Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Temple University – Of The Commonwealth System of Higher Education

2. **Reporting Period (start and end date of grant award period):** 1/1/2010-12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Germaine A Calicat, MA

4. **Grant Contact Person's Telephone Number:** 215.204.7655

5. **Grant SAP Number:** 4100050909

6. **Project Number and Title of Research Project:** Project 22: Integrin-mediated adhesion of osteoblasts to CTGF induces intracellular signaling and differentiation

7. **Start and End Date of Research Project:** 7/1/2011 – 6/30/2013

8. **Name of Principal Investigator for the Research Project:** Steven N. Popoff, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 42,873.16

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Hendesi, Honey	Graduate Assistant	100%	

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_X_____ No_____

If yes, please indicate the source and amount of other funds:

NIH, R01 AR47432

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No____X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

Data will be used to support competing renewal application for R01 AR47432 (Popoff, PI).

12. Future of Research Project. What are the future plans for this research project?

We plan to continue and expand the scope of the project to examine the effects of varying CTGF expression levels on osteoblast attachment to known extracellular matrix proteins. We will use genetically engineered cells (osteoblast) that express no CTGF, normal levels of CTGF or dramatically increased levels of CTGF.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White			1	
Black				
Asian				
Other				
Unknown				
Total			**1**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes___X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

Experiments completed as part of this project helped train a clinician scientist and generated novel information regarding CTGF-specific mechanisms of action in osteoblasts. These findings have implications that can be useful in developing new therapeutic strategies to treat bone loss (osteoporosis).

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Background

Previous studies have established that CTGF can function as an ECM-associated (matricellular) protein and that some of its biological activities reside in the unique interactions with specific integrin subtypes expressed by a given target cell to initiate intracellular signaling cascades that can regulate cytoskeletal organization, gene transcription, and cell differentiation. The activation of integrin signaling pathways provides a mechanistic interpretation for some of the biological activites of CTGF. In preliminary experiments, we demonstrated that osteoblasts can attach to

an immobilized CTGF substrate via the αvβ1 integrin and that cell surface HSPGs act as co-receptors in this integrin/CTGF interaction. Osteoblast attachment to CTGF resulted in cell spreading (formation of pseudopodia and filopodia), the formation of focal adhesion complexes and cytoskeletal reorganization (formation of stress fibers), and activation (phosphorylation) of focal adhesion kinase (FAK) and the MAPK, Erk1/2 (preliminary data). Recent studies have demonstrated that FAK signaling provides a link between activation of Erk1/2 by the ECM, and stimulates subsequent phosphorylation of the Runx2/Cbfa-1 transcription factor that controls gene expression and FAK signaling is required for osteogenic differentiation of mesenchymal stem cells cultured on laminin. In long term cultures, we also demonstrated enhanced osteoblast differentiation as evidenced by significant increases in alkaline phosphase activity and mineralization when cells were cultured on an immobilized CTGF substrate (preliminary data). Our data support the hypothesis that CTGF acts as a matricellular protein that binds to specific cell surface integrins on osteoblasts to initiate integrin-activated signaling, cytoskeletal reorganization, and regulate cell differentiation.

Specific aims

The aims of this proposal are to: 1) identify the integrin binding site in the fourth domain of the CTGF protein ($CTGF_4$); and 2) conduct a comparative analysis of the effects of the full-length protein ($CTGF_{1-4}$), the fourth domain only ($CTGF_4$), and the peptide within the fourth domain ($CTGF_4$-P) that supports integrin-mediated adhesion. Proposed experiments are expected to generate novel information regarding CTGF-specific mechanisms of action in osteoblasts. With the exception of aim 1, the objectives and aims as listed above and in the original proposal were successfully achieved during the project period. Aim 1 was not successful despite multiple attempts to generate and test various peptides derived from the sequence of this fourth domain. The results were inconclusive at this time. However, there were a number of experiments added to complement and expand upon the confirmation of the preliminary studies, in a manner that provided sufficient data for complete publishable unit (peer-reviewed publication). A detailed report of the methods and findings of the study follows.

Methodology

Cell culture

MC3T3-E1 subclone 4, a mouse pre-osteoblast cell line, was purchased from ATCC® (Manassas, VA). Cells were cultured in alpha minimal essential medium (Corning Cellgro, Manassas, VA) containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Corning Cellgro). Cells were incubated at 37 °C and 5% CO2.

Western blotting

MC3T3-E1 culture dishes were washed with cold 1X PBS and cells were lysed in 1X RIPA lysis buffer (Millipore, Billerica, MA) containing 1% protease inhibitor (Sigma-Aldrich, St. Louis, MO) then incubated for 1 hour at 4°C. Protein concentration of lysates was determined using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Membranes were blocked with 5% BSA/PBS- 0.1%Tween20 or LI-COR® Blocking Buffer (LiCore Biosciences, St. Lincoln, NE) for 1 hour at room temperature and then incubated with the following primary antibodies: 1:500 rabbit polyclonal anti-phospho-FAK (Tyr925) (Cell Signaling Technology,

Danvers, MA), 1:500 rabbit polyclonal anti- FAK (Cell Signaling Technology), 1:500 rabbit monoclonal anti-phospho-ERK1/2 (Cell Signaling Technology), 1:500 rabbit monoclonal anti-ERK1/2 (Cell Signaling Technology) and 1:1000 rabbit polyclonal anti-actin (Sigma-Aldrich) overnight at 4°C . Membranes were washed with 1X PBS- 0.1%Tween20 and incubated with 1:5000 horseradish peroxidase conjugated to a donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) or 1:5000 IRDye® 800cw, donkey anti-rabbit secondary antibody from Odyssey® infrared imaging system (LiCor Biosciences). Primary and secondary antibodies were diluted in blocking buffer. Bands were visualized with using a chemiluminescence detection system (Thermo Scientific, Rockford, IL) or LI-COR® infrared imaging system.

