made available by Dr. Archana Sanjay (Temple University School of Medicine, Philadelphia, PA). Anti-p38, anti-Erk, anti-Smad 2, 3 or 4, anti-Jnk and anti-pTyr416 Src antibodies were from Cell Signaling; anti-actin from Sigma; anti-Src from Upstate; anti-CTGF and anti-Smad 2/3 from SantaCruz. The Src inhibitor PP2 and TGF- β 1 were obtained from Calbiochem. Smad 2, 3, 4 siRNAs and non-silencing siRNA control were obtained from Qiagen. The MAPK inhibitor for Erk (PD98059) was obtained from Calbiochem.

TGF- β 1 Stimulation

Recombinant TGF- β 1 (Calbiochem) was diluted to a concentration of 1 μ g/ml in sterile 4mM HCL containing 0.1% BSA and stored at -20° C. Osteoblasts were serum deprived for 24hrs prior to treatment. Cells were treated with a dose of 5ng/ml of TGF- β 1, as determined in previous experiments [25], for a 24hr period unless otherwise stated.

Protein Isolation and Western Blotting

Western blot analysis was performed as previously described [50]. Briefly, cell monolayers were lysed at 4°C in protein extraction buffer (RIPA buffer) consisting of 50mM Tris-HCL (pH 7.5), 135mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 2mM EDTA, 50mM NaF, 2mM sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1mM PMSF. Total protein concentration was measured using the BCA Protein Assay Reagent Kit (Pierce) according to the manufacturer's instructions. Samples were subjected to sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% acrylimide gel and transferred to PVDF filters by electroblotting. Blots were incubated with either anti-CTGF (1:100), anti-Smad 2/3/anti-phospho Smad2/3 (1:200), anti-p38/anti-phospho p38 (1:1000), anti-Erk/anti-phospho Erk (1:1000), anti-Jnk/anti-phospho Jnk (1:1000), anti-Smad 2 (1:1000), anti-Smad 3 (1:1000), anti-Smad4 (1:1000), anti-Src (1:1000), anti-pTyr416 Src (1:1000) or anti-actin (1:5000) primary antibody, and then with HRP-conjugated secondary antibody (1:5000). Antigens were detected using the Pierce chemiluminescent substrate system.

For Western blots of total and phospho Smad2/3, Erk1/2, Jnk1/2 or p38, osteoblasts were plated in six well plates (1.2×10^5 cells/well), serum starved for 24 hours and treated with TGF- β 1 (5ng/ml) for 5, 15, 30 and 60 minutes prior to homogenization in protein extraction buffer. For Western blots of total and phospho Erk1/2 using the Erk inhibitor (PD98059), cells were plated in 6 well plates (1.2×10^5 cells/well), serum starved overnight and treated with indicated doses (5, 10, 20 μ M) PD98059 for 1hr prior to TGF- β 1 (5ng/ml) or control treatment (TGF- β 1 diluent) for 30 min prior to homogenization in protein extraction buffer. For Western blots of CTGF using Smad specific siRNAs, osteoblasts were plated in 6 well culture plates (1.2×10^5 cells/well) and transfected for 4 hours with 1.0 μ M of either Smad 2, 3 or 4 siRNA (Qiagen). Following transfection, the cells were serum starved overnight and then treated with TGF- β 1 (5ng/ml) or control (diluent only) for 24 hrs prior to homogenization in protein extraction buffer. These same protein isolates were used to determine the specificity and efficiency of each Smad siRNA in blocking the expression of its corresponding Smad. For Western blots of CTGF using the Erk inhibitor, PD98059, or the Src family kinase inhibitor, PP2, osteoblasts were plated in 6 well culture plates (1.2×10^5 cells/well), serum starved overnight and treated with PD98059 (20 μ M) or with PP2 (10 μ M) for 1 hour prior to TGF- β 1 treatment for an additional 24 hours. All Western blots were probed for β -actin which served as a loading/transfer control.

Coimmunoprecipitation

Cell cultures were washed twice with ice-cold PBS and lysed in buffer (20mM Hepes /NaOH (pH 7.4), 150mM NaCl, 0.05% NP-40, 10% glycerol, 10mM EDTA, 1mM Na₃VO₄, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1mM phenylmethanesulfonyl fluoride). After 30min on ice, cell lysates were cleared by centrifugation at 12,000g for 20min. The protein concentration in each sample was quantified using Bradford protein assay reagent (Pierce) and immunoprecipitation was performed using an Immunoprecipitation kit (#11719394001, Roche) following the manufacturers instructions. Briefly, 500 μ g of protein from total cell lysate was incubated with 2 μ g of antibody for 2h on ice, and subsequent to this incubation 20 μ g of protein-G agarose was added. After incubation for 1h at 4 C with mixing, the immune complex was recovered by centrifugation at 12,000g for 1min and washed four times with wash buffer (20 mM Hepes /NaOH (pH 7.4), 150mM NaCl, 0.02% NP-40, 10mM EDTA, 1mM Na₃VO₄, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1mM phenylmethanesulfonyl fluoride). The immunoprecipitates were then subjected to SDS /PAGE and Western blotting.

Smad Protein Capture

The SBE (S) and mutated SBE (Δ S) probes were 3' end labeled with biotin using the 3' biotin labeling kit according to the manufacturer's instructions (BioRad). The biotin labeled SBE probe was then annealed to Dynal M-289 streptavidin coated beads according to the manufacturer's instructions (Invitrogen). Nuclear lysates purified from primary osteoblasts treated with TGF- β 1 were then incubated with either the SBE or the Δ SBE probe coated Dynal M-289 beads and proteins that bound the SBE were separated and run on an SDS PAGE gel. Western blot analysis was used to identify whether Smads 2, 3 and 4 bound to the SBE.

