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):** 01/01/2009 – 12/31/2012

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

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

5. **Grant SAP Number:** 4100047651

6. **Project Number and Title of Research Project:** 23 - The Role of Osteoactivin (OA) as a Downstream Mediator of Osteoblast Differentiation Induced by BMP2

7. **Start and End Date of Research Project:** 11/18/2009 – 6/30/2011

8. **Name of Principal Investigator for the Research Project:** Fayez Safadi, 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:

   $ 25,000

   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	Position Title	% of Effort on Project	Cost
Maneet Singh	Grad. Student	100%	$25,000

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	Position Title	% of Effort on Project
Fabiola Del Carpio-Cano	Research scientist	10%
Roshanak Razampour	Technician	2%
M. Alexandra Monroy	Res. Assistant Professor	5%
Fayez F. Safadi	Professor, PI	35%

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 (part of an RO1 application)

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? See attached

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:
None	☐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:

NIH RO1 renewal

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

To examine the role of epigenetics on Osteoactivin methylation in osteoporosis

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				
Black				
Asian			1	
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.

Development of new techniques for DNA ChiP assay

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.

ABSTRACT

Bone morphogenetic protein-2 (BMP-2) stimulates osteoblast differentiation either through structural coupling between transcription factors, Runx2 and Smads1/4 or through their independent binding to the promoter region of osteogenic genes. Osteoactivin (OA) is a crucial anabolic factor required for osteoblast differentiation. Our previous work indicated that OA protein expression is regulated by BMP-2 activated Smad1. In addition, multiple Smads1/4 binding elements were found in the OA promoter. In the present study, we observed that Runx2 knockout cells express very low levels of OA protein. The precise mechanism of OA transcriptional regulation by Runx2 and Smads1/4 is still not known. Here, we hypothesized that BMP-2 stimulated Runx2 and Smads1/4 regulate OA transcription in osteoblasts. Potential consensus Runx2 and Smads1/4 binding sites were identified in the proximal 0.96kb OA promoter region. The key Runx2 and Smads1/4 binding sites that regulate BMP-2 induced OA transcription were determined by OA promoter deletion and mutagenesis studies. The up-regulatory roles of Runx2, Smad1 and Smad4 proteins in the OA gene transcription were examined by over expression and siRNA knockdown studies. *In vitro* DNA-protein binding assays demonstrated that BMP-2 stimulated binding of Runx2, Smad1 and Smad4 to the OA promoter. Using a Chromatin immunoprecipitation assay, we show that BMP-2 robustly enhanced the recruitment of Runx2 to the OA promoter during the proliferation and matrix maturation stages of osteoblast differentiation. However, during matrix mineralization stage of osteoblast differentiation, BMP-2 treated osteoblasts showed a differential OA promoter association pattern. BMP-2 treatment in mineralized osteoblasts resulted in a maximal Smad1 occupancy, and this was accompanied by a significant decline in Runx2 association with the OA promoter. Collectively, our results demonstrate that BMP-2 induced OA transcription is regulated by independent binding of Runx2 and Smads1/4, and not through a coupled interaction during osteoblast differentiation.

INTRODUCTION

Osteoactivin (OA) also known as glycoprotein nonmelanoma protein B (GPNMB) was initially identified in the long bones of osteopetrotic rats (Safadi et al. 2001). OA expression is enhanced during osteoblast differentiation with maximal expression in terminally differentiating osteoblasts (Abdelmagid et al. 2007). Our group has demonstrated that OA stimulates differentiation of osteoblasts *in vitro* (Abdelmagid et al. 2008), and is also expressed during fracture repair (Abdelmagid et al.). OA is involved in regulation of cell proliferation, differentiation, adhesion and in biosynthesis of extracellular matrix proteins in bone cells, osteoblasts (Abdelmagid et al. 2007) and osteoclasts (Sheng et al. 2008), as well as in muscle (Ogawa et al. 2005), liver (Onaga et al. 2003) and immune cells (Ripoll et al. 2007). OA is also reported to be associated with cancer progression (Rich et al. 2003; Rose et al. 2007; Ghilardi et al. 2008). Bone morphogenetic protein-2 (BMP-2) stimulates OA protein expression through Smad-1 signaling (Abdelmagid et al. 2007). Using functional blocking antibody to OA or an OA antisense, we previously demonstrated that OA acts as a downstream mediator for BMP-2 induced osteoblast differentiation and function (Selim et al. 2003; Abdelmagid et al. 2007; Abdelmagid et al. 2008).

BMP-2 promotes osteoblast differentiation through Smad1/4 signaling (Yamamoto et al. 1997) and by stimulating activity and also expression of Runx2 transcription factor (Bae et al. 2007). The role of Runx2 in osteoblast differentiation is well established, since the mice with homozygous deletion of Runx2 fail to form osteoblasts and a mineralized skeleton (Otto et al. 1997; Komori 2005; Bae et al. 2007). Runx2 binds to the consensus sequence PuACCPuCA or its complement sequence TGPyGGTPy on the promoter regions of collagen type I (Kern et al. 2001), osteocalcin (Ducy and Karsenty 1995; Frendo et al. 1998; Javed et al. 1999; Hoffmann et al. 2000; Zaidi et al. 2001; Sierra et al. 2003; Shin et al. 2006; Yu et al. 2008) and bone sialoprotein (Javed et al. 2001; Prince et al. 2001; Barnes et al. 2003; Lamour et al. 2007), as well as on its own gene for auto-regulation (Hassan et al. 2006). Runx2 stimulates commitment and differentiation of osteoblast cells by inducing expression of genes involved in osteoblast differentiation including alkaline phosphatase, osteocalcin and bone sialoprotein (Lee et al. 1999; Lee et al. 2000; Banerjee et al. 2001; Lee et al. 2002; Lee et al. 2003; Miyazono et al. 2004; Jeon et al. 2006; Phimphilai et al. 2006; Gersbach et al. 2007). Smad1 and Smad4 transcription factors through their DNA binding domain interact with Smad binding elements (SBE) and BMP responsive elements (BREs). SBE and BRE are GC rich sequences that include GCCGnCGC, CAGAC, GCAT, AGNC and GTCT (Dennler et al. 1998; Shi et al. 1998; Zawel et al. 1998; Johnson et al. 1999; Stroschein et al. 1999; Kusanagi et al. 2000; Hullinger et al. 2001; Lopez-Rovira et al. 2002). These regulatory elements are found in the promoter regions of genes activated by Smad1 signaling (Jonk et al. 1998; Henningfeld et al. 2000; Katagiri et al. 2002; Lopez-Rovira et al. 2002; Ghosh-Choudhury et al. 2006). Although, Smads have an intrinsic DNA binding affinity, their interactions with other transcription factors, transcriptional co-activators and co-repressors through their transactivation domain increase the affinity and specificity of Smad1 induced transcriptional regulation (Nojima et al.; Lian et al. 2003). Several studies suggest that BMP-2 induced Smad1 transcriptional activity is mediated by its interaction with Runx2 (Hanai et al. 1999; Afzal et al. 2005; Schroeder et al. 2005; Phimphilai et al. 2006). According this school of thought, following stimulation with BMP-2, Smad1 forms co-regulatory complexes with Runx2 and these BMP-2 induced-Smad1-Runx2 interactions are major regulators of osteoblast differentiation. Multiple reports indicate that BMP-2 fails to stimulate osteoblast differentiation in the absence of Runx2-Smad1 interactions (Zhang et al. 2000; Choi et al. 2001; Afzal et al. 2005; Javed et al. 2009). In addition, Runx2 has been reported to enhance osteogenic effects of BMP-2-Smad1 signaling by forming Runx2-Smad1 complex that can bind either to Smad1 or Runx2 binding sites, or both the sites simultaneously on the promoter region of osteogenic genes and stimulate osteoblast differentiation (Liu et al. 2007; Javed et al. 2008). Conflicting reports of BMP-2 induced osteoblast differentiation regulation through independent binding of BMP-2 activated Runx2 and Smad1 transcription factors and not Runx2-Smad1 complex are also indicated (Lee et al. 2002; Phimphilai et al. 2006).

