Published in final edited form as:

J Cell Biochem. 2010 May 15; 110(2): 477–483. doi:10.1002/jcb.22561.

CONNECTIVE TISSUE GROWTH FACTOR (CTGF) TRANSACTIVATES NUCLEAR FACTOR OF ACTIVATED T CELLS (NFAT) IN CELLS OF THE OSTEOBLASTIC LINEAGE

Anna Smerdel-Ramoya¹, Stefano Zanotti¹, and Ernesto Canalis^{1,2}

¹Department of Research, Saint Francis Hospital and Medical Center, Hartford, CT

²The University of Connecticut School of Medicine, Farmington, CT

Abstract

Connective Tissue Growth Factor (CTGF), a member of the Cyr 61, CTGF, Nov (CCN) family of proteins, regulates multiple cellular functions. Overexpression of CTGF *in vivo* causes osteopenia, but *in vitro* CTGF can induce osteoblastogenesis. To investigate mechanisms involved in the effects of CTGF on osteoblastic cell differentiation, we examined whether CTGF modifies the activity of nuclear factor of activated T cells (NFATc) 1, a transcription factor that cooperates with osterix in the formation of new bone. CTGF increased the transactivation of a transiently transfected reporter construct, where 9 NFAT binding sites direct the expression of luciferase (9xNFAT-Luc) and the activity of the *Regulators of calcineurin 1 exon 4* (Rcan1.4) promoter, an NFAT target gene. We postulated that CTGF could modify the phosphorylation of NFAT by regulating glycogen synthase kinase 3 β (GSK3 β). CTGF increased the mRNA levels of *Protein kinase cyclic guanosine monophosphate (GMP) dependent type II* (*Prkg2*), the gene encoding for cGMP dependent protein kinase II (CGKII) which phosphorylates GSK3 β . Accordingly, CTGF induced GSK3 β phosphorylation and decreased the active pool of GSK3 β , a kinase that phosphorylates NFAT and leads to its nuclear export. As a consequence, CTGF favored the nuclear localization of NFATc1. Down regulation of PRKG2 by RNA interference reversed the effect of CTGF on the transactivation of the 9xNFAT reporter construct and the Rcan 1.4 promoter, confirming the role of cGKII in the activation of NFAT by CTGF. In conclusion, CTGF enhances NFAT signaling through the induction of cGKII and the phosphorylation of GSK3 β .

Keywords

CTGF; NFAT; GSK3 β ; Osteoblasts

INTRODUCTION

CTGF is a member of the CCN family of cysteine-rich (CR) secreted proteins, which includes cysteine-rich 61 (Cyr 61), connective tissue growth factor (CTGF), nephroblastoma overexpressed (Nov), and Wnt inducible secreted protein (WISP) 1, 2, and 3 (Brigstock, 2003; Brigstock et al., 2003). CTGF is expressed in bone and cartilage, and in osteoblasts CTGF is induced by bone morphogenetic protein (BMP), transforming growth factor β (TGF β), Wnt and cortisol, suggesting a possible intermediary role in the activity of these agents in bone (Luo et al., 2004; Parisi et al., 2006; Pereira et al., 2000). Our previous studies have shown that CTGF induces osteoblastogenesis *in vitro* by mechanisms independent of

BMP or Wnt signaling. In addition, CTGF activated Nuclear Factor of Activated T-cells (NFAT) signaling in osteoblasts, although the mechanisms were not explored (Smerdel-Ramoya et al., 2008).

NFAT are five transcription factors (NFATc1 to c4 and NFAT5) involved in vertebrate development and in the growth and differentiation of multiple cell types (Aliprantis et al., 2008; Mammucari et al., 2005; Heit et al., 2006). In unstimulated cells, NFATc1 to c4 are highly phosphorylated and reside in the cytoplasm. Activation of the phosphatase calcineurin dephosphorylates specific serine residues in the SRR and SPXX repeat motifs of the regulatory domain of NFAT. This induces NFAT translocation to the nucleus and activation of transcription of NFAT target genes (Okamura et al., 2000). NFAT phosphorylation by protein kinases, such as glycogen synthase kinase 3 β (GSK3 β), induces the nuclear export of NFAT preventing its transactivation (Chow et al., 2008; Hogan et al., 2003; Shen et al., 2007). Activity of GSK3 β is suppressed by phosphorylation on Serine-9, which is a target of protein kinases, such as cyclic guanosine monophosphate (cGMP) dependent protein kinase II (cGKII), the product of the *Protein kinase cGMP-dependent type II (Prkg2)* gene (Kawasaki et al., 2008). cGKII activity is induced by cGMP, and it is sustained by auto-phosphorylation on Serine 126. NFATc1 and NFATc2 are expressed during osteoblast growth and differentiation (Koga et al., 2005). The function of the calcineurin/NFAT pathway in cells of the osteoblastic lineage is controversial, and both stimulatory and inhibitory effects on osteoblastic differentiation and function have been described (Choo et al., 2009; Sun et al., 2005; Tang et al., 2002; Winslow et al., 2006b; Winslow et al., 2006a; Yeo et al., 2007b; Yeo et al., 2007a). Similarly, both stimulatory and inhibitory effects of CTGF on osteoblastic differentiation have been reported (Abreu et al., 2002; Luo et al., 2004). These studies suggest that modulation of NFAT transactivation may play a role in these apparently divergent effects of CTGF on cells of the osteoblastic lineage. Consequently, mechanisms involved in the activation of NFAT by CTGF may be central to the actions of CTGF in osteoblasts. In the present study, the mechanisms responsible for NFAT transactivation by CTGF were explored.