Luciferase Assays

Luciferase activity was determined using the Dual-Glo luciferase assay (Promega) according to the manufacturer's instructions. Primary osteoblasts were plated in a 96 well microplate (2.4×10^4 cells/well), transfected with 0.4 μ g of a luciferase reporter vector (as indicated for each experiment) and co-transfected with 0.2 μ g of a Renilla (internal control) luciferase vector. Following transfection, the cells were serum starved overnight and treated as indicated below. Luciferase activity was measured using a Wallac 1420 fluorometer. Relative luciferase activity was expressed as a ratio of firefly/renilla luminescence values. All samples were normalized to an untreated (cells only) or mock treated (empty vector or diluent only) control reaction.

For experiments to assess the dose-response of TGF- β 1 on CTGF promoter activity, osteoblasts transfected with the luciferase constructs were subsequently treated with increasing doses of TGF- β 1 (0.5 to 20ng/ml) for 24 hours under serum free conditions. To examine the temporal pattern of CTGF promoter inducibility, transfected cells were treated with TGF- β 1 (5ng/ml) for varying periods of time ranging from 0.5 to 48 hours prior to measurement of luciferase activity. For experiments using the promoter deletion or site-directed mutant approaches, each construct was generated as a luciferase reporter vector. Following co-transfection with the Renilla (internal control) luciferase vector, the cells were serum starved overnight and treated with TGF- β 1 (5ng/ml) for an additional 24 hours. Luciferase activity was then assessed.

For luciferase experiments using the Erk inhibitor (PD98059) or the Src family kinase inhibitor, PP2, the cells were serum starved overnight and treated with indicated doses of PD98059 (5, 10, 20 μ M) or PP2 (10 μ M) for 1hr prior to TGF- β 1 (5ng/ml) or control (TGF- β 1 diluent) treatment for an additional 24 hrs prior to measurement of luciferase activity. For luciferase assays using Smad siRNAs, dominant-negative constructs or kinase-dead vectors, primary osteoblasts were co-transfected with either a specific Smad siRNA (1.0 μ M), DN-p38, DN-Erk or DN-Jnk (0.2, 0.4 or 0.8 μ g), or one of two Src kinase dead expression constructs (0.4 μ g) along with the luciferase reporter vector pGL3-W787 and pRL-Renilla as described above. An empty vector (0.8 μ g) or non-silencing siRNA (1.0 μ M) were used as negative controls as indicated in each experiment. Following transfection the cells were serum starved overnight and treated with TGF- β 1 (5ng/ml) or control treatment (TGF- β 1 diluent) for an additional 24 hrs prior to measurement of luciferase activity.

Cell Viability Assay

Cell Viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's directions. Briefly, cells were plated in a 96 well microplates (2.4×10^4 cells/well) and either treated with an inhibitor (Erk inhibitor or Src kinase inhibitor) or transfected with a dominant negative expression vector (DN-Erk, DN-Jnk, DN-p38) under conditions identical to those described for each experiment. After treatment or transfection, the cell cultures were equilibrated to RT and subsequently lysed in CellTiter-Glo reagent for 10 min with mild agitation. The samples were then measured using a Wallac 1420 fluorometer, normalized to a blank reaction and graphed as relative luminescence being compared to the appropriate controls.

Electro-Mobility Shift Assay

Nuclear extracts were prepared from TGF- β 1 treated and mock treated (TGF- β 1 diluent) osteoblast cultures using a modified Dignam protocol [51]. Briefly, cells were harvested and resuspended (10^7 cells/100.1 buffer) in a sucrose buffer containing: 0.32M Sucrose, 10mM Tris HCL (pH 8.0), 3mM CaCl₂, 2mM MgOAc, 0.1mM EDTA, 0.5% NP-40, 1mM DTT and 0.5mM PMSF. The nuclei were pelleted at 500g (4°C) and washed in sucrose buffer without NP-40. The cell pellet was then resuspended in low salt buffer (30 μ l/ 10^7 cells) containing 20mM HEPES (pH 7.9), 1.5mM MgCl₂, 20mM KCl, 0.2mM EDTA, 25% glycerol, 1% NP-40,

0.5mM DTT, 0.5mM PMSF. An equal volume of high salt buffer (20mM HEPES (pH 7.9), 1.5mM MgCl₂, 800mM KCl, 0.2mM EDTA, 25% glycerol, 1% NP-40, 0.5mM DTT, 0.5mM PMSF 4μg/ml leupeptin, aprotinin and pepstatin) was added to this and the sample was incubated at 4°C for 45 min with agitation. The sample was then centrifuged at 14,000g for 10 min, the supernatant containing the nuclear lysates was retained and protein concentration was determined using a Bradford assay. Oligonucleotide probes were labeled with [γ -³²P] ATP (Amersham) and T4 polynucleotide kinase (NEB). Oligonucleotides used in electrophoretic mobility shift experiments (EMSA) are shown in Figure 8. Binding reactions contained 5μg of nuclear extract, 1X binding buffer [5X binding buffer: 50mM Tris HCL (pH 8.0), 750mM KCL, 2.5mM EDTA, 0.5% Triton-X 100, 62.5% glycerol and 1mM DTT], poly-dIdc (1μg/ml), ~10,000 cpm of labeled probe, and a 50 molar excess of competitor oligonucleotides where indicated. The binding reaction was incubated at RT for 30 min and the entire sample was loaded on a 4% acrylamide, 60:1 acrylamide:bisacrylamide gel containing 0.5X TBE.

Data Analysis

For all quantitative data, analysis of variance (ANOVA) was employed to evaluate the effect of one variable on two or more independent groups. In the event of a significant group effect, individual pairs of means were compared using the Bonferroni post-hoc test. Data were calculated as mean + SEM, and in some cases, converted to percent of control. A value of $p < 0.05$ was used to determine whether differences were statistically significant.