OA expression is regulated by multiple factors including BMP-2 in osteoblasts (Abdelmagid et al. 2007), receptor activator of nuclear factor kappa-B ligand (RANKL) in osteoclasts (Sheng et al. 2008), and interferon gamma (IFN-γ) in macrophages (Metz et al. 2005).We previously reported that OA promoter region has multiple Smad1 binding sites, however, the mechanism for transcriptional regulation of OA expression is not yet known (Abdelmagid et al. 2007). Here, we hypothesize that OA transcription in osteoblasts is regulated by BMP-2 induced Runx2 and Smads1/4 transcription factors. In this study for the first time, we demonstrated that BMP-2

induced transcriptional activation of the OA gene in osteoblasts is mediated by Runx2, Smad1 and Smad4 transcription factors. Furthermore, we show that BMP-2 stimulated differential recruitment of Runx2, Smad1 and Smad4 transcription factors to the OA promoter during various stages of osteoblast differentiation. These findings provide novel insights into the molecular regulation of OA transcription by BMP-2induced osteoblast differentiation.

MATERIALS AND METHODS

Cell Cultures

Primary osteoblast cells were isolated from the calvaria of neonatal Norway-Hooded rats (4-5 days old) by five sequential digestions with collagenase P (0.1%, Roche Applied Science, Indianapolis, IN) and trypsin (0.25%, Mediatec, Manassas, VA) at 37°C as described previously (Abdelmagid et al. 2007). Osteoblast enriched cell populations obtained from the fifth digestion were plated at a density of $5X10^5$ cells/100mm dish in α- minimal essential medium (MEM, Mediatec) supplemented with fetal calf serum (FCS, 10%) and 10 international units (IU) penicillin/streptomycin (P/S). Osteoblasts were fed every third day with α-MEM containing β-glycerol phosphate (10mM) and ascorbic acid (50µg/ml) for the osteoblast differentiation studies. MC3T3-E1 subclone 4 osteoblasts were maintained in α-MEM media, while C2C12 osteoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatec). Both α-MEM and DMEM were supplemented with FCS (10%) and P/S (10 IU). Runx2 knockout osteoblast cells and their wild type osteoblasts were kindly provided by Dr. Jane Lian, Department of Cell Biology, University of Massachusetts Medical School, Massachusetts. They were cultured in α-MEM without ascorbic acid (Hyclone, Logan, UT) containing L-glutamine (1%), P/S (1%) and FCS (10%), as previously described (Liu et al. 2007).

OA Promoter Luciferase Vectors

The Rat OA promoter -962/+50 (p962) was amplified from the BAC clone CH230-65K1 that contains the 5' flanking sequence of rat OA gene (Gene Bank ™ accession number: AB218617) using primers cited in Table 1A. Rat genomic DNA was used as a template. The OA promoter was cloned into a promoter-less pGL3 basic luciferase (LUC) vector (Promega Corporation, Madison, WI). Forward and reverse primers were flanked by KpnI and BglII restriction sites, respectively, to insert the OA promoter into the pGL3-basic-LUC vector. Deletion mutants of the OA promoter vector p835 (-835/+50), p685 (-685/+50), p285 (-285/+50), p135 (-135/+50), p105 (-105/+50) were synthesized using p962 promoter region as a template DNA. Similar to the primers used for p962, the primers used for OA promoter deletion constructs (shown in Table 1A) contained KpnI restriction sites in the forward primer and a common reverse primer with a BglII restriction site. The nucleotide sequence of the OA promoter inserted into pGL3-basic vector was confirmed by sequencing. QuickChange Lightning Site-directed mutagenesis kit (Stratagene, LA Jolla, CA) was used to generate mutations in Runx2 binding sites at -779, -746, -735, and -585 and in Smad binding site at -248 of the OA gene using OA promoter-LUC as a backbone vector. The primers (Table 1B) for site-directed mutagenesis were designed as per the manufacturer's instructions (Sigma, St. Louis, MO). The nucleotide sequence of the mutated clones was confirmed by sequencing.

Protein Isolation, Western Blotting and Immunohistochemistry analyses

Cell monolayers were washed with ice-cold-PBS and lysed in RIPA buffer (50mM Tris-HCl (pH 7.5), 135mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 2mM EDTA, 50mM NaF, 2mM

sodium orthovanadate, 10µg/ml aprotonin, 10 µg/ml leupeptin and 1mM PMSF) over night at 4°C with gentle shaking. Total protein concentration was quantified using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gel and transferred to an Immobilon-P^{SQ}transfer membrane (Millipore, Billerica, MA) using semi-dry transfer apparatus. The membranes were blocked in Odyssey Blocking buffer (Licor Biosciences, Lincoln, NE) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies specifically, anti-HA-Tag (6E2) antibody (Cell Signaling, Danvers, MA), anti-Runx2 Antibody (Sigma, St. Louis, MO), anti-Smad1 antibody (Cell Signaling), anti-Smad4 antibody (Cell Signaling) or anti-β-Actin (Sigma) antibody (used to as a loading control). The next day, membranes were incubated at room temperature for 1 hour with gentle shaking with host specific fluorescent secondary antibodies, IRDye 800CW Goat anti-Rabbit (Licor Biosciences) and IRDye 680LT Goat anti-Mouse IgG (Licor Biosciences). Subsequently, the fluorescent signal was assayed using an Odyssey Infrared Imaging system (Licor Biosciences).

For measuring OA protein levels, a primary anti-OA antibody that was raised against peptide corresponding to amino acids 551-568 of rat OA protein in chicken (Cambridge Research Biochemicals, Cleveland, UK) was used, as previously described (Abdelmagid et al. 2007). Subsequently, the membrane was incubated with HRP-conjugated donkey anti-chicken IgY secondary antibody (Jackson Immunoresearch, West Grove, PA) for 1 hour and the signal was visualized by adding chemiluminescent substrate (Pierce, IL) and detected on XL-exposure films (Pierce, IL). For OA immunohistochemistry, wild type and Runx2 knockout limb sections harvested at E17.5 were de-paraffinized, antigen retrieved in a pressure cooker for 5 minutes in EDTA solution (pH 8.0), stained with mouse anti-human OA antibody (R&D Systems, Minneapolis, MN) using M.O.M Kit (Vector Laboratories, Burlingame, CA) and visualized using DAB chromogen (Sigma). Sections were lightly counterstained with hematoxylin (Surgipath, Richmond, IL).

Over-expression of proteins

MC3T3-E1 cells cultured in 6-well plates (75% confluent) were transfected with Smad1 vector (pCMV-HA-Smad1) (generously provided by Dr. C. Labrie, Oncology and Molecular Endocrinology Research Center, Laval University, Quebec, Canada), Smad4 vector (pCMV5-Smad4) (kindly provided by Dr. Rik Derynck, Department of Growth and Development, University of California at San Francisco, California) and Runx2-Isoform type II vector (pSG5-Runx2-II, kindly provided by Dr. Masahiro Iwamoto, Department of Orthopaedic Surgery, Thomas Jefferson Medical College, Philadelphia, PA) or their respective empty vectors using Fugene HD transfection reagent (Promega). Cell lysates were harvested forty-eight hours post-transfection and protein levels of Smad1, Smad4 and Runx2 were analyzed by Western blotting methods.

RNA interference (siRNA)

Nearly confluent (75%) MC3T3-E1 osteoblasts were transfected with 50 and 100nM of mouse Smad1 siRNA (sc-36507, Santa Cruz Biotechnology Inc., Santa Cruz, CA) or Smad1 scrambled siRNA-A (sc-37007, Santa Cruz); mouse Smad4 siRNA (sc-29485, Santa Cruz) or Smad4 scrambled siRNA (sc-44230, Santa Cruz); RUNX2 siGENOME SMARTpool (Dharmacon,

Lafayette, CO) or Runx2-scrambled siGENOME Non-Targeting siRNA #1 (Dharmacon) using a Fugene HD transfection reagent (Promega). Knockdown efficiency was analyzed by Western blotting 48 hours after transfection.