EXPERIMENTAL PROCEDURES

Vectors and Packaging Cell Lines

A 1,046 base pair (bp) DNA fragment containing the murine *Ctgf* coding sequence (R.P. Ryseck, Princeton, NJ), with a FLAG epitope tag on the C-terminal end (American Type Culture Collection, Manassas, VA) (ATCC) was cloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA) for use in acute transfection experiments, or cloned into the retroviral vector pLPCX (Clontech, Palo Alto, CA) for the creation of transduced cell lines. In both vectors, a cytomegalovirus (CMV) promoter directs the constitutive expression of CTGF. pLPCX control and pLPCX-CTGF vectors were transfected into Phoenix packaging cells (ATCC) by calcium phosphate/DNA co-precipitation and glycerol shock, and cells were selected for puromycin resistance (Sigma-Aldrich, St. Louis, MO), as described (Sciaudone et al., 2003). Retrovirus-containing conditioned medium was harvested, filtered through a 0.45 micron membrane and used to transduce ST-2 cells.

Cell Culture

ST-2 cells, cloned stromal cells isolated from bone marrow of BC8 mice, were grown in a humidified 5% CO₂ incubator at 37° C in α -minimum essential medium (α -MEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) (Otsuka et al., 1999; Sudo et al., 1983). For the creation of cell lines, ST-2 cells were transduced with pLPCX vector or with pLPCX-CTGF by replacing the culture medium with retroviral conditioned medium from Phoenix packaging cells in the presence

of 8 µg/ml polybrene (Sigma-Aldrich) followed by incubation for 16–18 h at 37° C (Sciaudone et al., 2003). The culture medium was replaced with fresh α -MEM, cells were grown, trypsinized, replated and selected for puromycin resistance. Transduced cells were plated at a density of 10⁴ cells/cm² and cultured in α -MEM supplemented with 10% FBS until reaching confluence (2–4 days). To examine for changes in reporter activity, subconfluent wild type ST-2 cells were transiently transfected with expression vectors, as described under transient transfections. To down regulate CTGF or PRKG2 expression, subconfluent wild type ST-2 cells were transfected with small interfering (si)RNAs as described under RNA interference (RNAi). In selected experiments, wild type ST-2 cells were cultured under the same conditions, and recombinant human CTGF (rhCTGF) protein (PeproTech, Rocky Hill, NJ) was added, as indicated in the text and legends.

Transient Transfections

To determine changes in NFAT signaling, a construct containing 9 copies of an NFAT response element, linked to a minimal α Myosin Heavy Chain promoter and cloned into pGL3 basic (9xNFAT-Luc; J.D. Molkentin, Cincinnati, OH) was tested (Dai et al., 2005). As a target gene for NFAT transactivation, a 900 base pair (bp) fragment of the *Regulators of calcineurin 1 exon 4 (rcan1.4)* promoter (Rcan1.4-Luc; B. Rothermel, University of Texas, Dallas, TX), which contains 15 NFAT consensus binding sites, cloned upstream of the *Luciferase* gene, was used. To test effects of CTGF, wild-type ST-2 cells were cultured to 70% confluence and transiently transfected with pcDNA-CTGF expression vector or pcDNA 3.1, and with the 9xNFAT-Luc or Rcan1.4-Luc construct using FuGENE6 (3µl FuGENE/2µg DNA), according to manufacturer's instructions (Roche, Indianapolis, IN). A CMV directed β -galactosidase expression construct (Clontech) was used to control for transfection efficiency. Luciferase and β -galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β -galactosidase activity.