Results

TGF-β1 induces CTGF promoter activity in primary osteoblasts (Figure 1)

We have previously reported that TGF-β1 is capable of inducing CTGF expression in primary osteoblasts [25]. The purpose of the present study was to characterize the signaling mechanisms involved in TGF-β1 regulated induction of CTGF in osteoblasts. To directly measure the effects of TGF-β1 on CTGF induction we used CTGF promoter activity, measured by luciferase activity, as an end point in this series of experiments. For these studies, we cloned the CTGF proximal promoter spanning nucleotides +1 to -787 into a pGL3-Luciferase reporter vector (W787) and used it to assay for TGF-β1 responsiveness (luciferase activity) in our primary osteoblast culture system (Figure 1A). In these experiments, a pGL3-SV40 vector containing the SV40 promoter was used as a non-TGF-β1 inducible control and the promoter-less pGL3-Basic vector were utilized as a negative control. Primary osteoblasts were transiently transfected with either the pGL3-W787, pGL3-SV40 or pGL3-Basic and all were co-transfected with pRL-Renilla. Luciferase assays revealed that TGF-β1 stimulated the activity of the CTGF promoter in a dose-dependent manner, with a maximal response occurring at a dose equal to or greater than 5ng/ml, representing an ~6.5-fold and 30-fold increase over the pGL3-SV40 and pGL3-Basic vectors, respectively (Figure 1B). These results were similar to our previous studies measuring CTGF mRNA and protein expression [25]. Since the standard dose of TGF-β1 previously used in many other cell/tissue culture systems is 5ng/ml [25,52, 53] and this represented the minimum dose required for maximal induction of CTGF promoter activity in osteoblast cultures, this dose was used in all subsequent experiments.

To examine the temporal pattern of CTGF promoter inducibility in TGF-β treated cultures, primary osteoblasts were treated with TGF-β1 for 0.5 – 48 hrs and the luciferase activity was determined. This experiment revealed a time dependent elevation of luciferase activity following TGF-β1 exposure through 48 hrs post-treatment (Figure 1C). Since the 24 hr time point represented the approximate mid-log phase of the response curve, we used a 24 hr treatment period for all subsequent experiments.

Smads are required for CTGF promoter activation and expression induced by TGF- β 1 (Figure 2)

Smads are known to play a role in mediating TGF- β 1 signaling, but the critical Smads involved in TGF- β 1 signaling vary depending on cell type [54], [55]. To determine the role of Smads in TGF- β 1 induction of CTGF in primary osteoblasts, we first assessed activation of Smad signaling by TGF- β 1 and then determined the requirement of specific Smad proteins for CTGF induction by TGF- β 1. Western blot analysis revealed that Smads 2 and 3 are activated (phosphorylated) when primary osteoblasts are treated with TGF- β 1 with initial activation occurring by 30 min post-treatment and persisting through 60 min (Figure 2A). To determine if Smad expression was necessary for CTGF promoter activation and expression, we utilized a Smad siRNA knockdown strategy. Primary osteoblast cultures were transfected with 1.0 μ M of either a specific Smad (2, 3 or 4) siRNA or a non-silencing control siRNA. The specific siRNAs used were able to effectively reduce the expression of the corresponding Smad by >80% (Figure 2C), and cell viability was not affected by any of the transfection reagents or the Smad siRNAs as determined by trypan blue exclusion counting and cell viability assay (data not shown). Transfection with either the Smad 3 or Smad 4 siRNA resulted in a significant inhibition (>75%) of TGF- β 1 induced CTGF promoter activity (luciferase assay; Figure 2B) and protein levels (Western blot; Figure 2C), with protein levels being similar to controls (without TGF- β 1) for cells transfected with the Smad 4 siRNA. In contrast, transfection with Smad 2 siRNA failed to inhibit TGF- β 1 induction of CTGF promoter activity and protein levels (Figure 2B and C). Although TGF- β 1 treatment induces the phosphorylation (activation) of Smads 2, 3 and 4, only Smads 3 and 4 are essential for CTGF induction by TGF- β 1 as blocking Smad 2 expression had no effect on CTGF promoter activation or protein expression.

Erk is required for CTGF induction by TGF- β 1 in osteoblasts (Figure 3 and Figure 4)

MAPKs (p38, Jnk, Erk) are known downstream signaling partners of TGF- β 1 [31,36,56], and more recently, MAPK signaling has been shown to directly regulate CTGF expression in fibroblasts [43]. In this series of experiments, we evaluated the ability of specific MAPKs to modify TGF- β 1 induced CTGF promoter activity and expression in primary osteoblasts. Since TGF- β 1 activation of MAPKs is cell type specific, we first evaluated the ability of TGF- β 1 to activate MAPKs in primary osteoblasts. Western blot analysis revealed that while Erk, Jnk and p38 all were activated in a time dependent manner by TGF- β 1 treatment, Erk and Jnk activation appeared to be more inducible than p38 with a maximal induction occurring 30 min post-treatment (Figure 3A). To determine which MAPKs were required for TGF- β 1 induction of CTGF, primary osteoblasts were transiently transfected with previously characterized DNMAPK expression constructs [57–59] and co-transfected with the luciferase reporter constructs pGL3-W787 and pRL-Renilla Figure 3B–D). Measuring the luciferase activity, we found that the expression of DN-Erk showed a significant inhibition of TGF- β 1 induced (~8-fold) and basal (~5-fold) CTGF promoter activity in a dose dependent manner (Figure 3D). In contrast, the expression of DN-p38 and DN-Jnk failed to inhibit activation of CTGF promoter activity (Figure 3B and C). These results suggest that only Erk, not p38 or Jnk, is involved in mediating TGF- β 1 induced, as well as, basal CTGF expression in osteoblasts. To rule out the possibility that this observation was the result of cytotoxicity, we evaluated the effects of these DN constructs on cell viability at the maximum concentration (0.8 μ g) used. The DN constructs did not cause a significant increase in cytotoxicity, with viability comparable to that of the empty vector control, as detected by trypan blue exclusion (data not shown) and the CellTiter-Glo luminescent cell viability assay (Figure 3E).