Luciferase Reporter Assay

Cells grown in 6-well plates (75% confluent) were transfected with a pGL3-Basic vector containing OA promoter insert (0.5µg per well) and transfection control pRTK4-SV40 renilla luciferase expression vector (50ng per well, Promega) using the Fugene HD transfection reagent. The following day, cells were treated with BMP-2 (100ng/ml, R&D Systems, MN) for different time points (4, 8, 12, 24, 48 hours) and cell lysates were collected in Passive cell lysis buffer (Promega). Luciferase activity in cell lysates was assayed using Dual-Glo TM Luciferase reporter Assay Kit (Promega) in a single tube luminometer Model 20/20 (Turner BioSystems, Madison, WI). Luciferase activity was normalized against renilla luciferase activity to determine relative promoter activity. Control vectors; pGL3-basic vector (Promega) and pGL3-control vector (Promega) were treated in a similar manner as described for a pGL3-Basic vector with a cloned OA promoter.

Nuclear Extraction

Osteoblasts (75% confluent) were serum starved overnight and treated with BMP-2 (100ng/ml) for 90 min, harvested and re-suspended in low salt buffer (10mM HEPES, pH 7.9; 10mM KCl, 2mM $MgCl_2$, 1mM DTT and complete Protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) for 15 minutes at 4ºC in an orbital shaker. NP-40 (10%) was added to rupture the cell membranes, nuclei were pelleted and incubated in a high salt buffer (10mM HEPES, pH 7.8; 2mM MgCl2; 1mM DTT, 50mM KCl, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 10% glycerol and complete protease inhibitor cocktail) for 20 min at 4ºC with gentle agitation. The samples were centrifuged at 12,000Xg for 10min and the nuclear fraction (supernatant) was collected. The protein concentration of the nuclear fraction was determined using Bradford's assay (BioRad, Hercules, CA).

Electrophoretic mobility shift assays (EMSA)

Double stranded wild type (W) and mutant (M) oligonucleotide sequences (Fig 4B and D) were synthesized with 3'sticky ends (Integrated DNA Technology, Coralville, IA). The 3'sticky ends of the oligonucleotide were labeled with Biotin using the Biotin 3'-end DNA labeling kit (Pierce Chemicals) according to the manufacturer's instruction. Nuclear extract from rat primary osteoblasts (6µg) for Smad EMSA and Runx2-type II isoform over-expressing MC3T3E1-subclone 4 (8µg) were incubated in 1X-binding buffer (50% glycerol, 100mM MgCl2, 1µg/µl Poly (dI-dC), 1% NP-40, 1M KCl, 200mM EDTA and 50pmol DNA probe) for 20 min at room temperature. The reaction mixture was loaded onto a 6% native polyacrylamide gel and run at 100V in 0.5X Tris borate-EDTA. The protein-DNA complex was then transferred to Hybond-N^+nylon membrane (Pierce Chemicals) using the Trans-Blot semi-dry method (BioRad, CA) and cross-linked (Spectrolinker XL-1000UV crosslinker, Spectroline, NY). Biotin labeled DNA was detected by LightShift chemiluminescent EMSA Kit (Pierce Chemicals) and visualized using charge-couple device camera (Fujifilm LAS 3000, USA). For competition assay, reaction mixture was pre-incubated with 100-fold excess of the cold non-labeled probe. Gel-supershift assays were performed by pre-incubating the nuclear extract with anti-Smad1 antibody (Smad1 (A4-X), Santa Cruz), Smad4 (Smad4 (C-20X), Santa Cruz) and Runx2 (Runx2 (M-70), Santa

Cruz) or control IgG antibody (Santa Cruz) for 20 min before addition of Biotin-oligonucleotide for another 15 min at room temperature. Reaction mixture was run on a 6% native polyacrylamide gel and visualized as described above.

Chromatin immunoprecipitation assay

Rat primary osteoblast ($2X10^7$) cells were cultured in osteoblast differentiation media on 150mm dishes and the cultures were harvested at day 7, day 14 and day 21. The three time points were derived from the same osteoblast isolations. Upon stimulation with BMP-2 (100ng/ml) for 2 hours, osteoblast cells were cross-linked with formaldehyde (1% final concentration) for 15 min at room temperature with gentle agitation. After cross-linking, the cells were washed in ice-cold PBS containing complete mini protease inhibitor cocktail (Roche Applied Sciences) and harvested in lysis buffer (50mM HEPES-KOH (pH 8.0), 0.1mM EDTA, 0.5M EGTA, 140mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100 and complete mini protease inhibitor cocktail) at 4ºC for 10 min on a rocker. The nuclei were centrifuged and resuspended in RIPA buffer (10mM Tris-HCl pH 8.0, 1% Triton-100, 0.1% SDS, 0.1% sodium deoxycholate, 1mM EDTA, 0.5mM EGTA, 140mM NaCl and mini protease inhibitor cocktail). Nuclei were sonicated (Sonifier Cell disrupter 350, Branson Sonic Power, Danbury, CT) and centrifuged. At this step, 5% of the supernatant was saved as Input at -20°C, while the rest of the supernatant was pre-cleared with protein G beads/sonicated salmon sperm DNA slurry and subsequently immunoprecipitated with 10µg of Smad1 (Santa Cruz), Smad4 (Santa Cruz) or Runx2 (Santa Cruz) antibody. Rabbit IgG (Santa Cruz) and polymerase II (Santa Cruz) were control antibodies. Immunocomplex was precipitated with a Protein G beads/sonicated salmon sperm DNA slurry, and washed sequentially with buffers from ChIP kit (Millipore). Antibody-bound chromatin fragments were eluted from the beads with 1% SDS in 0.1M $NaHCO_3$. Cross-links were reversed by incubating samples overnight in water bath at 65°C and DNA was recovered by phenol-chloroform extraction and precipitated. DNA was then subjected to quantitative Real-Time PCR (7500 Applied Biosystems, Carlsbad, CA) with OA primers (Table 1C). PCRs (in triplicates) for each sample were mixed with 2XSYBR Green PCR master mix (Applied Biosystems) and a standard curve of known target DNA (GAPDH) was run in parallel to calculate the relative amount of target DNA in the sample. The mRNA of interest and GAPDH were amplified with equal efficiencies by real time PCR analysis. Control ChIP assay with non-specific IgG and Polymerase II was performed in each experiment. ChIP data is presented as relative promoter occupancy calculated using the following equation: (amount of DNA immunoprecipitated by antibody of interest) – (amount of DNA immunoprecipitated by control IgG) / (amount of Input DNA), where input DNA was an aliquot of sheared chromatin sample collected before immunoprecipitation.

Statistical analysis

One way analysis of variance (ANOVA) with Bonferroni post-hoc tests were used to compare the statistical difference between BMP-2 treated and untreated cultures and adjusted p-values are reported. In certain cases, percentage increase in OA promoter activity with over expression of Runx2, Smad1 or Smad4 proteins in comparison to empty vector transfected osteoblasts in BMP-2 treated osteoblasts is reported. Relative OA promoter activity and relative promoter occupancy (data for ChIP assay) are expressed as mean ±SEM for five independent experiments with three replicates per experiment.

RESULTS

BMP-2 mediated transcription of the OA gene requires Runx2

Our previous work has shown that BMP-2 enhances the expression of OA protein during osteoblast differentiation (Abdelmagid et al. 2007). To investigate the effects of BMP-2 on OA transcription, we cloned the proximal 0.96kb rat OA promoter region into a luciferase reporter vector and examined the effects of BMP-2 on OA promoter activity in rat primary osteoblasts. A significant increase in OA promoter activity was observed upon treatment with BMP-2 in a concentration-dependent manner (Fig 1A). The maximum response was observed at a concentration of 100ng/ml of BMP-2 (6.5-fold increase compared to untreated osteoblasts). The response on OA promoter was specific to BMP-2, since the osteoblast cells expressing luciferase vector alone or SV-40 promoter did not elicit a response (Fig 1A). Next, we established the time-dependent effect of BMP-2 (100ng/ml) on OA promoter activity for time periods ranging from 4-48 hours (Fig 1B). A temporal increase in OA promoter activity was observed with a maximal response achieved after 24 hours of BMP-2 treatment. For our subsequent experiments, we treated osteoblasts with 100ng/ml of BMP-2 for 24 hours.