Adhesion assay

Ninety-well non-tissue culture treated plates (Falcon® Becton Dickinson, Franklin Lakes, NJ) were coated with fibronectin (Sigma-Aldrich), recombinant CTGF (ProSpec, Ness-Ziona, Israel), or 1% BSA (Fisher Scientific, Pittsburgh, PA) in PBS and were left to dry in a tissue culture hood. 1% BSA was added to the wells and the plates were incubated at 4°C for 1 hour to block non-specific binding sites. BSA was discarded and wells were washed with 1X PBS prior to adding 2 $x10^4$ MC3T3-E1 cells (passage 5 to 10) to the wells and incubation at 37°C for 45 minutes. To block the various integrins of interest, cells were incubated with 5 μg/ml BioLegend® (San Diego, CA) monoclonal integrin antibodies against αv, α2, α5, β1, β3, β5 or normal IgG for 30 minutes at 37°C prior to seeding in 96-well plates. Wells were washed with 1X PBS. CyQuant® NF dye (Invitrogen, Carlsbad, Ca) was added to each well and the plates were incubated at 37°C for 1 hour. Fluorescence was measured using a microplate reader with excitation at ~485 nm and emission detection at ~530nm. Relative fluorescence units (RFUs) were converted to cell number using standard curve made by performing adhesion assay for different cell numbers.

Immunofluorescence staining

Glass chamber slides (Nalge Nunc International, Rochester, NY) were coated with 2 μg/ml recombinant CTGF (ProSpec), 2 μg/ml fibronectin (Sigma-Aldrich) or 1% BSA (Fisher Scientific) in PBS and were left to dry in a tissue culture hood. $2x10^3$ MC3T3-E1 cells were added to chambers and incubated at 37°C for 24 hours. Cells were fixed with 4% paraformaldehyde in phosphate buffer (Affymetrix, Santa Clara, CA) for 15 minutes, washed 3 times with washing buffer (1X PBS containing 0.05% Tween20) and then blocked with blocking solution (2.5% BSA in PBS) for 30 minutes at room temperature. After fixation, 1:200 primary antibody rabbit polyclonal anti-αvβ1 (Bioss, Woburn, MA) and 1:500 mouse monoclonal anti-vinculin (Millipore) diluted in blocking solution, were added to the chambers, which were then incubated overnight at 4°C. After 3 washes with washing buffer, fluorescence secondary antibodies conjugated to DyLight™549 or DyLight™ 488 (Jackson Immunoresearch) and diluted 1:2000 in blocking solution, or TRITC-conjugated phalloidin (Millipore) diluted 1:5000 in blocking solution, were added to the chambers, which were then incubated for 1 hour at room temperature. After 3 washes with washing buffer, the chamber slides were cover slipped using Vectashield ® mounting medium with Dapi fluorescence (Vector, Burlingame, CA). Fluorescence imaging was performed using a Nikon E1000. Cell spreading areas were measured using Image J.

Rac activity assay

Rac activity assay was performed on MC3T3-E1 cells cultured for 2 hours on dishes coated with recombinant CTGF (ProSpec), fibronectin (Sigma-Aldrich), 1%BSA (Fisher Scientific), or uncoated dishes as a negative control. A Rac1 Activation Assay Biochem Kit™ (Cytoskeleton, Denver, CO) was used to pull down active Rac1. Then, 300 μg of total cell protein was incubated with 10 μg PAK-PBD beads, and incubated for 1 hour at 4°C. Beads were washed once with the kit's washing buffer. After centrifugation, 20 μl of Laemmli sample buffer containing 5% β-mercaptoethanol was added to the beads and samples were boiled for 2 minutes. Western Blot analysis was performed on the samples using a mouse monoclonal anti-Rac1 antibody (Cytoskeleton).

Co-Immunoprecipitation

When the MC3T3-E1 cells in the culture dishes were 70% confluent culture dishes, they were harvested and the cells were lysed with1X RIPA lyses buffer (Millipore) containing 1% protease inhibitor (Sigma-Aldrich). Rabbit polyclonal anti-αvβ1 antibody (Bioss), rat monoclonal anti-α2β1 antibody (US BioLogical, Swampscott, MA), rat monoclonal anti-α5β1 antibody (US BioLogical), and normal IgG as a negative control (Santa Cruz Biotechnology, Santa Cruz, CA) were used to immunoprecipitate different integrin heterodimers, using the Catch and Release kit (Millipore). In spin columns, 500 μg of cell lysate, 5μg of primary antibody or IgG and 10 μl of Antibody Capture Affinity Ligand were incubated at room temperature for 30 minutes. Spin columns were centrifuged, the flow-through was discarded, and the beads in columns were washed 3 times with the kit's washing solution. To elute the protein of interest, 1X Denaturing Elution Buffer containing β-mercaptoethanol was added to columns and heated at 97°C for 3 minutes. Denatured proteins were collected by centrifugation. For Western blot analysis, a goat polyclonal anti-CTGF antibody (Santa Cruz Biotechnology) diluted 1:200 in blocking buffer, and then a horseradish peroxidase conjugated donkey anti-goat secondary antibody (Jackson Immunoresearch) diluted 1:5000 in blocking buffer were applied as described previously.

Alkaline phosphatase staining and activity

Forty eight-well plates (Falcon® Becton Dickinson) were coated with 2 μg/ml recombinant CTGF (ProSpec) or 1% BSA (Fisher Scientific) in PBS. 1.2 x 10^4 MC3T3-E1 cells suspended in α-MEM containing 10%FBS and 1% penicillin/streptomycin were seeded in each well. At day 3 of culture, cells were treated with osteogenic medium (50 μg/ml Ascorbic acid+ 10 mM β-glycerophosphate in α-MEM+ 10%FBS and 1% penicillin/ streptomycin), which was changed every 3 days. Alkaline phosphatase staining was performed on day 14 of culture using a Leukocyte Alkaline Phosphatase kit and its solutions (Sigma-Aldrich). After washing cells in 1X HBSS, they were fixed in citrate buffer containing acetone and formaldehyde. After fixation, cells were washed with ddH2O and stained with a solution containing sodium nitrate, FRV-alkaline solution and napthol AS-BI alkaline solution for 25 minutes at room temperature. Cells were washed with ddH2O, air-dried and images were taken using a Nikon Eclipse TE300 inverted microscope.

Alkaline phosphtase activity was quantified using a Quantichrome™ Alkaline Phosphatase kit (BioAssays Systems, Hayward, CA). On day 14 of culture, cells from additional cultures were digested with ddH2O + 0.2% Triton X-100 and incubated at room temperature for 20 minutes. Cells were scraped, centrifuged, supernatant collected and assay was performed

according to the manufacturer's protocol. Alkaline phosphatase activity was normalized to total amount of protein, determined using a BCA assay kit (Pierce Biotechnology).