To confirm that Erk is required for TGF- β 1 induced CTGF expression, we next utilized a pharmacological inhibitor approach. Osteoblast cultures were treated with an inhibitor of Erk, PD98059, and its ability to inhibit Erk activation, CTGF promoter activity and CTGF protein expression were then assessed. Western blot analysis revealed that the specific Erk (PD98059)

inhibitor prevented the activation of ERK1/2 in a dose-dependent manner (Figure 4A). This effect was specific for the inhibitor alone as treatment with similar concentrations of DMSO (inhibitor diluent) had no effect on Erk activation (Figure 4B). When the luciferase activity of the CTGF proximal promoter (W787) was assessed in similarly treated cultures, we found that PD98059 effectively prevented TGF- β 1 induction of the CTGF promoter (Figure 4C). Western blot analysis confirmed that the Erk inhibitor prevented the induction of CTGF expression by TGF- β 1 (Figure 4D). To rule out the possibility that the observed inhibition by PD98059 might be the effect of cytotoxicity, we evaluated the effects of this inhibitor on cell viability using the maximum dose (20 μ M). Neither the inhibitor or the DMSO (diluent) had any adverse effect on cell viability as assessed by trypan blue exclusion (data not shown) and the CellTiter-Glo luminescent cell viability assay (Figure 4E).

Src kinase activity is a necessary requirement for TGF- β 1 induction of CTGF and activation of Erk in primary osteoblasts (Figure 5 and Figure 6)

Studies have shown that Src can also act as a downstream signaling effector for TGF- β 1 and can function upstream of Erk in some cell types [52,53,60–63]. To determine if Src was activated by TGF- β 1 in osteoblasts, we utilized a coimmunoprecipitation strategy. To assess Src activation, we used an antibody that specifically recognizes phosphorylated tyrosine 416 (pTyr416), the autophosphorylation site of c-Src. Osteoblasts were treated with TGF- β 1 for 0, 15, 30 and 60 minutes, the cells were lysed, Src was immunoprecipitated using a Src specific antibody and Src activity was determined by Western blot using pTyr416. TGF- β 1 treatment caused activation of Src reaching maximal levels at 30 minutes post treatment (Figure 5A). To determine if Src plays a role in CTGF induction by TGF- β 1 in osteoblasts, we inhibited Src function using either an inhibitor, PP2, or two independent kinase dead Src dominant negative constructs, Src-KD or Src-KDYF. Primary osteoblasts were treated with PP2 (10 μ M) or diluent alone (control) prior to TGF- β 1 (5ng/ml) treatment for 24 hrs. In osteoblasts transfected with a CTGF promoter reporter construct (W787), we found that CTGF promoter activity was significantly reduced when cultures were pretreated with PP2 (Figure 5B). Similarly, Western blot analysis revealed that PP2 treatment inhibits the induction of CTGF expression by TGF- β 1, with CTGF levels comparable to those observed in non-TGF- β 1 treated cultures (Figure 5C). To confirm these results, we also assessed CTGF reporter activity after osteoblasts were transfected with two independent Src kinase-dead constructs (Src-KD, Src-KDYF). As seen in Figure 5D, CTGF promoter activity was dramatically reduced in the presence of both DN constructs thereby confirming the requirement of Src for TGF- β 1 induction of CTGF promoter activity and expression in osteoblasts.

To determine if Src was required for Erk activation by TGF- β 1, we inhibited Src using either PP2 or kinase-dead Src (Src-KD). The results of these experiments are summarized in Figure 6. Primary osteoblasts were treated with PP2 (10 μ M) or diluent alone (DMSO) prior to TGF- β 1 (5ng/ml) treatment for 30min. Western blot analysis revealed that PP2 treatment inhibits TGF- β 1 induced activation of Erk 1/2. Similar results were seen in osteoblasts transfected with kinase-dead Src (Figure 6B) confirming the requirement of Src for TGF- β 1 induced activation of Erk.

The SBE and TRE are required for TGF- β 1 induction of CTGF (Figure 7 and Figure 8)

Several previous studies have identified putative and functional regulatory motifs in the CTGF proximal promoter that are required to confer TGF- β 1 responsiveness [40,41]. Previously CTGF induction has largely been attributed to the presence of two proximal promoter elements, the TGF- β 1 response element (TRE) and the Smad binding element (SBE) [41]. The location of these two regulatory motifs in the proximal promoter is shown in Figure 7A. The relative contributions of these two elements in CTGF induction by TGF- β 1 vary in different cell types [41,45]. In these experiments, we evaluated whether the TRE or the SBE were required to

confer TGF- β 1 responsiveness to the CTGF promoter in primary osteoblasts. For these experiments, we created the promoter deletion constructs pictured in Figure 7B. The T129 construct contains only the first 129 nucleotides of the CTGF promoter and lacks the TRE and SBE, the S164 construct contains the first 164 nucleotides of the CTGF promoter and includes only the TRE, and the P314 construct contains the first 314 nucleotides of the CTGF promoter and includes both the SBE and TRE. The W787 construct, containing the entire CTGF proximal promoter, was used as a positive control and the promoter-less pGL3 construct was used as a negative control. As summarized in Figure 7C, the pGL3-Basic construct did not exhibit TGF- β 1 inducibility compared to the TGF- β 1 responsive W787 construct. With respect to TGF- β 1 inducibility of the CTGF promoter constructs, the T129 and S164 constructs demonstrated minimal inducibility upon TGF- β 1 treatment, demonstrating that inclusion of the TRE alone was not sufficient to confer TGF- β 1 responsiveness as seen in the positive (W787) control (Figure 7C). While the P314 construct, which contained both the SBE and TRE, was 3-fold more responsive to TGF- β 1 treatment than the other deletion constructs, this response was still significantly lower (by ~70%) compared to that of the full length proximal promoter (W787) construct (Figure 7C). In addition, for all deletion constructs a significant decrease in the basal level of CTGF promoter activity was observed compared to the W787 construct. These results suggested that: 1) other elements upstream of the SBE may be required for TGF- β 1 responsiveness, 2) due to the relative proximity of the SBE and TRE, the deletion strategy itself may sterically inactivate these sites.