Next, we established BMP-2 induced OA transcription in osteogenic cell types and found that increasing concentrations of BMP-2 resulted in gradual increment of OA promoter activity with a maximal response at 100ng/ml of BMP-2 (Fig 1C). Interestingly, rat primary osteoblasts exhibited a maximal induction (with BMP-2 treatment) in OA promoter activity compared with the other cell types (Fig1C). In order to determine the role of Runx2 in OA transcription, OA promoter activity was assayed in Runx2 knockout and their wild type osteoblasts. Runx2 knockout osteoblasts showed a significantly decreased BMP-2 induced OA promoter activity in comparison to their wild type osteoblasts, indicating that Runx2 is required for OA promoter regulation. A high expression of OA protein was observed in wild type osteoblasts, while minimal to low OA expression in Runx2 knockout osteoblasts, even with BMP-2 stimulation (Fig 1D). Similarly, decreased OA staining (brown stain in osteoblasts and chondrocytes) was observed in bones harvested at E18.5 from Runx2 knockout mice in comparison to WT mice (Fig 1F). These findings suggest that Runx2 is involved in OA protein expression both *in vitro* and *in vivo*. Runx2 knockout and their wild type osteoblasts displayed similar expression levels of Smad1 and Smad4 protein (Fig 1E). Taken together, these results suggest that BMP-2 stimulates OA transcription and Runx2 plays crucial role in OA transcription and protein expression.

Requirement of Smad1, Smad4 and Runx2 binding sites in the OA promoter region.

To examine whether Smad1, Smad4 and Runx2 transcription factors are involved in OA transcription, we analyzed the proximal 0.96kb OA promoter region for consensus Smad1, Smad4 and Runx2 binding sites. Twenty-two Smad (both Smad1 and Smad4) binding sites and four Runx2 binding sites are present in the proximal 0.96kb OA promoter sequence. To characterize the roles of Smad1, Smad4 and Runx2 binding sites, a series of 5' deletions of OA promoter were constructed (Fig 2A and B) and analyzed for the OA promoter activity. Interestingly, BMP-2 stimulated the activity of all OA promoter deletion constructs, except p135 and p105-OA promoter constructs (Fig 2C). Loss of all four Runx2 and distal eighteen Smad1/4 binding sites (in p285-OA promoter construct) from the proximal 0.96kb OA promoter region decreased the BMP-2 induced OA promoter activity by approximately 80%, suggesting that major BMP-2 response elements are located between -962 to -285 of the OA gene. In

comparison to p285, p135 showed a 68.5% decrease in BMP-2 induced OA promoter activity with loss of -248 SBE, suggesting that -248 SBE could be crucial for BMP-2 induced OA promoter activation (Fig 2C).

Next, we investigated the specific roles of consensus four Runx2 binding sites and -248 SBE in the proximal 0.96kb OA promoter region by introducing mutations in these sites (Fig 2D and E, respectively). Mutations in -779 (pMR1), -746 (pMR2), -735 (pMR3) and -585 (pMR4) Runx2 binding sites decreased the BMP-2 stimulated OA promoter activity, especially mutation in -585 Runx2 binding site (pMR4) resulted in robust decrease (71% decrease compared with wild-type, p962) in BMP-2 induced OA promoter activity (Fig 2D). Similarly, mutation in -248 SBE (pMSBE) reduced the BMP-2 induced OA promoter activity of wild-type (p962) OA vector by 63% (Fig 2E). Mutation and deletion studies in the OA promoter region suggest that all four Runx2 binding sites, especially -585 Runx2 and -248 SBE are crucial for BMP-2 induced OA promoter activation.

Roles of Smad1, Smad4 and Runx2 proteins in OA transcription

To confirm the involvement of Smad1, Smad4 and Runx2 proteins in the regulation of OA gene expression, we knocked down endogenous Smad1, Smad4 and Runx2 proteins using specific siRNAs and assayed for their effects on OA promoter activity and OA protein expression in MC3T3-E1 osteoblasts. A dose-dependent decrease in Smad1, Smad4 and Runx2 proteins was observed with increasing concentrations of corresponding siRNAs (Fig 3A-C). Smad1, Smad4 and Runx2 siRNAs at 100nM concentrations were most effective for down-regulating Smad1, Smad4 and Runx2 protein levels, respectively. This concentration of specific siRNAs was used for OA promoter assays described in Fig 3D-F. The knockdown of Smad1, Smad4 and Runx2 proteins significantly reduced the BMP-2 induced OA promoter activity (Fig 3D-F). In comparison to Smad1-siRNA, Runx2-siRNA and Smad4-siRNA resulted in more robust decrease in BMP-2 induced OA promoter activity. Furthermore, simultaneous knockdown of Runx2 and Smad4 proteins resulted in approximately 90% decrease in BMP-2 induced OA promoter activity in comparison to 80% decrease with Runx2-siRNAs and 60% decrease with Smad4 siRNAs alone. Similarly, decrease in OA protein levels were observed in osteoblasts treated with specific siRNAs for Smad1, Smad4 and Runx2 (Fig 3A-C).

In addition to knockdown studies, the specific effects of Smad1, Smad4 and Runx2 proteins on OA transcription were determined by analyzing the effect of over expression of these proteins on BMP-2 induced OA promoter activity in MC3T3-E1 osteoblasts. The over expression of Smad1, Smad4 and Runx2 proteins was confirmed by Western blotting (Fig 3G-I). Smad1 or Runx2 over expression significantly enhanced BMP-2 induced OA promoter activity in comparison to Smad4 over expression (Fig 3J-L). Furthermore, co-expression of Smad1 and Runx2 proteins further increased (191% increase compared to empty vector transfected osteoblasts) the BMP-2 induced OA promoter activity relative to Smad1 (85% increase) or Runx2 (108% increase) over expression alone (Fig 3K). Taken together, both knockdown and over expression studies suggest that Smad1, Smad4 and Runx2 proteins are required for BMP-2 induced OA transcriptional regulation and these transcription factors up-regulate OA transcription.

BMP-2 stimulates the binding of Smad1, Smad4 and Runx2 proteins to OA promoter

To evaluate the role of -248 SBE site in Smad1 and Smad4 regulated effects on BMP-2 induced

OA transcription, -248 SBE in the OA promoter region was disrupted and the effects of Smad1 or Smad4 over expression on BMP-2 induced OA promoter activity was analyzed in MC3T3-E1 osteoblasts (Fig 4A). Disruption of -284 SBE significantly decreased both Smad1 and Smad4 up-regulated OA promoter activity, suggesting that -248 SBE is a crucial site for both Smad1 and Smad4 mediated effects on OA promoter activation. *In vitro* association of Smad1 and Smad4 proteins with the OA promoter region was visualized by mobility shift assays (Fig 4B). Wild type (W) and mutant (M) oligonucleotide were synthesized from the OA promoter region containing -248 SBE (Fig 4B, top panel). A mobility shift observed (Fig 4B, lower panel) indicated that Smad1 and Smad4 proteins associate with the W-SBE (-248 SBE probe, Fig 4B top panel) in basal (Fig 4B, Lane 6) and BMP-2 treated (Fig 4B, Lane 7) nuclear lysates. However, no interaction indicative of Smad1/4 association was observed with M-SBE in basal (Fig 4B, Lane 4) and BMP-2-treated (Fig 4B, Lane 5) nuclear extracts. The specificity of the complex was tested by competition and supershift assays. Addition of 100X-cold-W-SBE competed with Biotin-W-SBE for binding to Smad1 and Smad4 proteins in nuclear extracts and resulted in weak shift band intensity (Fig 4B, Lane 8). To further confirm the specific binding of Smad1 and Smad4 proteins to W-SBE, a supershift assay was performed using anti-Smad1 or anti-Smad4 antibodies in BMP-2 treated nuclear extracts. The appearance of high molecular weight supershift complex confirmed the binding of Smad1 and Smad4 proteins to -248 SBE in OA promoter region (Fig 4B, Lane 9 and 10, respectively).