RNA Interference

To down regulate CTGF and PRKG2 expression, small interfering (si)RNAs were obtained commercially (CTGF, 21 bp; PRKG2, 20 bp, both from Applied Biosystems, Foster City, CA). A scrambled 19 bp siRNA with no homology to known mouse or rat sequences was used as a control (Applied Biosystems) (Sharp, 2001; Elbashir et al., 2001). CTGF, PRKG2 or scrambled siRNA at 20–40 nM were transfected into 60% confluent ST-2 cells, using siLentFect lipid reagent, in accordance with manufacturer's instructions (Bio-Rad, Hercules, CA) and allowed to recover for 18 h. To test for the effect of CTGF down regulation on NFAT or Rcan1.4 promoter transactivation, cells were transfected with a 9xNFAT-Luc reporter or an Rcan1.4-Luc promoter construct in the presence of CTGF or scrambled siRNA. To test for the effects of CTGF on NFAT transactivation and the activity of Rcan1.4 promoter in the presence of PRKG2 down regulation, cells were co-transfected with pcDNA-CTGF or pcDNA 3.1 expression vectors and with 9xNFAT-Luc reporter or Rcan1.4-Luc promoter construct in the presence of PRKG2 or scrambled siRNA. To ensure adequate down regulation, total RNA was extracted in parallel cultures, and CTGF and PRKG2 mRNA levels determined by real time RT-PCR.

Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted, and CTGF, Rcan1.4 and PRKG2 mRNA levels were determined by real-time RT-PCR (Nazarenko et al., 2002a; Nazarenko et al., 2002b). For this purpose, 1–10 µg of RNA were reverse-transcribed using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen), according to manufacturer's instructions and amplified in the presence of 5'-CACTCCGGGAAATGCTGCAA GGAG[FAM]G-3' and 5'-GTTGGGTCTGGGCCAAATGT-3' primers for CTGF; 5'-

CAAGCGCAAAGGAACCTCCAGC[FAM]TG-3' and 5'-GGCAGACGCTTAACGAACGA-3' for rcan1.4; 5'-CGGCACAGAGAACTTAATTCTGGATGC[FAM]G-3' and 5'-GCCAATCTTCTTAGCGAATCCAA-3' primers for PRKG2 and 5'-CGAACCGGATAATGTGAAGTTCAAGGTT[FAM]G-3' and 5'-CTGCTTCAGCTTCTCTGCCTTT-3' primers for ribosomal protein L38 (RPL38), and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 54°–60°C for 45 cycles. Gene copy number was estimated by comparison with a standard curve constructed using CTGF (R.P. Ryseck), Rcan1.4 (ATCC) and PRKG2 (B. Hogema, Erasmus University Medical Center, Rotterdam, The Netherlands) DNA and corrected for RPL38 (ATCC) copy number (Tso et al., 1985). Reactions were conducted in a 96-well spectrofluorometric thermal iCycler (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step.

Western Immunoblot Analysis

The level of glycogen synthase kinase 3 β (GSK3 β) phosphorylation was determined in the cell layer of ST-2 cells transduced with pLPCX or pLPCX-CTGF washed with phosphate buffered saline (PBS) and extracted in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors, as described (Schreiber et al., 1989; Gazzero et al., 2005). The activity of cGKII was induced by the addition of 100 μ M cGMP (Promega), 1mM adenosine triphosphate (ATP) and kinase assay buffer (both from Cell Signaling Technology), and incubation at 30° C for 30 min, as described (Kawasaki et al., 2008). Protein concentration was determined by the DC protein assay, and 20 μ g of total cellular protein were fractionated by electrophoresis in 10% polyacrylamide gels under reducing conditions, and transferred to Immobilon P membranes. The membranes were blocked with 3% bovine serum albumin (BSA) in PBS and exposed to an antiphospho-GSK-3 β antibody or to a rabbit polyclonal antibody to unphosphorylated GSK3 β (Cell Signaling Technology). Blots were exposed to anti-rabbit or anti-mouse IgG antiserum conjugated to horseradish peroxidase and developed with a chemiluminescence detection reagent (Perkin Elmer Life Sciences, Boston, MA).

Immunofluorescence Staining and Laser Confocal Scanning Microscopy

To assess the effects of CTGF overexpression on the cellular localization of NFATc1, wild-type ST-2 cells were grown to confluence, serum deprived for 6 h, and treated with rhCTGF (1 μ g/ml) for 24 h. The translocation of NFAT was induced by the addition of 1 μ M ionomycin (Sigma) for 5 min before the take down, and was inhibited by the addition of 1 μ M of the calcineurin inhibitor FK506 (Alexis Biochemicals, San Diego, CA) for one hour before treatment with rhCTGF. Cells were grown on glass cover slips to confluence, washed with PBS and fixed in 4% formaldehyde in PBS containing 0.2% Triton X-100 (Sigma-Aldrich), for 30 min on ice. After three washes with PBS, cells were incubated overnight with blocking buffer containing 3% normal rabbit IgG (Invitrogen) in PBS, 3% BSA and 20 mM Hepes (Sigma-Aldrich). For the detection of NFATc1, cells were incubated for 1 h at 40C with anti-NFATc1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:25 in blocking buffer, washed and incubated with Alexa 488 conjugated streptavidin (1:100 dilution, Invitrogen). Cells were washed with PBS and mounted in 50% glycerol. Confocal scanning images were obtained using an LSM 510 confocal system (Carl Zeiss, Thornwood, NY).