In order to overcome the potential steric hindrance of the deletion strategy and to explore whether other TGF- β 1 responsive sites in the promoter were involved, a mutagenesis approach was utilized and is summarized in Figure 8A. Our approach included using previously characterized mutations of the SBE or TRE to BamHI sites [36]. Multiple mutation constructs were generated containing a mutation of either the SBE (Δ SBE), the TRE (Δ TRE) or both the SBE and TRE (Δ SBE/ Δ TRE) (Figure 8A and B). When we assessed TGF- β 1 inducible promoter activity, the Δ SBE, Δ TRE and the Δ SBE/ Δ TRE all exhibited a marked decrease in TGF- β 1 inducibility (ranging from 70 to 90%) when compared to the full length proximal promoter (W787) construct (Figure 8C). Furthermore, for all mutation constructs a significant decrease in basal CTGF expression was detected. These results demonstrate for the first time that the presence of both the SBE and TRE are required for basal and TGF- β 1 induced CTGF expression in osteoblasts.

The TRE and SBE bind TGF- β 1 inducible complexes from primary osteoblast nuclear lysates (Figure 9)

To determine if proteins from osteoblast nuclear lysates specifically bound to the TRE and SBE motifs, electro-mobility shift assays were conducted as summarized in Figure 9. For these experiments, probes were generated that contained the SBE and TRE (S-T), the TRE alone (T) or the SBE alone (S). A second set of probes were generated containing mutated SBE and TRE (Δ S-T), mutated TRE alone (Δ TRE) or mutated SBE (Δ SBE) (see Figure 9A). These probes were subsequently utilized to determine the ability of TGF- β 1 induced or mock-treated nuclear lysates to bind to a normal versus mutated promoter element. We found that nuclear lysates could bind to each probe (Figure 9B, lanes: 1, 5, 9) and that this binding was TGF- β 1 inducible (Figure 9B, Lanes: 1 vs. 2, 5 vs. 6, 9 vs. 10). This binding was also specific as binding could be competed out by adding excess unlabeled oligomer (Figure 9B), lanes: 4, 8, and 12). Importantly, some of the binding complexes could be abolished by mutating the SBE and the TRE consensus sequences in each probe (Figure 9B, lanes: 3, 7, 11) providing direct evidence that these motifs are required for normal complex binding and subsequent TGF- β 1 induced CTGF expression. It is also important to note that when S-T probes were used (lane 1) more bands (complexes) were present and with greater intensity compared to either the T (lane 5) or the S (lane 9) probes alone, providing additional evidence that both motifs together are

required to allow proper complex formation resulting in TGF- β 1 induced CTGF expression in osteoblasts.

To demonstrate that the SBE in the CTGF promoter was a true Smad binding element and could bind Smad proteins, we used SBE coated magnetic beads to capture SBE binding proteins from nuclear cell lysates from TGF- β 1 treated osteoblasts (Figure 9C). After adsorption to the beads, proteins were run on SDS-PAGE gels and blotted with antibodies to Smad 2, 3 or 4. As seen in Figure 9C, Smad 2, 3 and 4 were all capable of binding to the SBE thereby establishing it as a functional Smad binding element.

Discussion

Previous studies have shown that CTGF acts as a downstream mediator of the cellular effects of TGF- β 1 in various cell types [15–24]. Among these, we recently demonstrated that CTGF plays an essential role in mediating TGF- β 1 induced extracellular matrix production in osteoblasts [25]. The purpose of this study was to examine molecular mechanisms involved in TGF- β 1 regulation of CTGF expression in osteoblasts.

The signaling pathways that mediate TGF- β 1 induction of CTGF have been shown to vary depending on the cell type being examined [14] and to date there have been no studies in osteoblasts. In general, TGF- β 1 signals through a generic Smad mediated pathway involving Smads 2, 3 and 4 [32]. However, 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 [31]. Thus, Smad signaling is required, but in most cases it is not sufficient by itself to achieve target gene activation. In this study, we examined the requirements of Smads as well as MAPK and Src kinases in TGF- β 1 induced up-regulation of CTGF expression in osteoblasts. Additionally, we also assessed the contributions of cis-regulatory elements, specifically the TRE and SBE of the CTGF promoter, in the induction of CTGF by TGF- β 1 in osteoblasts.

Our results showed that treatment of osteoblasts with TGF- β 1 initiates the activation (phosphorylation) of Smads 2 and 3, consistent with other studies that have demonstrated the requirement of Smad signaling to achieve CTGF induction in fibroblasts [64,65], mesangial cells [44] and airway smooth muscle cells [66]. However, previous studies have demonstrated differential roles of Smads 2 and 3 for TGF- β 1 signaling [67,68]. For example, TGF- β 1 induced collagenase-3 expression is mediated by Smad 3 and does not require Smad 2 in fibroblasts [54], yet in osteoblasts, only Smad 2 is required for collagenase-3 promoter activation [55]. Since the critical Smads involved in TGF- β 1 signaling vary depending on cell type, we used an siRNA approach to inhibit specific Smads (2, 3 and 4). Our results demonstrated that Smads 3 and 4, but not Smad 2, are required for the induction of CTGF expression and promoter activity by TGF- β 1 in osteoblasts.

MAPKs represent another group of downstream signaling transducers of TGF- β 1 that have been shown to directly regulate CTGF expression in some cell types [43]. However, studies have shown that there are differences in the requirement of specific MAPKs for TGF- β 1 induction of CTGF expression and this variability appears to be cell type dependent. Studies in renal fibroblasts and mesangial cells demonstrated the requirement of Erk for TGF- β 1 induced CTGF expression [44,69,70]. However, in smooth muscle cells both Erk and Jnk are required for CTGF induction by TGF- β 1 [66]. In another study using lung fibroblasts, it was determined that CTGF expression was dependent on Jnk, not p38 or Erk [64]. Based on the cell dependent differences in the MAPKs that are essential for TGF- β 1 induction of CTGF expression, we characterized the role of the MAPKs, Erk1/2, p38 and Jnk, in CTGF induction by TGF- β 1 in osteoblasts. Although treatment of osteoblasts with TGF- β 1 activated all three

MAP kinases, we used dominant negative mutants and specific pharmacological inhibitors to demonstrate that only Erk was required for TGF- β 1 induction of CTGF expression and promoter activity.