The regulatory roles of Runx2 binding sites (R1, R2, R3 and R4) in mediating BMP-2 induced OA promoter activation was examined by comparing the activity of the wild type p962-OA promoter vector with OA promoter vectors containing mutations in Runx2 binding sites (pMR1, pMR2, pMR3, pMR4) in Runx2 over expressing MC3T3-E1 osteoblasts (Fig 4C). Runx2 over expression was confirmed by Western blotting as indicated in Fig 3I. Runx2 over expression enhanced BMP-2 induced activity of Runx2 mutant OA promoter constructs (Fig 4C). pMR4 (vector with mutation in R4 Runx2 binding site) showed minimal enhancement with Runx2 over expression (in BMP-2 treated osteoblasts) compared with other Runx2 mutant-OA promoter vectors, indicating R4 to be crucial for Runx2 binding. Thus, we next performed mobility shift assay with OA promoter oligonucleotide containing R4 Runx2 binding site (Fig 4D). W-Runx2 and M-Runx2 oligonucleotide used for mobility shift assay are shown in Fig 4D, top panel. Low expression levels of endogenous Runx2 in rat primary osteoblasts made the detection of Runx2 by shift assays technically challenging. For this reason, we over expressed the Runx2 isoform II in MC3T3-E1 cells and isolated their nuclear lysates for gel shift assays. The binding of Runx2 to W-Runx2 (Fig 4D, Lane 5) was abolished by mutation in -585 Runx2 (M-Runx2) as shown in lane 3. BMP-2 treatment enhanced binding of Runx2 to W-Runx2 (Fig 4D, Lane 6); however, Runx2 did not bind to M-Runx2 even with BMP-2 stimulation (Fig 4D, Lane 4). Displacement of biotin-labeled-W-Runx2 with a 100-fold excess of cold-W-Runx2 (Fig 4D, Lane 7) displayed the specificity of the W-Runx2 oligonucleotide. Furthermore, appearance of a band shift (Fig 4D, Lane 8) was abolished by incubation with anti-Runx2 antibody (Fig 4D, Lane 9), confirming that the shifted band was Runx2. Taken together, mobility shift assays demonstrated binding of Smad1 and Smad4 proteins to -248SBE and Runx2 association with -685 Runx2 (R4) binding site on the OA promoter region.

Occupancy of Smad1, Smad4 and Runx2 proteins to the OA promoter during osteoblast differentiation

To gain further insight into the physiological roles of Smad1, Smad4 and Runx2 on the OA promoter regulation during osteoblast differentiation, ChIP assays were performed. Smad1, Smad4 and Runx2 occupancy was first determined in -780 to -575 region of the OA promoter. This region contains five potential Smads1/4 and four Runx2 binding sites (Fig 5A). In untreated osteoblasts, association of Smad1 with the OA promoter was enhanced during osteoblast differentiation until day 14, when a maximal Smad1 occupancy on OA promoter was observed. Subsequently at day 21, a modest decrease in Smad1 binding was observed. BMP-2 treatment in proliferating osteoblasts (at day 7) significantly enhanced the recruitment of Smad1 to the OA promoter. In comparison to day 7, BMP-2 treatment at day 14 decreased the binding of Smad1 to the OA promoter. Notably, BMP-2 treated osteoblasts during matrix maturating stage of differentiation (at day 14) displayed a 3.8-fold reduction in Smad1 occupancy on OA promoter relative to untreated osteoblast cultures. Finally, BMP-2 treatment in terminally differentiated osteoblasts (at day 21) resulted in an 11-fold increase in Smad1 occupancy on OA promoter (in comparison to untreated cells). Under basal conditions, Smad4 transcription factor showed a modest increase in OA promoter occupancy during osteoblast differentiation. BMP-2 treatment resulted in similar temporal pattern for Smad4 occupancy on OA promoter as observed with Smad1 (Fig 5B).

In comparison to Smad1 and Smad4 proteins, Runx2 transcription factor showed differential association with the OA promoter (in both BMP-2 treated and untreated cells) during osteoblast differentiation (Fig 5B). Untreated osteoblasts showed a minimal Runx2 occupancy on OA promoter region at days 7 and 14 (Fig 5B). Notably at day 21, a 9-fold increase in Runx2 recruitment was observed in comparison to untreated osteoblast cultures terminated at days 7 and 14. BMP-2 treatment at day 7 and day 14 resulted in 171-fold and 673-fold increase in Runx2 occupancy, respectively relative to untreated cultures (Fig 5B). However at day 21, BMP-2 did not affect Runx2 association with the OA promoter relative to untreated osteoblast cultures. Also, BMP-2 treatment had no significant affect on the binding of non-specific controls, IgG and Polymerase II during osteoblast differentiation.

Next, we assayed for OA promoter occupancy in region -410 to -240 nucleotides of the OA gene. This OA promoter region has four potential Smad1/4 binding sites, including -248SBE, which is crucial for binding Smads1/4 proteins to the OA promoter (Fig 5C). Notably, this promoter region does not have any potential Runx2 binding site. Under basal conditions, Smad1 binding was unaffected until day 14, however, at day 21 a significant increase (3.5-fold increase relative to day 14) in Smad1 binding to OA promoter was observed (Fig 5D). Proliferating osteoblasts (at day 7) displayed a 34-fold increase in Smad1 binding to the OA promoter with BMP-2 treatment relative to untreated cultures. BMP-2 treated osteoblasts at day 14 showed a significantly decreased Smad1 association with OA promoter in comparison to day 7 terminated cells. In addition, BMP-2 treatment at day 14 decreased the binding of Smad1 to the OA promoter by 1.6-fold relative to untreated osteoblast cultures, as observed in Fig 5B. Terminally differentiated osteoblasts cultures (at day 21) displayed a robust increase (69-fold increase) in Smad1 binding with the OA promoter region relative to day 14 with BMP-2 treatment (Fig 5D). In comparison to untreated cultures, BMP-2 treatment enhanced (30-fold increase) Smad1 binding to the OA promoter in osteoblast cells in cultures for 21 days (Fig 5D).

Under basal conditions, a maximal Smad4 occupancy to the OA promoter was observed at day 7, relative to days 14 and 21. BMP-2 enhanced the Smad4 occupancy on the OA promoter at day 7, 14 and 21 by 10.6-fold, 3.8-fold and 2.6-fold, respectively relative to untreated osteoblasts. Interestingly, we did not observe any significant change in Runx2 binding to the OA promoter during osteoblast differentiation with or without BMP-2 treatment in the region that lacks Runx2 binding site (Fig 5D). Collectively, OA ChIP studies indicate that Smads1/4 and Runx2 proteins bind independently on OA promoter and not as a complex of BMP-2 induced Runx2-Smad1 interactions.

DISCUSSION

Mechanism for BMP-2 induced OA transcription in osteoblasts

Our earlier findings indicated vital roles of OA in mediating BMP-2 induced osteoblast differentiation (Abdelmagid et al. 2007). In our present study, we investigated the mechanism of OA gene expression by BMP-2 in osteoblasts. Our results demonstrated that BMP-2 induced maximal stimulation of OA promoter activity in rat primary osteoblasts and minimal in Runx2 knockout osteoblasts, suggesting that Runx2 is required for BMP-2 induced OA transcription (Fig 1C). The elements of OA promoter between -962 and -285 nucleotides are crucial for regulation of OA transcription (Fig 2C) and contain multiple BMP-2 responsive elements (Fig 2D, E). However, the promoter region between -135 to +50 of the OA gene did not respond much to BMP-2 stimulation, indicating that this region has little to minimal functional control of the promoter (Fig 2C). A significant decrease in BMP-2 induced OA promoter activity in Runx2-siRNA treated (Fig 3E) and Runx2 knockout cells (Fig 1C) suggest that Runx2 is crucial for BMP-2 stimulated OA transcription. In comparison to Smad1-siRNA, Smad4-siRNA showed a robust decrease in BMP-2 induced OA promoter activity (Fig 3D). Since, loss of Smad4 will inhibit nuclear translocation of all Receptor-regulated Smads namely, Smad1, Smad5 and Smad8 (Chen et al. 2004), this suggests that Smad5 and Smad8 could also be involved in BMP-2 induced OA transcription. A residual promoter activity in BMP-2-treated osteoblasts was observed even with the loss of both Smad4 and Runx2 proteins (Fig 3F), which could due to contribution of other transcription factors like homeodomain proteins Dlx3 and Dlx5 (Singh et al.). Homeodomain proteins could function independently or in combination with Smad1 and Runx2 transcription factors to regulate OA transcription. Future studies are warranted to test this hypothesis. Similar observations are reported for contribution of Dlx3 and Dlx5 in the regulation of osteocalcin promoter (Hassan et al. 2006). Smad1 and Runx2 proteins when co-expressed showed additive effects on OA transcription in response to BMP-2 stimulation (Fig 3K). Since, we did not observe synergistic increase in OA promoter activity with both Smad1 and Runx2 proteins, we ruled out the possibility of functional interaction between Smad1 and Runx2 proteins, as observed between PEB2αC (a member of the Runx family of proteins) and Smad3 for the transcriptional activation of the germline IgCα promoter (Hanai et al. 1999) .