Alizarin red staining

MC3T3-E1 osteogenic cells were cultured on recombinant CTGF (ProSpec) or 1% BSA (Fisher Scientific) coated 48-well plates (Falcon® Becton Dickinson) and terminated on day 40 to evaluate osteoblast mineralization. Cells were washed with ddH_2O and fixed with 10% paraformaldehyde in phosphate buffer for 15 minutes at room temperature. After washing cells with 1X PBS, 40 mM Alizarin red stain (Sigma-Aldrich) was added to wells and incubated at room temperature for 15 minutes. Cells were washed with ddH_2O and air-dried. Images were taken using a Nikon Eclipse TE300 inverted microscope. ImageJ software was used to measure nodule area.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed on MC3T3-E1 cells cultured on recombinant CTGF (ProSpec) coated, 1%BSA (Fisher Scientific) coated or un-coated culture dishes, and treated for 7 days with 50 μg/ml Ascorbic acid + 10 mM β-glycerophosphate in α-MEM+ 10%FBS and 1% penicillin/streptomycin. Cultured cells were cross-linked with 1% paraformaldehyde (Affymetrix) in phosphate buffer for 10 minutes at room temperature. Cells were scraped and collected in 1% PBS, centrifuged, and pellets were resuspended in lysis buffer (50mM Hepes-KOH [pH 8.0], 1mM EDTA, 140mM NaCl, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100, 1mM PMSF) containing 1% protease inhibitor (Sigma-Aldrich) and incubated at room temperature for 10 minutes. Samples were centrifuged and pellets were resuspended in dilution buffer (10mM Tris-HCl [pH 8.0], 1% triton X-100, 0.1% SDS, 1mM EDTA, 140 mM NaCl, 1mM PMSF and 1% protease inhibitor). Lysates were sonicated 8 times, each time for 10 seconds at 4°C. After high-speed centrifugation (10 minutes, 13,000 x g), 25μl of supernatants were stored for input of ChIP reaction. The remainder of the supernatants were incubated with protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 hour at 4°C to pre-clear samples. Runx2 antibody (Cell Signaling Technology), Acetyl-Histone4 antibody (Millipore) as a positive control and normal rabbit IgG (Santa Cruz Biotechnology) as a negative control were added to pre-cleared supernatants and incubated overnight at 4°C. Immuno-complexes were isolated by adding 60 μl of protein A/G-agarose beads, incubated for 1 hour at 4°C, then centrifugation for 1 minute at 2,000 x g. The beads were washed with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100. 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), LiCl immune complex wash buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, [pH 8.1]) and TE buffer (10mM Tris-HCl, 1mM EDTA, [pH 8.0]). The immunocomplexes were eluted by resuspending the beads in 500 μl of elution buffer (1% SDS, 0.1 M NaHCO3 [pH 8.0]). After centrifugation, supernatants were collected, 20 μl of 5M NaCl was added to reverse cross-links, and samples were heated overnight at 65°C. Next, 10 μl of 1M Tris-HCl [pH 6.5], 10 μl of 0.5 M EDTA [pH 8.0], and 2 μl of 10mg/ml proteinase K (Sigma) were added to the eluates, which were incubated for 1 hour at 45 °C. DNA fragments were extracted with 500 μl of phenol chloroform (Acros Organics) and centrifuged for 3 minutes at maximum speed at room temperature. Following centrifugation, 50 μl of 3 M potassium acetate [pH 5.5], 2 μl Glycogen glycogen (Roche, Basel Switzerland) and 1 ml 100% ethanol were added to the samples and incubated on dry ice for 2 hours. Samples were

centrifuged and pellets were washed with 70% ethanol and resuspended in ddH20. DNA that was isolated from the immuno-complexes and DNA from the input (25 μl of lysate after sonication) were subjected to Quantitative PCR.

Quantitative PCR

The quantification of ChIP-DNA by quantitative PCR was performed using primers from a previous study. For detection of the Runx2 binding site on the mouse osteocalcin gene promoter (OSE2b; -611/-605) F': TGCCTCCATAGATCCGGTT and R': CCCACAATGGGCTAGGCTC, and for negative control primer that does not produce a PCR product with ChIP-DNA, F': CTGCCAGGCTTCCTGCTAGT and R': TACAGATGCCAAGCCCAGC were used. OSE2b promoter occupancy by Runx2 transcription factor was determined using Syber Green Master Mix (Applied Biosystems), loading equal amount of DNA, and running cycles of 2 min at 50 °C, 10 min at 95°C, and 40 cycles of 15s at 95 °C and 1 min at 60 °C. PCR was performed using an Applied Biosystems 7500 Real-time PCR System SDS v1.2.

Statistical analysis

One-factor analysis of variance (ANOVA) was performed to evaluate the effect of each variable on two or more independent groups. In the event of a significant group effect, a Bonferroni post-test was performed to compare selected pairs of group means. Adjusted p values are reported. For comparisons between two group means in which the response was affected by a single variable, an unpaired *t*-test was performed. Any difference with a probability value less than 0.05 was considered statistically significant.

Results

CTGF as a substrate for integrin-dependent osteoblast adhesion

To evaluate whether CTGF can serve as a matrix for osteoblast attachment, we conducted an adhesion assay using MC3T3-E1 osteoblasts that were seeded onto culture plates coated with various concentrations of CTGF. This assay demonstrated a significant, concentration-dependent increase in the attachment of cells plated onto the CTGF substrate (ranging from 0.25 to 2 μg/ml) compared to BSA (negative control), with cell attachment reaching a plateau at 2 μg/ml CTGF (Fig. 1A). Next, we compared the attachment of osteoblasts to CTGF and fibronectin, a well-documented bone matrix protein that promotes osteoblast adhesion. While osteoblasts adhered to both fibronectin and CTGF (2 μg/ml), the number of cells that attached to fibronectin was greater than to CTGF under identical assay conditions (Fig. 1B). Next, we investigated which domain of CTGF provides the binding site for osteoblast adhesion. For these experiments, we coated wells with the third domain of CTGF, the fourth domain of CTGF, or the full length CTGF protein and performed adhesion assays. Osteoblast adhesion to the fourth domain was comparable to adhesion to the whole CTGF protein (Fig. 1C), while adhesion to the third domain was not significantly different from BSA (the negative control). To further determine if the fourth domain of CTGF plays an essential role for osteoblast adhesion to CTGF, we incubated osteoblasts with recombinant domain four for 30 minutes at room temperature prior to seeding cells in wells coated with full length CTGF. Pre-incubation of cells with the fourth domain of CTGF blocked adhesion to full length CTGF with levels comparable to the negative control (BSA) (Fig. 1D).