While TGF- β 1 activation of MAPKs is cell type dependent [36], they can act independent of the Smad signaling pathway or can interact to potentiate or inhibit Smad signaling [37,38]. In mink epithelial cells, TGF- β 1 induced Jnk activation is required for Smad 3 activation [71], and in fibroblasts, transcriptional activation of fibronectin occurs through Smads in a MAPK-dependent mechanism [56]. Recently an interaction between MAPK and Smad signaling subsequent to their activation by TGF- β 1 has been characterized in osteoblastic cells. In these studies, TGF- β 1 induced the activation of Erk1/2, p38, and Jnk and this occurred in a Smad independent manner [72]. Inactivation of Erk1/2 and Jnk with their specific inhibitors antagonized the inhibitory effects of TGF- β 1 on alkaline phosphatase (ALP) activity and mineralization in MC3T3-E1 cells and enhanced the transcriptional activity of Smad 3, as well as ALP activity and mineralization induced by Smad 3 [73], suggesting that TGF- β 1 induced Smad signaling is regulated by MAPKs in osteoblasts. While these studies are very novel, they offer a new paradigm for understanding TGF- β 1 signaling, where TGF- β 1 induced cellular responses are determined by the interaction of Smad and MAPK signaling pathways. A more detailed analysis of the Erk and Smad pathways, their interactions as well as their independent functions, is required to fully understand how CTGF is regulated by TGF- β 1 in osteoblasts, and the functional consequence of each pathway in mediating the effects of TGF- β 1 on osteoblasts.

Previous studies have demonstrated that the non-receptor tyrosine kinase, Src, can also be activated following TGF- β 1 treatment in certain cell types [52,53,60–63]. Src activation following TGF- β 1 treatment can occur as a direct result of TGF- β receptor activation [60,63] or indirectly as a result of enhanced integrin-mediated cell attachment induced by TGF- β 1 [52,53,61,62]. In this study we demonstrated for the first time that Src is activated in primary osteoblasts following TGF- β 1 treatment. We also examined whether Src played a role in CTGF induction by TGF- β 1. Use of the Src kinase inhibitor, PP2, or two independent Src kinase-dead constructs (Src-KD, Src-KDYF), prevented the induction of CTGF expression and promoter activity by TGF- β 1. These results are the first to demonstrate a critical role for Src in the regulation of CTGF expression by TGF- β in any cell type. Src has been shown to function as an upstream modulator of Erk activation in some cell types including osteoblastic like cells [60–63,74]. When we assessed the ability of TGF- β 1 to activate Erk 1/2 signaling in Src compromised osteoblasts we found that Src was required for Erk 1/2 activation demonstrating the involvement of the Src as an upstream mediator of Erk signaling in primary osteoblasts. Given the importance of the Src/Erk signaling cascade for TGF- β 1 induction of CTGF, a more detailed analysis of Src and/or Erk signaling and their potential crosstalk with Smads is warranted.

The cis-regulatory elements required for TGF- β 1 induction of CTGF expression vary depending on the cell type being investigated and include the TGF- β 1 response element (TRE) and/or the Smad binding element (SBE) [40,41]. To characterize the cis-regulatory motifs involved in CTGF induction by TGF- β 1, we used a combination of promoter deletion and site-directed mutant constructs and demonstrated that both the TRE and SBE are required for basal and TGF- β 1 induced CTGF expression in osteoblasts. This is the first study in which both elements together have been shown to be necessary for induction of CTGF expression by TGF- β 1. Previous studies in other cell types have demonstrated divergent roles for each of these elements in either basal or TGF- β 1 induced regulation of CTGF[40,41]. For example, in scleroderma fibroblasts and the chondrocytic cell line (HCS-2/8), only the TRE is required for TGF- β mediated CTGF induction [41,75]. 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 [41].

Despite the unique cooperation of both elements in CTGF induction in osteoblasts, it is not uncommon for multiple transcriptional elements to be involved in the context of Smad mediated signaling involving the SBE. Smads themselves have an intrinsically weak DNA binding affinity and 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 [76]. For example, multiple transcriptional motifs have been shown to be required for Smad3-mediated TGF- β 1 induced transcription of p15 [77,78]. While the SBE in the p15 promoter has low affinity for Smad3 by itself, the close proximity of the Sp1 motif results in a much greater affinity, demonstrating the requirement of both the SBE and Sp1 to achieve high affinity binding and subsequent p15 expression [77, 78].

Based on our results we hypothesize that in osteoblasts the TRE acts as a cognate motif in juxtaposition to the SBE to achieve maximal induction of CTGF expression by TGF- β 1. When we used a probe that contained both the TRE and SBE, more bands (complexes) were present and with greater intensity in comparison to using probes with either the TRE alone or SBE alone. These binding patterns imply that both elements together attract a greater number of proteins/complexes to form and that protein/complex binding is greater when both elements are present in comparison with either element by itself. Additional studies are underway to confirm this interaction and elucidate the signaling proteins that bind to the TRE and SBE to regulate CTGF induction by TGF- β 1 in primary osteoblasts.

In conclusion, our study demonstrates that the induction of CTGF promoter activity by TGF- β 1 requires both the TRE and SBE in osteoblasts. It also demonstrates that specific components of the Smad and MAPK/Src kinase signaling pathways are required for TGF- β 1 mediated activation of the CTGF promoter. Additional studies are required to further elucidate the signaling pathways that regulate TGF- β 1 induction of CTGF expression, and to understand how specific signaling components interact to regulate CTGF expression. These studies will not only enhance our understanding of the molecular mechanisms of TGF- β 1 and CTGF in osteoblasts, but may also help to identify novel therapeutic targets to control early stages of osteoblast differentiation and increase bone formation. Additionally, in future studies, it will be important to see if the signaling mechanisms demonstrated in this study can be confirmed using osteoblasts derived from different species including mouse and human.