Interaction of OA promoter with Smad1, Smad4 and Runx2 transcription factors during osteoblast differentiation.

BMP-2 treated proliferating osteoblast cultures displayed a high occupancy of Smad1, Smad4 and Runx2 transcription factors on OA promoter; however, during matrix maturation stage of osteoblast differentiation, BMP-2 reduced the binding of Smad1 and Smad4 to the OA promoter (Fig 5B). We hypothesize that the decreased Smad1 binding in matrix maturating osteoblasts

could be due to interactions of BMP-2 induced Smad1 with other regulatory proteins like Tob, an anti-proliferative protein reported to co-localize with Smad1 following BMP-2 stimulation (Yoshida et al. 2000). These Tob-Smad1 interactions have been shown to suppress the activity of BMP-2 stimulated Smad1 in osteoblasts. Cas-interaction zinc finger protein (CIZ) is another regulatory protein that has been shown to negatively regulate BMP-2 induced Smad1 activity in osteoblasts (Shen et al. 2002). There is also a possibility of protein-protein interactions between Smad1 and co-repressor proteins including Smad1 interacting protein (SIP-1) (Verschueren et al. 1999), fork head activin signal transducer-2 (FAST-2) (Yeo et al. 1999) and ubiquitin-mediated degradation of Smad1 and Smad4 by smad ubiquitin regulatory factor, Smurf1 (Zhao et al. 2004) and jun activating binding protein 1 (Jab1) (Wan et al. 2002), that could down-regulate Smad1 activity and expression. Finally, histone deacetylase (HDAC) and /mSin3A induced changes in chromatin conformation could decrease the binding of Smad1 and Smad4 proteins from transcriptionally active sites (Kim and Lassar 2003). A similar association pattern of Smad1 and Smad4 with the OA promoter region during osteoblast differentiation with BMP-2 stimulation (Fig 5B & D) and also binding of both Smad1 and Smad4 to the -248SBE on the OA promoter (Fig 4B), indicates that both these proteins are recruited as a complex of Smads1/4, which binds the common SBEs on the OA promoter.

BMP-2 robustly recruited Runx2 to the OA promoter region in proliferating and matrix maturating osteoblasts (Fig 5B). Likewise, enhanced association of Runx2 with the promoter regions of collagen type I (Kern et al. 2001), osteocalcin (Hoffmann et al. 2000) and also Runx2 (Hassan et al. 2006) gene are reported during proliferation and matrix maturation stages of osteoblast differentiation. This enhanced Runx2 occupancy at OA promoter could be due to high expression levels of Runx2 protein during matrix maturating stage. Evidence indicates that recruitment of homeodomain proteins, Dlx3 and Dlx5 to the Runx2 promoter stimulates Runx2 expression (Hassan et al. 2006). Interestingly, during terminal stages of osteoblast differentiation, BMP-2 had no effect on Runx2 binding, rather BMP-2 treatment robustly recruited Smad1 and Smad4 proteins to the OA promoter region (Fig 5B). In comparison to osteoblasts in proliferation and matrix maturation stages of differentiation, osteoblasts during their terminal stages of differentiation displayed a decreased Runx2 association with the OA promoter in response to BMP-2 treatment, which we propose could be due to multiple reasons described below. Runx2 is shown to regulate its expression by binding to its own promoter and by this auto-regulatory mechanism decreased Runx2 expression is observed in terminally differentiated osteoblasts (Hassan et al. 2006). Also, protein-protein interactions between Runx2 and transcription factors including homeodomain protein, Msx2 result in sequestration of Runx2 by Msx2 (Shirakabe et al. 2001) and dissociation of Runx2 from osteocalcin promoter (Sierra et al. 2004). Transcriptional co-repressors like histone deacteylase (HDAC) (Westendorf et al. 2002; Schroeder et al. 2004), transducin-like enhancer of split (TLE) protein (Javed et al. 2000; Westendorf 2006), mSin3a (Fenrick et al. 1999) and yes-associated proteins (YAP) (Zaidi et al. 2004) are reported to alter the chromatin structure and decrease the affinity of Runx2 for DNA. Additional studies are warranted to examine the effects of Msx2 and other transcriptional co-repressors in OA promoter regulation during terminal stages of osteoblast differentiation. Alternatively, cytoplasmic sequestration of Runx2 by STAT1 could decrease amount of Runx2 protein available at the transcriptionally active nuclear foci (Kim et al. 2003). Degradation of the Runx2 protein via Smurf1 ubiquitin-mediated proteosome could also reduce Runx2 expression levels in terminally differentiated osteoblasts (Zhao et al. 2004). Both cAMP-dependent and

independent Smad ubiquitin regulatory factor1 (Smurf1) mediated ubiquitination of Runx2 has also been reported (Zhao et al. 2003). Finally, the activity of Runx2 is modulated by phosphorylation/dephosphorylation events controlled by MAPK (Franceschi et al. 2009; Ge et al. 2009) and PI3K-AKT signaling pathways (Fujita et al. 2004; Mukherjee and Rotwein 2009). Taken together, any of the above suggested mechanisms could sequester Runx2 from the OA promoter during terminal stages of osteoblast differentiation.

BMP-2 induced Smads1/4 and Runx2 regulatory interactions.

The generic BMP-2 induced Smad1 signaling pathway for its complete functionality requires association of other transcription factors, transcriptional co-activators and co-repressors. BMP-2 induced interactions of Smad1 with Runx2 have been demonstrated to synergistically stimulate osteoblast differentiation in C2C12 mesenchymal progenitor cells (Lee et al. 2000). Notably, this interaction between Smad1 and Runx2 is observed in response to BMP-2 stimulation (Zhang et al. 2000). In addition, Runx2 protein has been shown to mediate transport of Smad1 to transcriptionally active nuclear foci through specific Runx2-Smad1 interactions (Hanai et al. 1999; Lee et al. 2000; Zhang et al. 2000; Afzal et al. 2005; Javed et al. 2008; Javed et al. 2009). Multiple conflicting indicate that BMP-2 induced osteoblast differentiation occur through independent binding of Runx2 and Smads and not through Runx2-Smad complex (Lee et al. 2002; Phimphilai et al. 2006). Thus, osteoblast differentiation induced by BMP-2 activated pathway occurs either through independent binding of Runx2 and Smads1/4 transcription factors and/ or through coupled interactions between Runx2-Smad1 proteins. Here, our results demonstrate that Runx2 and Smad1 regulation of OA gene is dependent on both proteins but not a coupled interaction (Fig 3K, 5B, D). Based on our findings, we propose that BMP-2 stimulates recruitment of Runx2, Smad1 and Smad4 proteins on the OA promoter region (Fig 6). However, future studies are warranted to elucidate the role of other transcription factors, co-activators and co-regulators/co-repressors that function along with and/ or independent of Smads1/4 and Runx2 transcription factors in OA transcription.