Previous studies have demonstrated that integrins serve as functional receptors for the adhesion of various cell types to CTGF. To examine whether the attachment of osteoblasts to CTGF was integrin-dependent, we examined two characteristics of integrin-dependent adhesion, namely the requirement of divalent cations for integrin activation and ligand binding, and the role of heparan sulfate proteoglycans as integrin co-receptors in cell adhesion. Treatment of cells with 5 mM EDTA chelated the divalent cations and impaired cell adhesion (Fig. 1E). When an excess amount of divalent cations (10 mM of $MgCl_2$ or $CaCl_2$) were added back to the EDTA treated cell suspensions, cell adhesion was restored (Fig. 1E). Next, we conducted an adhesion assay in which 0.1 μg/ml of heparin was added to the cell suspension. The heparin was bound to the heparin- binding site (within the fourth domain) of CTGF, thereby making it unavailable for cell surface heparan sulfate proteoglycans to bind to CTGF. Heparin treatment significantly impaired osteoblast adhesion to CTGF (Fig. 1F). Collectively, these adhesion assays demonstrate that CTGF can serve as a substrate for osteoblast adhesion and that the adhesive interaction is likely to involve the binding of cell surface integrins (as receptors) and heparan sulfate proteoplycans (as co-receptors) to the fourth domain of CTGF.

Integrin $\alpha_v\beta_1$ is the primary receptor mediating osteoblast adhesion to CTGF

To determine which integrin mediates osteoblast adhesion to CTGF, we used blocking antibodies directed against the six primary integrin monomer subunits expressed on osteoblasts. Cells were incubated with 20 μg/ml of the appropriate blocking antibody for 30 minutes at 37°C prior to performing the adhesion assay. We observed a significant decrease in osteoblast adhesion to CTGF when α_v, α_2, α_5, or β_1 integrins were blocked, while blocking β_3 or β_5 integrins did not have any effect on osteoblast adhesion (Fig. 2A). To further examine the specificity of the CTGF-integrin interaction in osteoblasts, we immunoprecipitated different integrin heterodimers ($\alpha_v\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$) from osteoblast cell lysates and examined CTGF levels in these integrin pull downs by Western blotting. These analyses confirmed that the most significant interaction occurs between the $\alpha_v\beta_1$ integrin and CTGF (Fig. 2B). Immunofluorescent staining of osteoblasts cultured on recombinant CTGF demonstrated actin stress fibers, clustering of $\alpha_v\beta_1$ integrin receptors and vinculin staining in the areas of focal adhesions (Fig. 2C). The actin stress fibers converged on the sites of focal adhesions, and $\alpha_v\beta_1$ integrin and vinculin staining was co-localized in these areas (Fig. 2C, insets). Collectively, these data demonstrate that $\alpha_v\beta_1$ integrin serves as the primary receptor for osteoblast adhesion to a CTGF matrix.

Adhesion to CTGF promotes cytoskeletal reorganization, cell spreading and Rac activation in osteoblasts

Regulation of cytoskeletal reorganization is an important aspect of integrin dependent cell adhesion to extracellular matrix proteins. To investigate the effects of osteoblast adhesion to CTGF on cytoskeletal reorganization and cell spreading, we compared cells that were cultured for eight hours on substrates of CTGF, BSA (negative control) or fibronectin (positive control) (Fig. 3A and B). Immunofluorescence staining of actin filaments and vinculin demonstrated that cells attached to CTGF had a more uniform, rounded shape, compared to cells attached to fibronectin, which exhibited a more polarized appearance (Fig. 3A). It is interesting to note that this difference in shape was more pronounced at earlier time points; many of the cells on CTGF had a more polarized appearance by 24 hours (see Fig. 2C). Quantification of cell area demonstrated that cells plated on CTGF spread well, compared to BSA, but not to the same extent as cells on fibronectin (Fig. 3B). We next examined if osteoblast adhesion/spreading on

CTGF results in the activation of Rac1, a key signaling molecule in the regulation of cell spreading. We performed Rac1 activation assays on cells cultured on dishes coated with BSA, fibronectin, or CTGF or on uncoated dishes for 2 hours. Activation of Rac1 occurred in cells cultured on fibronectin and CTGF compared to BSA or uncoated controls, with highest levels of active Rac1 observed in cells cultured on fibronectin (Fig. 3C). Total Rac levels were similar in under all conditions (Fig. 3C). Collectively, these data support a role for CTGF as matrix protein that promotes cytoskeletal reorganization, spreading and Rac activation in osteoblasts.

CTGF matrix enhances osteoblast differentiation and matrix mineralization

Previous *in vivo* and *in vitro* studies have demonstrated a role of CTGF in osteogenesis and early stages of osteoblast differentiation. To examine the effect of osteoblast adhesion to a CTGF matrix on subsequent differentiation, we cultured preosteoblastic cells (MC3T3-E1) on a matrix of CTGF or BSA (negative control) coated plates under osteogenic conditions (see Methodology for details). After 14 days, we assessed osteoblast maturation by alkaline phosphatase (ALP) staining (Fig. 4A) and activity (Fig. 4B). The results demonstrated a more robust staining for ALP and significantly higher enzyme activity in cells cultured on CTGF compared to BSA (Fig. 4A and B). Next, we evaluated bone nodule formation and matrix mineralization at day 35 of culture. Staining for Alizarin red showed large, well-formed nodules in cells cultured on the CTGF matrix compared to BSA (Fig 4C). The number (Fig. 4D) and area (Fig. 4E) of nodules were also significantly greater on CTGF compared to BSA. These data demonstrate that preosteoblasts grown on a CTGF matrix results in enhanced osteogenic differentiation, bone nodule formation and matrix mineralization.

Osteoblasts cultured on a CTGF matrix activate an important osteoblast differentiation signaling pathway

The MAPKinase signaling pathway (including FAK and ERK1/2), which activates Runx2, is a well-known signaling pathway leading to osteoblast differentiation. To examine if osteoblasts cultured on CTGF results in activation of these signaling molecules, we cultured cells on CTGF or BSA (negative control) for a period of 7 days and compared expression of activated and total FAK and ERK1/2 proteins (Fig. 5A-F). Western blot analyses demonstrate that osteoblasts on CTGF expressed higher levels of both activated and total FAK and ERK (Fig. 5A-F). We also performed Western blots to examine the short term activation of FAK and ERK (up to 2 hours post-plating) after seeding onto CTGF or BSA, and observed a rapid (within 30 minutes) increase in activation of both signaling molecules (data not shown).