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Figure 1. Effect of TGF- β 1 on CTGF promoter activity

(A) Structures of the promoter constructs: pGL3-W787, pGL3-SV40 and pGL3-Basic. (B) Primary osteoblasts were plated in 96 well plates, transfected with 0.4 μ g of either the pGL3-W787 (CTGF proximal promoter +1/- 787), pGL3-SV40 or pGL3-Basic firefly luciferase vector and co-transfected with 0.2 μ g of Renilla luciferase expression vector (internal control). The cells were serum starved overnight and then treated with indicated doses of TGF- β 1 for 24 hrs. Relative luciferase activity was expressed as a ratio of firefly/renilla luminescence values. Data expressed as mean $\pm$ SEM (n=5). * p<0.001 compared to pGL3-SV40 and pGL3-Basic vectors. (C) Osteoblasts were cotransfected with 0.4 μ g pGL3-W787 and 0.2 μ g

Renilla luciferase expression vectors, serum starved and treated with TGF- β 1 (5ng/ml) for indicated times. Data shown as mean $\pm$ SEM (n=5).

Figure 2. The requirement of Smads 3 and 4 for TGF-β1 induced CTGF promoter activity and expression

(A) Primary osteoblasts were plated in six well plates, serum starved for 24 hrs and subsequently treated with TGF-β1 (5ng/ml). At the indicated times, cell protein lysates were harvested and assessed for activation of Smad signaling by Western blot analysis. This experiment was repeated three times with similar results. (B) Osteoblasts were plated in 96 well plates and co-transfected with 0.4 μg of W787 luciferase vector, 0.2μg of Renilla (internal control) luciferase vector and 1.0μM of either Smad 2, 3, 4 or control siRNA (as indicated). The cells were serum starved overnight and then treated with 5ng/ml of TGF-β1 for 24 hrs. Relative luciferase activity was assessed and expressed as a ratio of firefly/renilla luminescence

values. Data shown as mean + SEM (n=6). (C) Primary osteoblasts were plated in six well plates and transfected with 1.0 μ M of either Smad 2, 3, 4 or control siRNA. The cultures were serum starved overnight and subsequently treated with TGF- β 1 (5ng/ml) or mock treated (TGF- β 1 diluent) for 24 hrs. Cell lysates were harvested and assessed by Western blot for corresponding Smad expression (i.e. cells transfected with Smad 2 siRNA probed with anti-Smad 2) and for CTGF expression. This experiment was repeated four times with similar results.

Figure 3. The role of MAPKs in TGF-β1 induced activation of CTGF promoter activity

(A) Primary osteoblasts were plated in six well plates and serum starved for 24 hrs prior to treatment with TGF-β1 (5ng/ml). At the indicated times, protein lysates were harvested and assessed for Erk, Jnk and p38 activation by Western blot analysis. This experiment was repeated three times with similar results. (B–D) Osteoblasts were plated in 96 well plates and co-transfected with 0.4μg of W787 luciferase vector, 0.2μg of Renilla (internal control) luciferase vector and with indicated concentrations of either dominant negative (DN) p38 (B), Jnk (C), Erk (D) or 0.8μg of an empty vector (negative control). Following transfection, the cells were serum starved overnight and then treated with 5ng/ml of TGF-β1 (black bars) or mock treated (diluent only, gray bars) for 24 hrs. Relative luciferase activity was assessed and expressed as

a ratio of firefly/renilla luminescence values. Data represent mean + SEM (n=6). A = $p < 0.001$ compared to mock treated empty vector; B = $p < 0.001$ compared to TGF- β 1 treated empty vector; C = $p < 0.001$ compared to TGF- β 1 treated empty vector and DN-Erk [0.2 μ g] transfected). (E) Osteoblasts were transfected with either an empty vector, DN-p38, DN-Jnk or DN-Erk and treated with TGF- β 1 as above. Cell number was assessed using CellTiter-Glo assay (Promega) where cell number is correlated directly to assessment of ATP activity (relative luminescence). Data represent mean + SEM (n=6).

Figure 4. The requirement of Erk for TGF- β 1 induced CTGF promoter activity and expression
(A) Primary osteoblasts were treated with indicated doses of the Erk (PD98059) inhibitor for 1 hr prior to TGF- β 1 treatment for 30 min. Erk activation (phosphorylation) was assessed by Western blot. For each blot an untreated (negative) and a TGF- β 1 treated (positive) control were also used. Experiment was repeated three times with similar results. **(B)** Activation of Erk (after 30 min) was not inhibited after treatment with increasing doses of DMSO (the inhibitor diluent) used in the previous experiment. Experiment was repeated three times with similar results. **(C)** Primary osteoblasts were plated in 96 well culture plates and co-transfected with 0.4 μ g of W787 and 0.2 μ g of Renilla (internal control) luciferase expression vectors. Following transfection, the cells were serum starved overnight and treated with indicated doses

of the Erk inhibitor PD98059 for 1 hr prior to TGF- β 1 (5ng/ml) or control (TGF- β 1 diluent) treatment for 24 hrs. Luciferase activity was assessed and expressed as a ratio of firefly/renilla luminescence values. Data represent mean + SEM (n=5). A = p<0.001 compared to TGF- β 1 treatment alone; B = p<0.01 compared to 5 and 10 μ M PD98059 treatment. **(D)** Osteoblasts were plated in six well plates, serum starved overnight and treated with the Erk (PD98059) inhibitor (20 μ M) for 1 hr prior to TGF- β 1 treatment for an additional 24 hrs. Cell lysates were harvested and assessed by Western blot for CTGF expression. Experiment was repeated four times with similar results. **(E)** Effects of PD98059 at highest concentration (20 μ M) on cell viability was assessed using CellTiter-Glo assay (Promega) where cell number is correlated directly to assessment of ATP activity (relative luminescence). Neither the inhibitor or DMSO (inhibitor diluent) affected cell viability. Data represent mean + SEM (n=6).