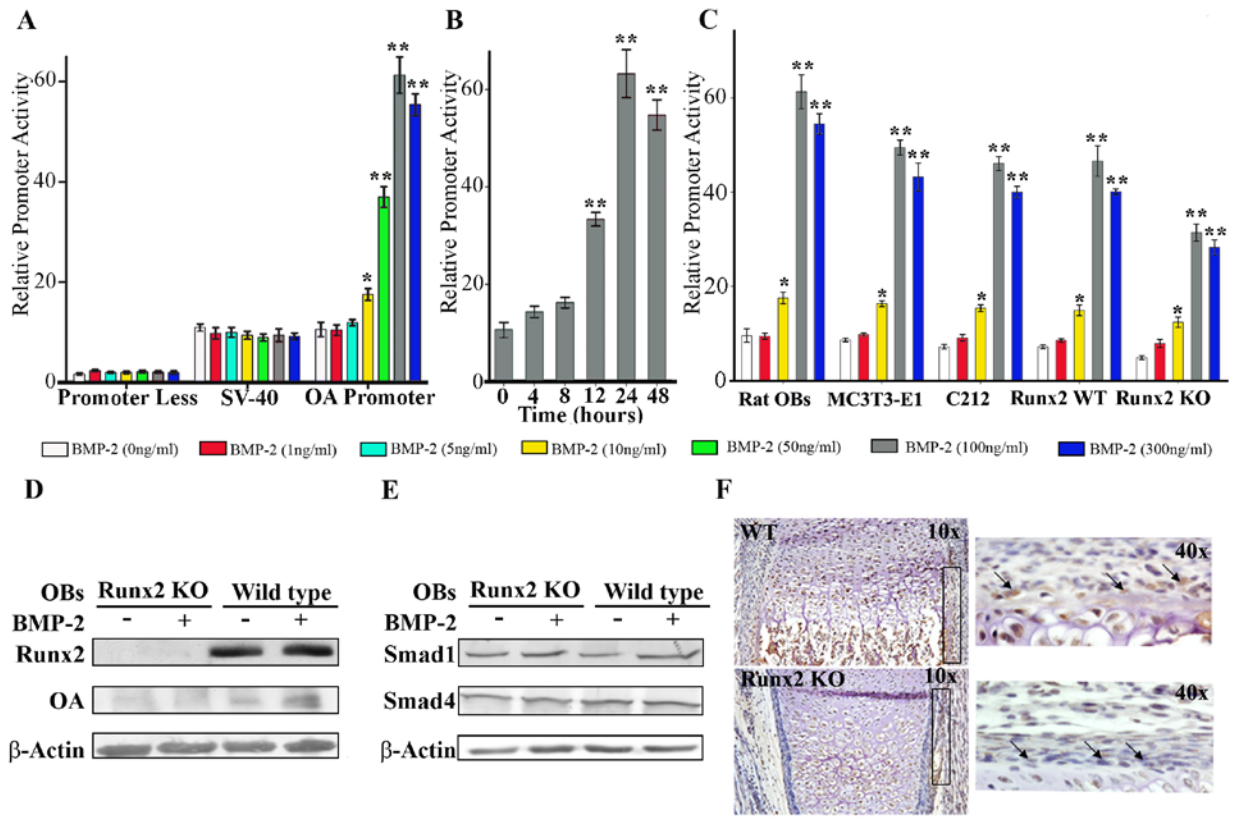
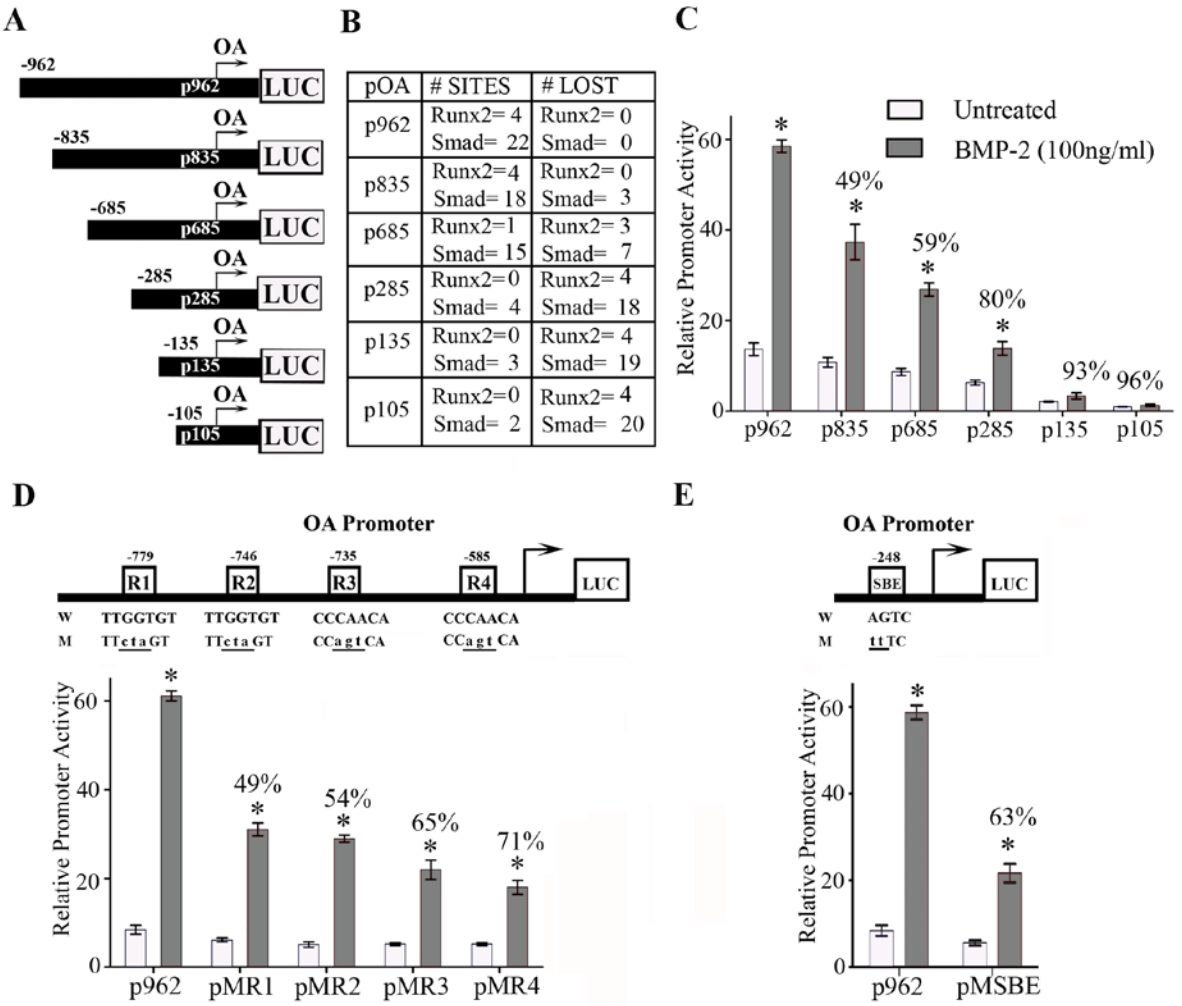


Figure 1. Regulation of OA promoter by BMP-2. A. OA promoter-LUC vector was co-transfected with renilla vector in rat primary osteoblasts. Osteoblasts were serum starved over night and then treated with BMP-2 at doses ranging from 1-300ng/ml. The next day, promoter activity was quantified and is expressed as relative promoter activity that represents luciferase activity normalized against renilla expression. Promoter-less-LUC vector (pGL3-Basic) and constitutively active SV-40 promoter-LUC vector were treated as described for OA-LUC vector. *p-value <0.05 represents OA promoter activity with BMP-2 (10ng/ml) compared with untreated (control) osteoblasts and ** p<0.001 (ANOVA) compares BMP-2 at 50ng/ml, 100ng/ml and 300ng/ml treatment with untreated (control) osteoblasts. **B.** Rat primary osteoblasts were transfected with OA-LUC vector and renilla vector as described in **(A)**. Twenty four hours post-transfection, osteoblasts were treated with BMP-2 (100ng/ml) for 4, 8, 12, 24, 48 hours and relative promoter activity was quantified.**p-value < 0.001 represents OA promoter activity with BMP-2 treatment for 12, 24 and 48 hours compared with 0 hours treatment. **C.** Rat primary osteoblasts, MC3T3-E1 subclone-4, C2C12, C3H10T1/2, Runx2 knockout osteoblasts and their wild type mouse osteoblasts were co-transfected with OA promoter-LUC vector and renilla vector as described in **A.** Relative OA promoter activity in different cell types were quantified and is shown on Y-Axis. *p-value <0.05 represents OA promoter activity with BMP-2 (10ng/ml) compared with untreated (control) osteoblasts in each cell type and ** p<0.001 compares BMP-2 at 50ng/ml, 100ng/ml and 300ng/ml treatment with untreated (control) osteoblasts in each cell type. **D & E.** Western blot for Runx2, OA, Smad1, Smad4 and β-actin in Runx2 knockout (KO) and their wild type (WT) osteoblasts untreated or treated with BMP-2 (100ng/ml) for 24 hours. **F.** OA immunohistochemistry (brown signal) in bones harvested from E18.5 WT and Runx2 KO mice. The periosteal region is shown in a black inset in 40X magnification.