Runx2 is a critical transcription factor for osteoblast differentiation that has been shown to regulate the transcription of other essential proteins such as osteocalcin. To investigate if osteoblasts cultured on a CTGF matrix have an effect on Runx2 transcriptional activity, we performed chromatin immunoprecipitation (ChIP) assays to analyze Runx2 binding occupancy on the osteocalcin gene promoter (Fig. 5G). Cells cultured on CTGF for 7 days showed a significant increase in Runx2 binding occupancy compared to cells on BSA or uncoated plates (Fig. 5G). Collectively, these data demonstrate that osteoblasts cultured on CTGF results in sustained up-regulation and activation of FAK and ERK, as well as increased Runx2 binding occupancy of the osteocalcin gene promoter. Increased transcriptional activity of the osteocalcin gene is consistent with terminal differentiation of osteoblasts and mineralization on the matrix that they produce.

Figures and Legends

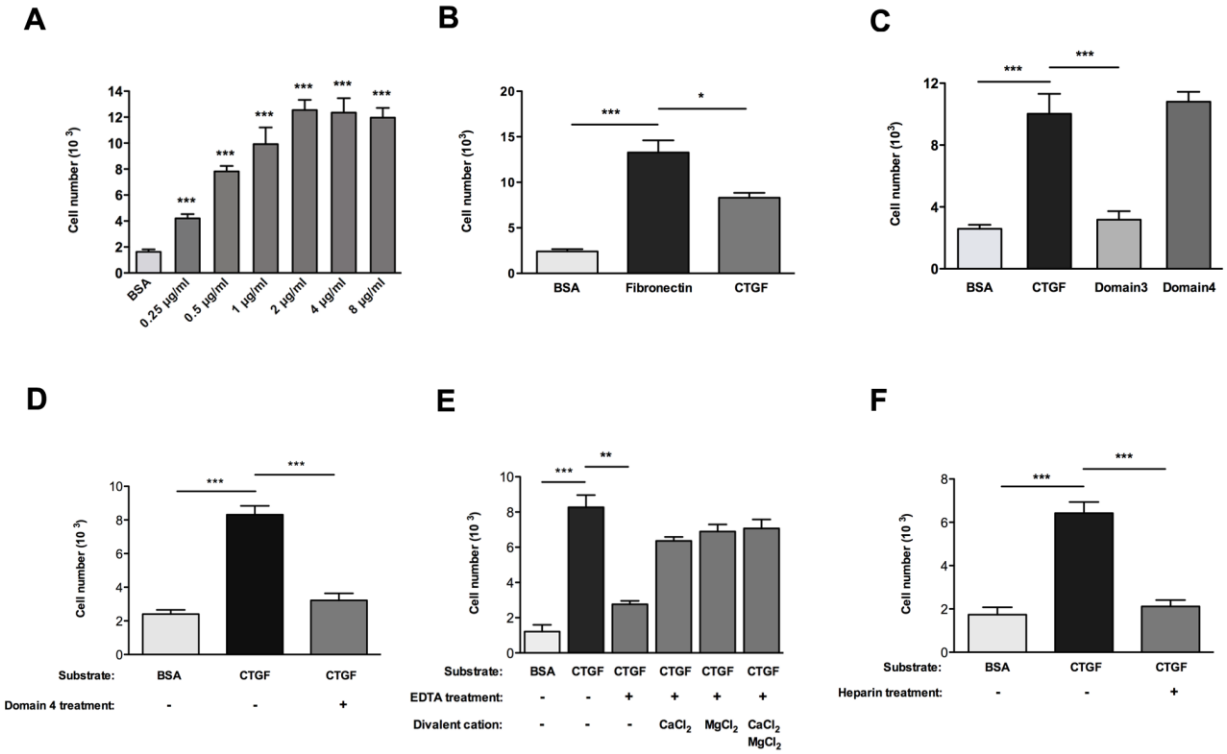

Figure 1. Osteoblast adhesion to CTGF is via integrin receptors and through fourth domain of CTGF.

(A) Adhesion assay to analyze osteoblast adhesion levels to various concentrations of recombinant CTGF, used to coat the wells. The number of cells adhered to CTGF is compared to cell adhesion to 1% BSA (negative control) **(B)** Adhesion assay comparing osteoblast adhesion levels to 2 µg/ml CTGF, 2 µg/ml fibronectin, or 1% BSA (negative control) coated wells. **(C)** Adhesion assay comparing osteoblast adhesion levels to 2 µg/ml of full-length CTGF, third domain of CTGF, fourth domain of CTGF, or 1% BSA (negative control) coated wells. **(D)** Adhesion assay analyzing effect of osteoblast treatment with fourth domain of CTGF prior to culture on full-length CTGF coated wells. **(E)** Adhesion assay analyzing effect of divalent cation chelation by EDTA on osteoblast adhesion to full-length CTGF, and reversing this effect by adding excessive amount of divalent cations to the culture. **(F)** Adhesion assay studying the effect of blocking heparin binding site of CTGF molecule via adding heparin to osteoblast cell suspension prior to culture on CTGF coated wells. n= 6, *$p<0.05$; **$p<0.01$; ***$p<0.001$. Adhesion assays were repeated three times with similar results.