Figure 5. The role of Src in TGF- β 1 induced CTGF promoter activity and expression

(A) Src was immunoprecipitated from osteoblasts treated with TGF- β 1 for indicated times and Western blotted with Src pTyr-416 antibody that detects the activated form Src. (B) Osteoblasts were plated in 96 well microplates and co-transfected with 0.4 μ g of W787 and 0.2 μ g of Renilla (internal control) luciferase expression vectors. Following transfection, cells were serum starved overnight and treated with either the Src family kinase specific inhibitor, PP2 (10 μ M), or diluent alone (control) for 1 hr prior to treatment with TGF- β 1 (5ng/ml) for an additional 24 hrs. Luciferase activity was assessed and expressed as a ratio of firefly/renilla luminescence values. Data represent mean + SEM (n=6). (C) Osteoblasts were plated in six well plates, serum starved overnight and treated with PP2 (10 μ M) for 1 hr prior to TGF- β 1 treatment for an additional 24 hrs. Positive and negative controls included cells cultured in the presence or absence of TGF- β 1 without prior PP2 treatment. Cell lysates were harvested and assessed by Western blot for CTGF expression. Experiment was repeated three times with similar results. (D) Osteoblasts were plated in 96 well microplates and co-transfected with 0.4 μ g of W787 luciferase vector, 0.2 μ g of Renilla (internal control) luciferase vectors, and 0.4 μ g of either one of two kinase dead (KD or KDYF) Src vectors or the empty vector. The cells were serum starved overnight and then treated with 5ng/ml of TGF- β for 24 hrs. Luciferase activity is expressed as a ratio of firefly/renilla values and the means + SEM are graphed as a percentage of the TGF- β 1 treated empty vector control.

Figure 6. Src is required for TGF- β 1 activation of Erk

(**A**) Osteoblasts were plated in six well plates, serum starved overnight and treated with PP2 (10 μ M) or diluent alone (control) for 1 hr prior to TGF- β 1 treatment for 30min. Erk activation (phosphorylation) was assessed by Western blot. Experiment was repeated three times with similar results. (**B**) Osteoblasts were plated in six well plates, serum starved overnight and transfected with 0.4 μ g of kinase dead (KD) Src vector or the empty vector. The cells were then treated with 5ng/ml of TGF- β or diluent alone (control) for 30min. Erk activation (phosphorylation) was assessed by Western blot. Experiment was repeated three times with similar results.

Figure 7. Deletion analysis of CTGF promoter luciferase constructs

(A) Position of the TRE and SBE (in red) relative to each other in the CTGF proximal promoter. (B) Deletion constructs used to test involvement of the TRE or SBE in CTGF promoter activity by TGF-β1 treatment in osteoblasts. Consensus sequences for the TGF-β1 response element (TRE) and the Smad binding element (SBE) are indicated. (C) Primary osteoblasts were plated in 96 well culture plates, transfected with 0.4 μg of either the pGL3-Basic, T129, S164, P314 or W787 luciferase expression vector and co-transfected with 0.2μg of Renilla (internal control) luciferase expression vector. The cells were serum starved overnight and treated with TGF-β1 (5ng/ml) or mock treated (TGF-β1 diluent) for an additional 24 hrs. Luciferase activity was assessed and expressed as a ratio of firefly/renilla luminescence values. Data represent

mean + SEM (n=8). A = $p < 0.001$ compared to TGF- β 1 treated pGL3, T129, S164; B = $p < 0.001$ compared to mock treated pGL3, T129, S164; C = $p < 0.001$ compared to TGF- β 1 treated P314.

Figure 8. Requirement of both the SBE and TRE to confer TGF-β1 responsiveness to the CTGF promoter in osteoblasts

(A) * indicates base substitutions in the SBE and TRE consensus sequences used to create a BamHI restriction sites in place of either the SBE, TRE or both. Mutations were confirmed by BamHI restriction digestion and sequencing. (B) Multiple luciferase mutation constructs were generated containing substitutions of the SBE alone (ΔSBE), the TRE alone (ΔTRE) or both the SBE and TRE (ΔSBE/ΔTRE). pGL3 (negative control) and W787 (positive control) were used as controls for luciferase activity. (C) Primary osteoblasts were plated in 96 well microplates and transfected with 0.4μg of either the ΔSBE, ΔTRE, ΔSBE/ΔTRE, pGL3 or W787 luciferase vector and co-transfected with 0.2μg of Renilla (internal control) luciferase

vector. The cells were serum starved overnight and treated with TGF- β 1 (5ng/ml) for an additional 24 hrs. Relative luciferase activity is expressed as a % of W787 (+ SEM, n=8). A = p<0.001 compared to W787.

Figure 9. Electro-mobility shift assay: Mutation of the SBE and TRE inhibits TGF-β1 induced nuclear protein binding
(A) Probes were generated containing either the SBE and TRE (S-T), the TRE alone (T) or the SBE alone (S). A second set of probes were generated containing a mutated SBE and TRE (ΔS-T), mutated TRE alone (ΔTRE) or mutated SBE alone (ΔSBE). These probes were subsequently utilized to determine the ability of TGF-β1 induced nuclear proteins to bind to a normal versus mutated promoter element. **(B)** Nuclear lysates were generated (see methods) from osteoblasts either treated with TGF-β1 (5ng/ml; +) or mock treated (TGF-β1 diluent; -). Nuclear protein binding was assessed using 5μg of indicated nuclear lysates and probes (see above). Ability of proteins to bind a mutated version of the probe was also compared. As a

control, some lanes contain lysates that were pretreated with a 50 molar excess of unlabeled probe (specific competitor) prior to binding (Comp 50X). (C) Probes containing the SBE were biotinylated and annealed to streptavidin coated magnetic beads and used to test if the SBE was capable of binding Smad proteins harvested from nuclear lysates of TGF- β 1 (5ng/ml) treated osteoblasts. Magnetically separated proteins were then run on SDS-PAGE gels and blotted using Smad 2, 3 and 4 antibodies and compared to bead only controls (C).