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical differences were determined by Student's *t* test or ANOVA.

RESULTS

In accordance with previous observations, forced expression of CTGF in ST-2 stromal cells enhanced the transactivation of an NFAT reporter construct, where 9 repeats of NFAT consensus sequences direct luciferase expression (Figure 1A). Accordingly, down regulation of CTGF by RNAi, resulted in decreased transactivation of 9xNFAT-Luc (Fig. 1B) (Smerdel-Ramoya et al., 2008). In addition, CTGF induced the activity of an Rcan1.4-Luc promoter fragment, which contains 15 copies of an NFAT binding site and is transactivated by NFAT, confirming the results obtained with the 9xNFAT-Luc reporter construct (Fig. 1C) (Yang et al., 2000). Moreover, Rcan 1.4 mRNA levels were increased in CTGF overexpressing cells, confirming the stimulatory effect of CTGF on NFAT transactivation (Fig. 1E). Accordingly, down regulation of CTGF by RNAi, resulted in a decrease in the activity of an Rcan1.4 -Luc promoter fragment (Fig. 1D) and in the level of Rcan1.4 mRNA (Fig. 1F).

In unstimulated cells, NFAT is phosphorylated and resides in the cytoplasm. Activation of the phosphatase calcineurin dephosphorylates NFAT, which translocates to the nucleus to regulate gene transcription, whereas GSK3 β phosphorylates NFAT inducing its nuclear export (Hogan et al., 2003). In accordance with the stimulatory effects on NFAT transactivation, rhCTGF induced the translocation of NFAT from the cytoplasm into the nucleus (Figure 2). The effect mimicked the effect of the calcineurin inducer ionomycin and was prevented by the calcineurin inhibitor FK506.

To determine the mechanism responsible for the translocation of NFAT in the presence of CTGF, we explored whether CTGF regulated GSK3 β , a kinase that phosphorylates NFAT and regulates its activity. In preliminary experiments, we explored the actions of CTGF on the expression of *Prkg2*, the gene encoding cGKII, a kinase that phosphorylates and inhibits GSK3 β (Kawasaki et al., 2008). PRKG2 mRNA levels were increased 4 fold in pLPCX-CTGF transduced cells when compared to pLPCX controls, demonstrating that CTGF overexpression induces PRKG2 transcript levels (Figure 3A). To test whether this effect was responsible for the induction of NFAT transactivation by CTGF, lysates from ST-2 cells transduced with pLPCX-CTGF or pLPCX as control were treated with cyclic GMP, a physiological activator of cGKII. Lysates were tested for the levels of total and GSK3 β phosphorylated at Serine-9 by Western blot analysis. CTGF induced the level of phospho-GSK3 β so that the pool of active GSK3 β was reduced (Figure 3B).

To confirm that induction of PRKG2 was relevant to the actions of CTGF on the activation of NFAT signaling, the effects of CTGF on NFAT transactivation were tested in the context of PRKG2 down regulation by RNAi. CTGF induced the transactivation of 9xNFAT-Luc reporter and Rcan 1.4 promoter, and the effect was decreased in cells transfected with pcDNA-CTGF following the down regulation of PRKG2 (Figure 4 A and C). In accordance with the results obtained in transiently transfected cells, ST-2 cells transduced with pLPCX-CTGF enhanced the transactivation of 9xNFAT-Luc reporter and Rcan 1.4 promoter, and the effect was decreased by the down regulation of PRKG2 (Figure 4 B and D). These results demonstrate that the up regulation of PRKG2 by CTGF is mechanistically relevant to the effects of CTGF on NFAT transactivation.

DISCUSSION

In the present study, we examined the role of CTGF in the regulation of NFAT transactivation in ST-2 stromal cells. We confirmed our previous observations demonstrating that forced overexpression of CTGF increases NFAT transactivation (Smerdel-Ramoya et al., 2008). We demonstrate that CTGF induces the transcription of

Rcan1.4 and increases the transcript levels of this NFAT target gene. Accordingly, down regulation of CTGF by RNAi caused a decrease in NFAT activity suggesting that CTGF plays a physiological role in the control of NFAT signaling. CTGF induced the nuclear localization of NFATc1 explaining the augmented NFAT activity in cells of the osteoblastic lineage.