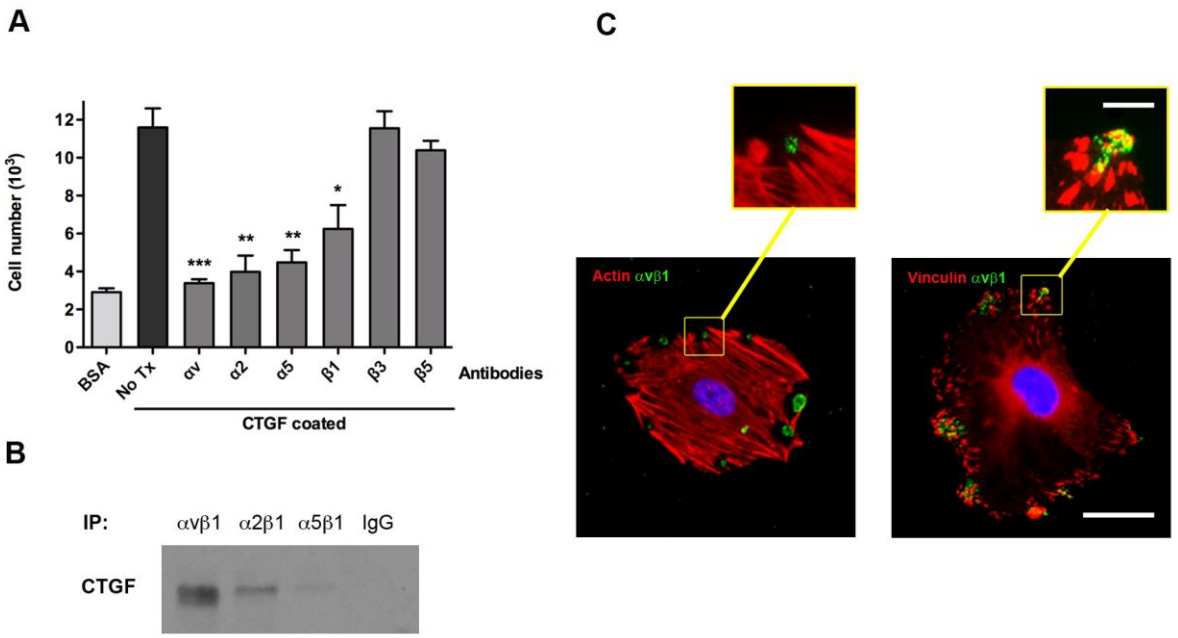

Figure 2. Osteoblast adhesion to CTGF is mediated by αvβ1 integrin receptor.

(A) Adhesion assay of osteoblasts treated with different blocking integrin antibodies prior to culture on CTGF or BSA (negative control) coated wells. The number of adhered cells in each treatment group was compared to the adhesion level of untreated cells. Adhesion to CTGF matrix after treatment with β3 or β5 integrin antibodies was not significantly different from untreated cells. **(B)** Western blot analyzing CTGF co-precipitation levels following immunoprecipitation of different integrins in osteoblast cell lysate. IgG was used as negative control of integrin antibodies. **(C)** Immunofluorescence staining of osteoblasts cultured on CTGF coated slides for 24 hours at 37 °C. Cells stained for αvβ1 (green), F-actin (red in left panel), or vinculin (red in right panel). Lower panel: 20X magnification, scale bar shows 50 μm. Upper panel: 40X magnification, scale bar shows 10 μm. n=6, *$p<0.05$; **$p<0.01$; ***$p<0.001$. Experiments were repeated three times with similar results

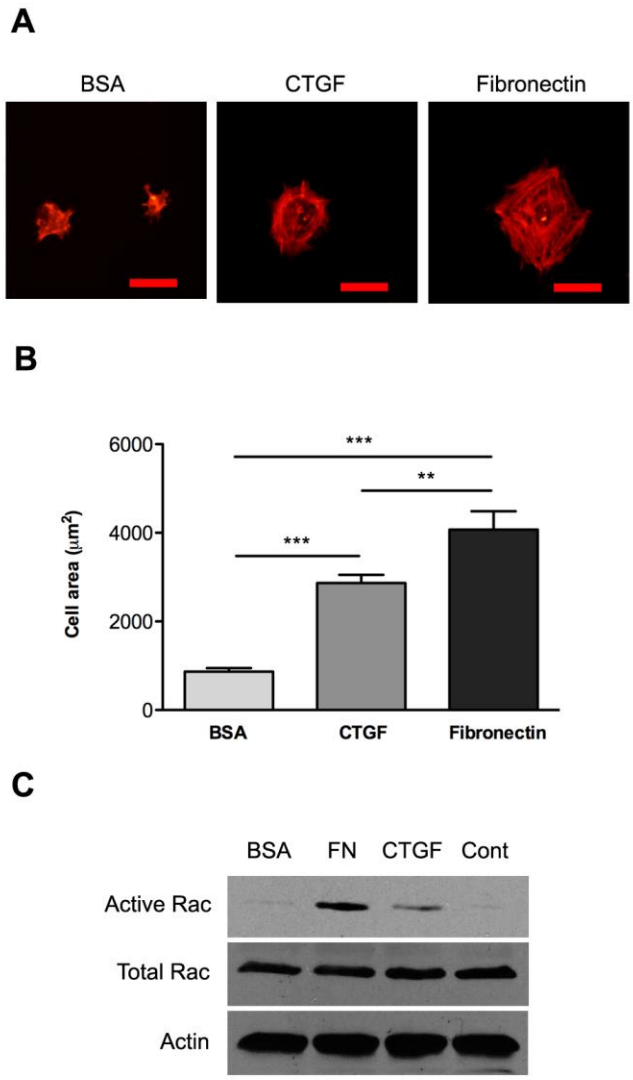

Figure 3. Osteoblast adhesion to CTGF matrix induces Rac activation and cell spreading.

(A) Imuunofluorescence staining of F-actin in osteoblasts cultured on 1% BSA, 2 μg/ml of CTGF or fibronectin coated slides for 8 hours at 37 °C. 20X magnification. Scale bar = 50 μm. **(B)** Cell spreading area of osteoblasts cultured on BSA, CTGF or fibronectin for 8 hours and imunofluorescence stained for actin were measured by ImageJ; n= 50. **p<0.01; ***p<0.001. **(C)** Western blot analysis of active Rac1, total Rac1 and actin to study Rac1 activation levels. Osteoblasts were cultured for 2 hours on uncoated plates or plates coated with BSA, CTGF or fibronectin. Abbreviations: fibronectin (FN) and negative control (Cont). Experiments were repeated three times with similar results.