We explored the mechanisms involved in the induction of NFAT transactivation by CTGF. We demonstrated that CTGF favors the nuclear accumulation of NFAT by inhibiting the activity of GSK3 β , a kinase that phosphorylates and inhibits NFAT by inducing export from the nucleus to the cytoplasm. The results obtained are in agreement with previous observations demonstrating a role of GSK3 β in NFAT phosphorylation and nuclear export (Zamurovic et al., 2004). The data presented indicate that CTGF increases the expression of *Prkg2*, which is the gene coding for cGKII, a kinase that phosphorylates GSK3 β on Serine-9 (Kawasaki et al., 2008). As a consequence, the pool of active GSK3 β decreases and its potential to phosphorylate NFAT is tempered.

In our previous work, we demonstrated that CTGF may activate NFAT through additional mechanisms. CTGF induced Hairy Enhancer of Split (Hes) 1, a basic helix-loop-helix transcription factor able to form homo- and hetero-dimers with related transcription factors. Hes related with an YRPW motif (Hey)-1 is a transcription factor related to Hes and a suppressor of NFAT transactivation (Zamurovic et al., 2004). CTGF induces Hes1 expression, and the formation of Hes1-Hey1 heterodimers, relieving the suppressive effect of Hey1 on NFAT transactivation (Smerdel-Ramoya et al., 2008). Calcineurin is a calcium/calmodulin dependent phosphatase that dephosphorylates NFAT and promotes its nuclear localization (Graef et al., 2001). In keratinocytes, Hes1 also suppresses the expression of calcipressin, an inhibitor of calcineurin, and as a consequence enhances calcineurin activity and NFAT activation (Iso et al., 2001). However, we found there is no evidence that this mechanism operates in cells of the osteoblastic lineage. Our findings indicate that CTGF enhances the activation of NFAT by at least 2 mechanisms, the induction of PRKG2 with a consequent decrease in the phosphorylation of GSK3 β and the induction of Hes1 with the consequent dimerization of Hey1. It is of interest that Hes1 and Hey1 are Notch target genes, and in cells of the osteoblastic lineage, Notch induces Hey1 to a greater extent than Hes1 (Iso et al., 2001). Notch decreases the transactivation of NFAT and CTGF decreases Notch signaling in osteoblasts (Smerdel-Ramoya et al., 2008; Zanotti et al., 2009), and this may serve as an additional mechanism to maintain NFAT activity in this cells by CTGF.

The consequences of NFAT signaling in osteoblastic cells are controversial and both stimulatory and inhibitory effects on osteoblastic differentiation and function have been reported (Tang et al., 2002; Koga et al., 2005; Sun et al., 2005; Winslow et al., 2006b; Yeo et al., 2007a; Yeo et al., 2007b; Choo et al., 2009). Recent studies from our laboratory have shown that the differentiation of ST-2 cells is not affected by the transduction of vectors expressing a constitutive active form of NFATc1 or NFATc2 (Zanotti et al, unpublished results). This would suggest that NFAT does not enhance osteoblastogenesis in ST-2 cells so that the activation of NFAT does not explain the stimulatory effects of CTGF on osteoblastogenesis in this cell line. However, they may be relevant to alternate actions of CTGF in osteoblastic cells. The induction of osteoblastic cell differentiation by CTGF can be explained by the suppression of Notch, a molecule that inhibits osteoblastogenesis and causes osteopenia (Canalis, 2008; Zanotti et al., 2008; Zanotti and Canalis, 2010).

In conclusion, CTGF induces transactivation and nuclear localization of NFAT by inducing the CGKII and the phosphorylation and degradation of GSK3 β .

Acknowledgments

This work was supported by Grants AR021707 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases and DK045227 from the National Institute of Diabetes Digestive and Kidney Diseases.

The authors thank Drs. J.D.Molkentin for 9xNFAT-Luc construct; B. Hogema for cGKII cDNA; B. Rothermel for Rcan1.4 promoter, R. P. Rysek for CTGF cDNA, Melissa Burton for technical help, and Mary Yurczak for secretarial assistance.

The abbreviations used are

ATCC	American Type Culture Collection
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
bp	base pair
cCGKII	cGMP-dependent protein kinase type II
cGMP	cyclic guanosine monophosphate
CMV	cytomegalovirus
CTGF	connective tissue growth factor
Cyr 61	cysteine-rich 61
FBS	fetal bovine serum
GSK3β	glycogen synthase kinase 3 β
kDa	kilodaltons
MEM	minimum essential medium
NFAT	nuclear factor of activated T-cells
Nov	Nephroblastoma overexpressed
PBS	Phosphate buffered saline
PRKG2	protein kinase cGMP-dependent, type II
Rcan1.4	regulators of calcineurin 1 exon 4
RNAi	RNA interference
RPL38	ribosomal protein L38
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TGF-β	transforming growth factor

REFERENCES

- Abreu JG, Ketpura NI, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. *Nat. Cell Biol.* 2002; 4:599–604. [PubMed: 12134160]
- Aliprantis AO, Ueki Y, Sulyanto R, Park A, Sigrist KS, Sharma SM, Ostrowski MC, Olsen BR, Glimcher LH. NFATc1 in mice represses osteoprotegerin during osteoclastogenesis and dissociates systemic osteopenia from inflammation in cherubism. *J. Clin. Invest.* 2008; 118:3775–3789. [PubMed: 18846253]
- Brigstock DR. The CCN family: a new stimulus package. *J. Endocrinol.* 2003; 178:169–175. [PubMed: 12904165]

- Brigstock DR, Goldschmeding R, Katsube KI, Lam SC, Lau LF, Lyons K, Naus C, Perbal B, Riser B, Takigawa M, Yeger H. Proposal for a unified CCN nomenclature. *Mol. Pathol.* 2003; 56:127–128. [PubMed: 12665631]
- Canalis E. Notch signaling in osteoblasts. *Sci. Signal.* 2008; 1:e17.
- Choo MK, Yeo H, Zayzafoon M. NFATc1 mediates HDAC-dependent transcriptional repression of osteocalcin expression during osteoblast differentiation. *Bone.* 2009; 45:579–589. [PubMed: 19463978]
- Chow W, Hou G, Bendeck MP. Glycogen synthase kinase 3 β regulation of nuclear factor of activated T-cells isoform c1 in the vascular smooth muscle cell response to injury. *Exp. Cell Res.* 2008; 314:2919–2929. [PubMed: 18675800]
- Dai YS, Xu J, Molkentin JD. The DnaJ-related factor Mrj interacts with nuclear factor of activated T cells c3 and mediates transcriptional repression through class II histone deacetylase recruitment. *Mol. Cell Biol.* 2005; 25:9936–9948. [PubMed: 16260608]
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 2001; 411:494–498. [PubMed: 11373684]
- Gazzerro E, Pereira RC, Jorgetti V, Olson S, Economides AN, Canalis E. Skeletal overexpression of gremlin impairs bone formation and causes osteopenia. *Endocrinology.* 2005; 146:655–665. [PubMed: 15539560]
- Graef IA, Chen F, Crabtree GR. NFAT signaling in vertebrate development. *Curr. Opin. Genet. Dev.* 2001; 11:505–512. [PubMed: 11532391]
- Heit JJ, Apelqvist AA, Gu X, Winslow MM, Neilson JR, Crabtree GR, Kim SK. Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. *Nature.* 2006; 443:345–349. [PubMed: 16988714]
- Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* 2003; 17:2205–2232. [PubMed: 12975316]
- Iso T, Sartorelli V, Chung G, Shichinohe T, Kedes L, Hamamori Y. HERP, a new primary target of Notch regulated by ligand binding. *Mol. Cell Biol.* 2001; 21:6071–6079. [PubMed: 11486044]
- Kawasaki Y, Kugimiya F, Chikuda H, Kamekura S, Ikeda T, Kawamura N, Saito T, Shinoda Y, Higashikawa A, Yano F, Ogasawara T, Ogata N, Hoshi K, Hofmann F, Woodgett JR, Nakamura K, Chung UI, Kawaguchi H. Phosphorylation of GSK-3 β by cGMP-dependent protein kinase II promotes hypertrophic differentiation of murine chondrocytes. *J. Clin. Invest.* 2008; 118:2506–2515. [PubMed: 18551195]
- Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrughe B, Nakashima K, Takayanagi H. NFAT and Osterix cooperatively regulate bone formation. *Nat. Med.* 2005; 11:880–885. [PubMed: 16041384]
- Luo Q, Kang Q, Si W, Jiang W, Park JK, Peng Y, Li X, Luu HH, Luo J, Montag AG, Haydon RC, He TC. Connective tissue growth factor (CTGF) is regulated by Wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells. *J. Biol. Chem.* 2004; 279:55958–55968. [PubMed: 15496414]
- Mammucari C, Tommasi dV, Sharov AA, Neilson J, Havrda MC, Roop DR, Botchkarev VA, Crabtree GR, Dotto GP. Integration of Notch 1 and calcineurin/NFAT signaling pathways in keratinocyte growth and differentiation control. *Dev. Cell.* 2005; 8:665–676. [PubMed: 15866158]
- Nazarenko I, Lowe B, Darfler M, Ikonomi P, Schuster D, Rashtchian A. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res.* 2002a; 30:e37. [PubMed: 11972352]
- Nazarenko I, Pires R, Lowe B, Obaidy M, Rashtchian A. Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. *Nucleic Acids Res.* 2002b; 30:2089–2195. [PubMed: 11972350]
- Okamura H, Aramburu J, Garcia-Rodriguez C, Viola JP, Raghavan A, Tahiliani M, Zhang X, Qin J, Hogan PG, Rao A. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol. Cell.* 2000; 6:539–550. [PubMed: 11030334]