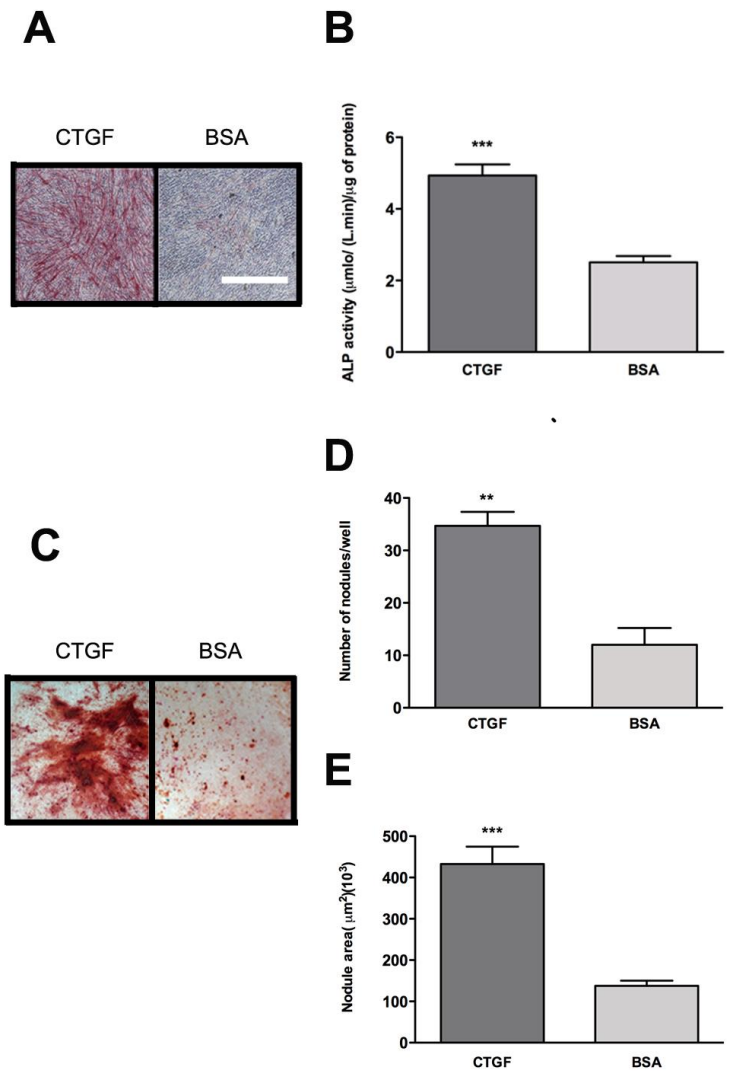

Figure 4. Osteoblast adhesion to CTGF matrix enhances osteoblast maturation and matrix mineralization.

(A) Alkaline Phosphatase (ALP) staining of osteoblasts cultured on 2 μg/ml CTGF or 1% BSA coated plates for 14 days. All pictures have 4X magnification. Scale bar = 2 mm; n=9. **(B)** ALP activity quantified at day 14 of culture and normalized to total protein content. **(C)** Alizarin red staining of osteoblasts cultured on 2 μg/ml CTGF or 1% BSA coated plates for 35 days; n=9 **(D)** Number of nodules formed after 35 days of culture. **(E)** Area of nodules measured by ImageJ software. **$p<0.01$; ***$p<0.001$. Experiments were repeated three times with similar results.

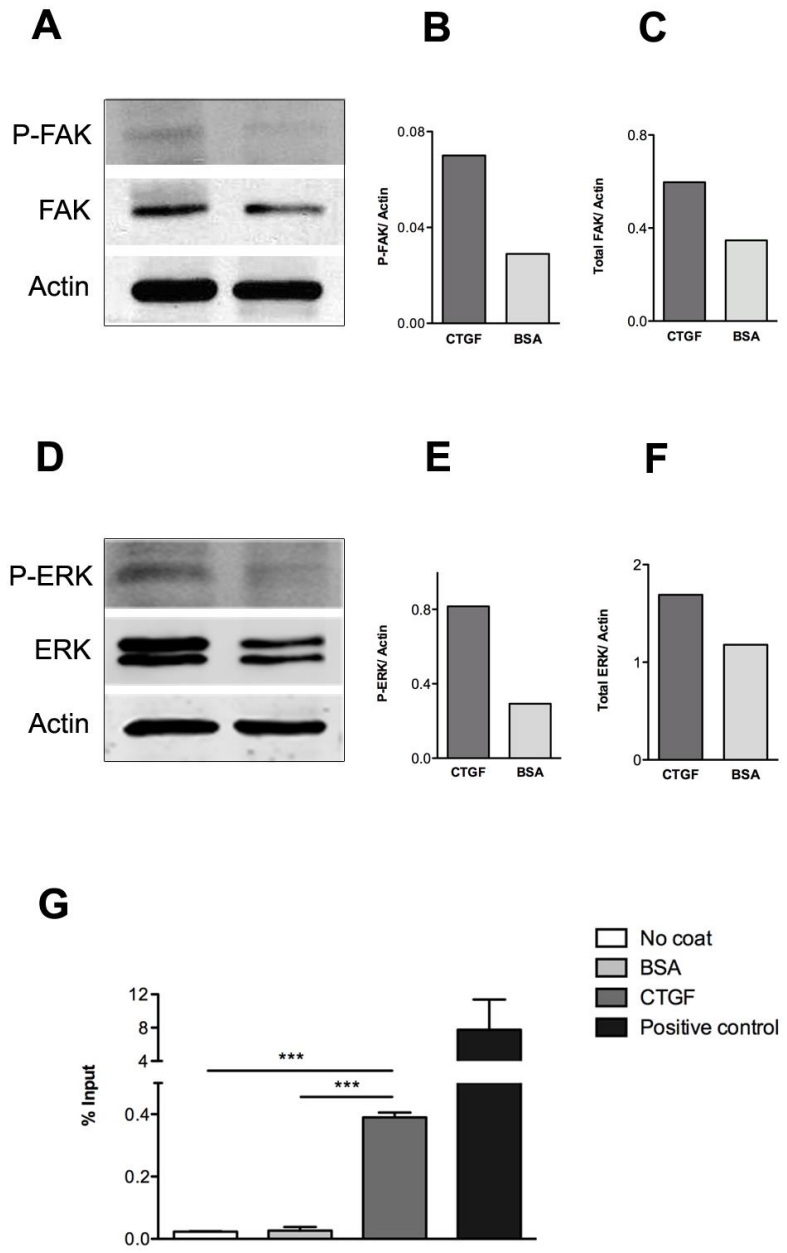

Figure 5. Osteoblast adhesion to CTGF matrix induces FAK, ERK and Runx2 activation.