- Otsuka E, Yamaguchi A, Hirose S, Hagiwara H. Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Am. J. Physiol.* 1999; 277:C132–C138. [PubMed: 10409116]
- Parisi MS, Gazzerro E, Rydziel S, Canalis E. Expression and regulation of CCN genes in murine osteoblasts. *Bone.* 2006; 38:671–677. [PubMed: 16311085]
- Pereira RC, Durant D, Canalis E. Transcriptional regulation of connective tissue growth factor by cortisol in osteoblasts. *Am. J. Physiol Endocrinol. Metab.* 2000; 279:E570–E576. [PubMed: 10950824]
- Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 1989; 17:6419. [PubMed: 2771659]
- Sciaudone M, Gazzerro E, Priest L, Delany AM, Canalis E. Notch 1 impairs osteoblastic cell differentiation. *Endocrinology.* 2003; 144:5631–5639. [PubMed: 12960086]
- Sharp PA. RNA interference--2001. *Genes Dev.* 2001; 15:485–490. [PubMed: 11238371]
- Shen T, Cseresnyes Z, Liu Y, Randall WR, Schneider MF. Regulation of the nuclear export of the transcription factor NFATc1 by protein kinases after slow fibre type electrical stimulation of adult mouse skeletal muscle fibres. *J. Physiol.* 2007; 579:535–551. [PubMed: 17185343]
- Smerdel-Ramoya A, Zanotti S, Deregowski V, Canalis E. Connective tissue growth factor enhances osteoblastogenesis in vitro. *J. Biol. Chem.* 2008; 283:22690–22699. [PubMed: 18583340]
- Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell Biol.* 1983; 96:191–198. [PubMed: 6826647]
- Sun L, Blair HC, Peng Y, Zaidi N, Adebajo OA, Wu XB, Wu XY, Iqbal J, Epstein S, Abe E, Moonga BS, Zaidi M. Calcineurin regulates bone formation by the osteoblast. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:17130–17135. [PubMed: 16286645]
- Tang L, Ebara S, Kawasaki S, Wakabayashi S, Nikaido T, Takaoka K. FK506 enhanced osteoblastic differentiation in mesenchymal cells. *Cell Biol. Int.* 2002; 26:75–84. [PubMed: 11779223]
- Tso JY, Sun XH, Kao TH, Reece KS, Wu R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.* 1985; 13:2485–2502. [PubMed: 2987855]
- Winslow MM, Gallo EM, Neilson JR, Crabtree GR. The calcineurin phosphatase complex modulates immunogenic B cell responses. *Immunity.* 2006a; 24:141–152. [PubMed: 16473827]
- Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, Crabtree GR. Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev. Cell.* 2006b; 10:771–782. [PubMed: 16740479]
- Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN, Bassel-Duby R, Williams RS. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. *Circ. Res.* 2000; 87:E61–E68. [PubMed: 11110780]
- Yeo H, Beck LH, McDonald JM, Zayzafoon M. Cyclosporin A elicits dose-dependent biphasic effects on osteoblast differentiation and bone formation. *Bone.* 2007a; 40:1502–1516. [PubMed: 17392048]
- Yeo H, Beck LH, Thompson SR, Farach-Carson MC, McDonald JM, Clemens TL, Zayzafoon M. Conditional disruption of calcineurin B1 in osteoblasts increases bone formation and reduces bone resorption. *J Biol. Chem.* 2007b; 282:35318–35327. [PubMed: 17884821]
- Zamurovic N, Cappellen D, Rohner D, Susa M. Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene *Hey1* inhibits mineralization and *Runx2* transcriptional activity. *J. Biol. Chem.* 2004; 279:37704–37715. [PubMed: 15178686]
- Zanotti S, Canalis E. Notch and the Skeleton. *Mol. Cell Biol.* 2010; 30:886–896. [PubMed: 19995916]
- Zanotti S, Smerdel-Ramoya A, Durant D, Canalis E. Hairy Enhancer of Split (Hes)-1 Inhibits Osteoblastic Function and Causes Osteopenia In Vivo. 2009
- Zanotti S, Smerdel-Ramoya A, Stadmeier L, Durant D, Radtke F, Canalis E. Notch Inhibits Osteoblast Differentiation And Causes Osteopenia. *Endocrinology.* 2008; 149:3890–3899. [PubMed: 18420737]

Figure 1.

Effect of CTGF on NFAT transactivation and on Rcan1.4 expression. In Panels A and C, ST-2 cells were cultured to subconfluence and transiently transfected with control pcDNA (white bars) or pcDNA-CTGF (black bars), and with 9xNFAT-Luc (Panel A) or with a 0.9 kb fragment of Rcan1.4 promoter construct (Rcan1.4-Luc) (Panel C), and CMV/ β -galactosidase expression vectors. In Panels B and D, ST-2 cells were transfected with CTGF (black bars) or scrambled (white bars) small interfering (si)RNAs and with 9xNFAT-Luc (Panel B) or Rcan1.4-Luc (Panel D) and CMV/ β -galactosidase reporter constructs, and harvested after 48 h. In Panel E, ST-2 cells transduced with pLPCX (white bars) and pLPCX-CTGF (black bars), and in Panel F, ST-2 cells transfected with CTGF (black bars)

or scrambled (white bars) small interfering (si)RNAs, were cultured to confluence and total RNA was extracted and subjected to real time RT-PCR for the determination of *Rcan1.4* mRNA. Data are expressed as *Rcan1.4* copy number corrected for *Rpl38* expression. Bars represent means $\pm$ SEM for 6 (Panels A, B, C and D) or 4 (Panel E and F) observations. Down regulation of CTGF was documented in RNA extracted from parallel cultures and subjected to real time RT-PCR. *Ctgf* copy number corrected for *Rpl38* was (means $\pm$ SEM; n = 4) 2.9 ± 0.7 in control and 1.6 ± 0.2 in cells transfected with CTGF siRNA ($p < 0.05$) (Panels B and E). *Significantly different between control and CTGF and between control and CTGF down regulated cells, $p < 0.05$.

Figure 2.

Effect of CTGF on the cellular localization of NFATc1. ST-2 cells were cultured to confluence, serum deprived for 6 h and treated with vehicle (left panels) or rhCTGF at 1 µg/ml (right panels) for 24 h. To control for nuclear translocation of NFAT, cells were treated with ionomycin at 1 µM to activate (middle panels), or with FK506 at 1 µM to inhibit (lower panels) nuclear translocation of NFAT in the absence or presence of rhCTGF. Cells were fixed and subjected to immunostaining for the detection of NFATc1 by confocal microscopy.

Figure 3.

Effect of CTGF overexpression on cGKII-dependent GSK3 β phosphorylation in ST-2 stromal cells. In Panel A, total RNA from confluent pLPCX (white bars) or pLPCX-CTGF (black bars) transduced cultures was reverse-transcribed, and amplified by real time RT-PCR in the presence of specific primers to detect *Prkg2* mRNA. Data are expressed as *Prkg2* copy number corrected for *Rpl38* expression. Bars represent means $\pm$ SEM for 4 observations. *Significantly different between cells expressing CTGF and control cells, $p < 0.05$. In Panel B, ST-2 cells transduced with pLPCX vector (-) or pLPCX-CTGF (+) were cultured to confluence and serum-deprived for 6 h. Total cell lysates were treated with ATP at 1 mM, with (+) or without (-) cGMP at 100 μ M. Lysates were resolved by gel electrophoresis and transferred to Immobilon P membranes, which were incubated either with antibodies to phosphorylated GSK3 β (pGSK3 β) or to unphosphorylated GSK3 β .

Figure 4.

Effect of CTGF on NFAT transactivation in the context of PRKG2 down regulation by RNA interference (RNAi) using siRNAs directed to PRKG2 in ST-2 stromal cells. In Panels A and C, ST-2 cells were transiently transfected with control pcDNA (white bars) or pcDNA-CTGF (black bars). In Panels B and D, cells were transduced with pLPCX (white bars) or pLPCX-CTGF (black bars). Cells were cultured to subconfluence and transiently transfected PRKG2 or scrambled siRNA, and after 18 h transfected with 9xNFAT-Luc (Panels A and B) or with a 0.9 kb fragment of *Rcan.41* promoter construct (Rcan1.4-Luc) (Panels C and D), and a CMV/ β -galactosidase expression vector, and harvested after 48 h. Bars represent means $\pm$ SEM for 6 observations. Down regulation of PRKG2 was documented in RNA extracted from parallel cultures and subjected to real time RT-PCR. *Prkg2* copy number corrected for *Rpl38* was (means $\pm$ SEM; n = 4) 7.3 ± 0.98 in cells transfected with scrambled and 1.2 ± 0.3 in cells transfected with PRKG2 siRNA ($p < 0.05$). *Significantly different between control and CTGF, $p < 0.05$. + Significantly different between control and PRKG2 down regulated cells, $p < 0.05$.