(A) Western blot analysis of p-FAK, total FAK and actin protein levels at day 7 of osteoblast culture on 2 μg/ml CTGF or 1% BSA coated plates while treated with osteogenic medium **(B)** P-FAK levels were normalized to actin. **(C)** Total FAK levels were normalized to actin. **(D)** Western blot analysis of p-ERK, total ERK and actin protein levels at day 7 of osteoblast culture on 2 μg/ml CTGF or1% BSA coated plates. **(E)** P-ERK levels were normalized to actin. **(F)** Total ERK levels were normalized to actin. **(G)** ChIP assay performed on osteoblasts cultured on CTGF coated, BSA coated or uncoated plates for 7 days while treated with osteogenic medium. Runx2 antibody or acetyl-Histone H4 antibody (positive control) used for chromatim immunoprecipitation. Quantitative PCR was performed using osteocalcin gene

promoter primers. n=3, ***p<0.001. Western blots and ChIP assay were repeated three times with similar results.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X____No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:

_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X___ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the

publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.Integrin mediated adhesion of osteoblasts to connective tissue growth factor (CTGF/CCN2) induces cytoskeletal reorganization and cell differentiation	Hendesi H, Barbe MF, Safadi FF, Monroy MA and Popoff S N	PLOS ONE	February, 2014	XSubmitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis,

or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

This project generated new information regarding the effects of CTGF on bone development and growth, specifically its mechanisms of action in regulating osteoblast differentiation and function. Understanding these effects and how CTGF acts to promote osteogenesis can be used to develop new therapeutic strategies to selectively enhance bone formation in patients with significant bone loss (e.g. generalized or localized osteoporosis).

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No___X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Steven N. Popoff	Professor and Chairperson of Anatomy and Cell Biology
eRA COMMONS USER NAME (credential, e.g., agency login)	
spopoff	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Loyola University of Chicago, IL	B.S.	1979	Biology
Loyola University Stritch School of Medicine, Maywood, IL	Ph.D.	1986	Anatomy/Cell Biology

A. Personal Statement – I have over 30 years of experience as a bone cell biologist studying the regulation of bone cell development and function. Current studies involve the characterization of the bone phenotype in genetically engineered mice in which the matricellular protein, connective tissue growth factor (CTGF), is either absent or over-expressed. The analyses include microCT, histomorphometry, immunohistochemistry, qPCR and Western blot analysis of bone markers, and ex vivo assays to test bone cell differentiation and function. Additional studies are designed to elucidate the mechanisms of action whereby CTGF regulates bone cell differentiation and function.

B. Positions and Honors

Employment/Experience:

1985-1988	Postdoctoral Fellowship, Cell Biology, University of Massachusetts Medical School
1985-1989	Instructor of Cell Biology, University of Massachusetts Medical School
1988-1993	Assistant Professor of Anatomy and Cell Biology, Temple Univ. School of Medicine
1993-1998	Associate Professor of Anatomy and Cell Biology, Temple Univ. School of Medicine
1997-present	Chairperson, Dept. of Anatomy and Cell Biology, Temple Univ. School of Medicine
1998-present	Professor of Anatomy and Cell Biology, Temple Univ. School of Medicine
2005-present	Medical Staff Scientist, Shriners Hospital for Children, Philadelphia
2006-present	Professor of Orthopaedic Surgery, Temple Univ. School of Medicine

Awards and Honors:

1975-1979	Loyola University Scholarship
1975-1979	Dean's List, Loyola University of Chicago
1979	Alpha Sigma Nu

1980	Graduated Summa Cum Laude, Loyola University of Chicago
1980	Lange Medical Publications Student Award
1985-1986	Basic Science Fellowship (Predoctoral), Loyola University
1986-1988	University of Massachusetts Medical School Scientific Council Research Award
1986-1988	National Institutes of Health Research Service Award
2001	Named the John Franklin Huber Chair of Anatomy and Cell Biology
2005-2008	Board of Directors (elected), Advances in Mineral Metabolism

C. Peer-Reviewed Publications (selected from a total of 88):

Ivkovic S, Yoon BS, **Popoff SN**, Safadi FF, Libuda DE, Stephenson RC, Daluiski A and Lyons KM (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* **130**:2779-2791.

Safadi FF, Xu J, Smock SL, Kanaan RA, Selim AH, Odgren PR, Marks SC, Jr, Owen TA and **Popoff SN** (2003) Expression of connective tissue growth factor in bone: Its role in osteoblast proliferation and differentiation *in vitro* and bone formation *in vivo*. *J. Cell. Physiol.* **196**:51-62.

Song JJ, Aswad R, Kanaan RA, Rico MC, Owen TA, Barbe MF, Safadi FF and **Popoff SN** (2007) CTGF acts as a downstream mediator of TGF-β1 in promoting condensation and regulating FN and Sox9 expression in mesenchymal stem cells. *J. Cell. Physiol.* **210**:398-410.

Arnott JA, Nuglozeh E, Rico MC, Arango-Hisijara I, Odgren PR, Safadi FF and **Popoff SN** (2007) Connective tissue growth factor (CTGF) is a downstream mediator for TGF-β1 induced extracellular matrix production in osteoblasts. *J. Cell. Physiol.* **210**:843-852.

Arnott JA, Zhang X, Sanjay A, Owen TA, Smock SL, Rehman S, DeLong WG, Safadi FF and **Popoff SN** (2008) Molecular requirements for Smad and Src/Erk signaling in the induction of CTGF expression by TGF-β1 in osteoblasts. *Bone* **42**:871-885.

Zhang X, Arnott JA, Rehman S, DeLong WG, Safadi FF and **Popoff SN** (2010) Src is a major signaling component for CTGF induction by TGF-β1 in osteoblasts. *J. Cell. Physiol.* **224**:691-701.

Arnott JA, Lambi AG, Mundy C, Hendesi H, Pixley RA, Owen TA, Safadi FF and **Popoff SN** (2011) The role of connective tissue growth factor (CTGF/CCN2) in skeletogenesis. *Crit Rev Eukaryot Gene Expr* **21**:43-69.

Geisinger MT, Astaiza R, Butler T, **Popoff SN**, Planey SL and Arnott JA (2012) Ets-1 is essential for connective tissue growth factor (CTGF/CCN2) induction by TGF-β1 in osteoblasts. *PLoS ONE* **7(4)**:e35258:1-9.

Lambi AG, Pankratz TL, Mundy C, Gannon M, Barbe MF, Richtsmeier JT and **Popoff SN** (2012) The context-dependent role of connective tissue growth factor in prenatal osteogenesis. *Develop Dynamics* 241:1944-1959 (published on-line October 16, 2012).

Mundy C, Gannon M and **Popoff SN** (2014) Connective tissue growth factor (CTGF/CCN2) negatively regulates BMP-2 induced osteoblast differentiation and signaling. *J Cell Physiol* **229(5)**:672-681 (published on-line October 11, 2013).

Hendesi H, Barbe MF, Safadi FF, Monroy MA and **Popoff SN** (2014) Integrin mediated adhesion of osteoblasts to connective tissue growth factor (CTGF/CCN2) induces cytoskeletal reorganization and cell differentiation. *PLoS ONE* (manuscript under revision).