pOA	# SITES	# LOST
p962	Runx2= 4 Smad= 22	Runx2= 0 Smad= 0
p835	Runx2=4 Smad= 18	Runx2= 0 Smad= 3
p685	Runx2=1 Smad= 15	Runx2= 3 Smad= 7
p285	Runx2=0 Smad= 4	Runx2= 4 Smad= 18
p135	Runx2=0 Smad= 3	Runx2= 4 Smad= 19
p105	Runx2=0 Smad= 2	Runx2= 4 Smad= 20

Figure 2. Role of Runx2 and Smads1/4 binding sites on OA promoter activation. A. Schematics of 5' deletions of OA-promoter construct namely p962, p835, p685, p285, p135, p105. Nucleotides are numbered relative to the transcription start site. Arrow represents transcription start site. Each of the OA promoter construct contains +50 nucleotides of the OA gene. **B.** Table illustrating the number of Runx2 and Smad1/4 binding sites in the OA promoter deletion mutants. Also, the number of Runx2 and Smad1/4 binding sites lost with deletion of OA promoter (p962) is shown. **C.** p962 or deletion mutants (p835, p685, p285, p135, p105) of OA promoter-LUC vector were co-transfected with renilla vector and treated with BMP-2 (100ng/ml) for 24 hours as described in Fig **(1A)**. **D.** Top panel, schematics representing position of Runx2 binding sites (R1, R2, R3 and R4) on the OA-promoter and the consensus Runx2 binding sequence is show in wild type (W). Mutated nucleotides in the Runx2 binding sites are in small case and underlined in Runx2 mutants (M). In the bottom panel, OA promoter-LUC vector (W, p962) or OA promoter LUC vector with mutations in Runx2 binding sites (pMR1, pMR2, pMR3 and pMR4) were treated as described in **(C). E.** Top panel, location of -248 SBE on the OA promoter region is shown. Consensus SBE sequence is shown as wild type (W) oligonucleotide. Mutated nucleotides in -248 SBE are underlined. Bottom panel, OA promoter (p962) or OA promoter with mutation in -248 SBE (pMSBE) was transfected and treated as described above in **(C).** Data is represented as relative promoter activity. Asterisks (*) represents the p-value<0.001 of BMP-2 treatment compared with untreated osteoblasts for each OA promoter mutant construct.

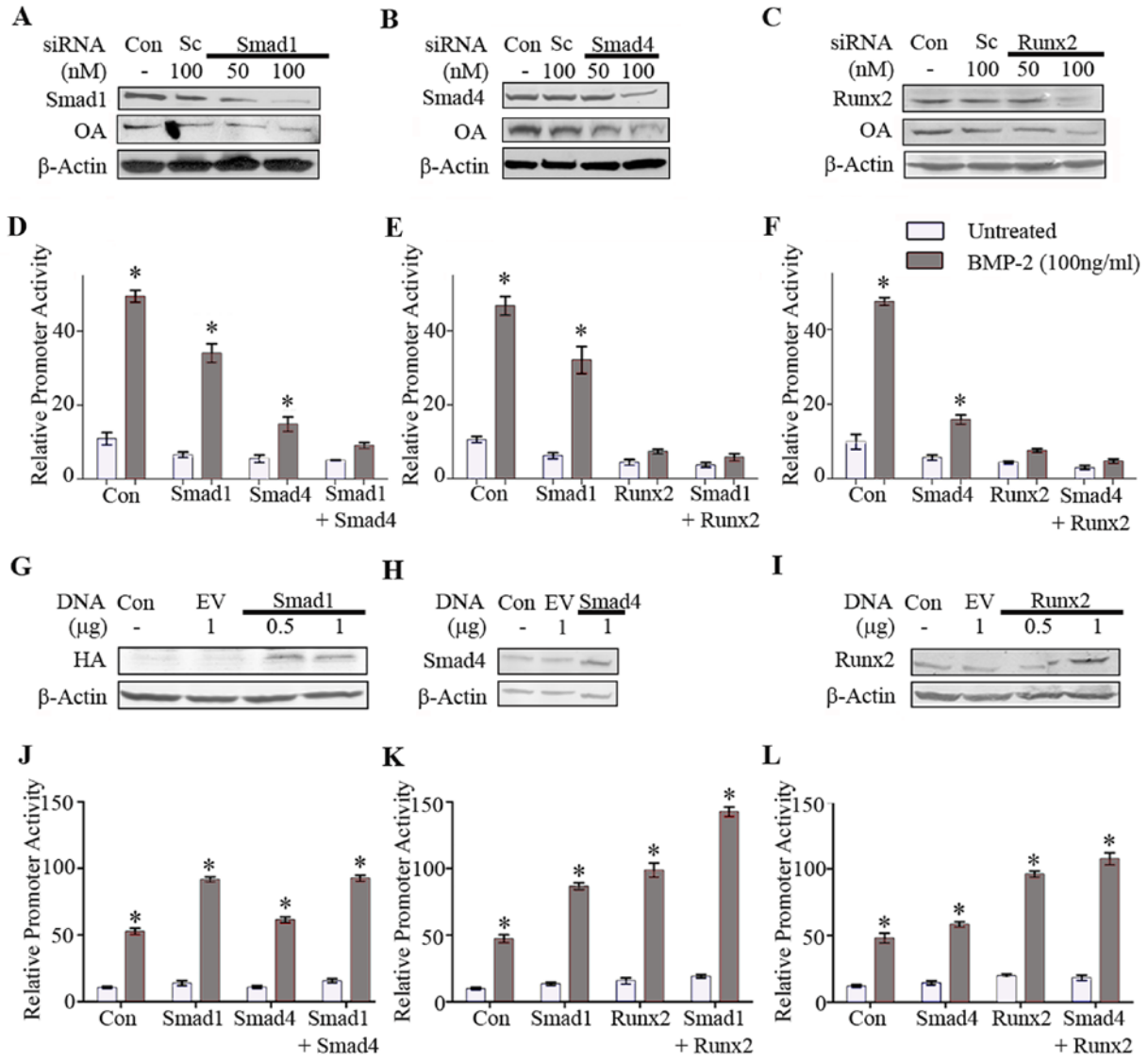


Figure 3. Role of Smad1, Smad4 and Runx2 proteins on OA promoter activation. A-C. MC3T3-E1 cells were transfected with Smad1-siRNA, Smad4-siRNA, Runx2-siRNA or their respective Scrambled (Sc)-siRNAs. The cell lysates were collected 48 hours after transfection and analyzed for knockdown of proteins by immuno-blotting for Smad1, Smad4 or Runx2 proteins, OA and β-actin. **D-F.** MC3T3-E1 osteoblasts were co-transfected with Sc-siRNAs or Smad1-siRNA (100nM), Smad4-siRNA (150nM), Runx2-siRNA (100nM) or combination of any two siRNAs and OA-promoter-LUC vector. Renilla vector was also transfected. The next day, serum starved osteoblasts were treated with BMP-2 (100ng/ml) for 24 hours and relative promoter activity was determined. Control (Con) represents untransfected cells. Transfection of Sc-siRNA for Smad1, Smad4 and Runx2 did not affect the OA promoter activity (data not shown). **D-F.** MC3T3-E1 osteoblasts were transfected with HA-tagged Smad1, Smad4, Runx2-Isoform II over-expression vectors or their respective empty vectors (EV) namely, pCMV, pCMV5 and pSG5 vector. Cell lysates were collected 48 hours after transfection and immuno-blotted for HA, Smad4, Runx2, OA and β-actin. **G-I.** MC3T3E1 cells were co-transfected with either empty vector (1µg), Smad1 (1µg), Smad4 (1µg), Runx2 over-expression vector (1µg) or a combination of any two over-expression

vectors and the OA-promoter-LUC vector. Transfected osteoblasts were treated with BMP-2 (100ng/ml) as described above and are expressed as relative promoter activity. Control (Con) represents untransfected cells. Empty vector did not affect the OA promoter activity (data not shown). Asterisks (*) represents the *p-value<0.001 of BMP-2 treatment compared with untreated osteoblasts for each condition.

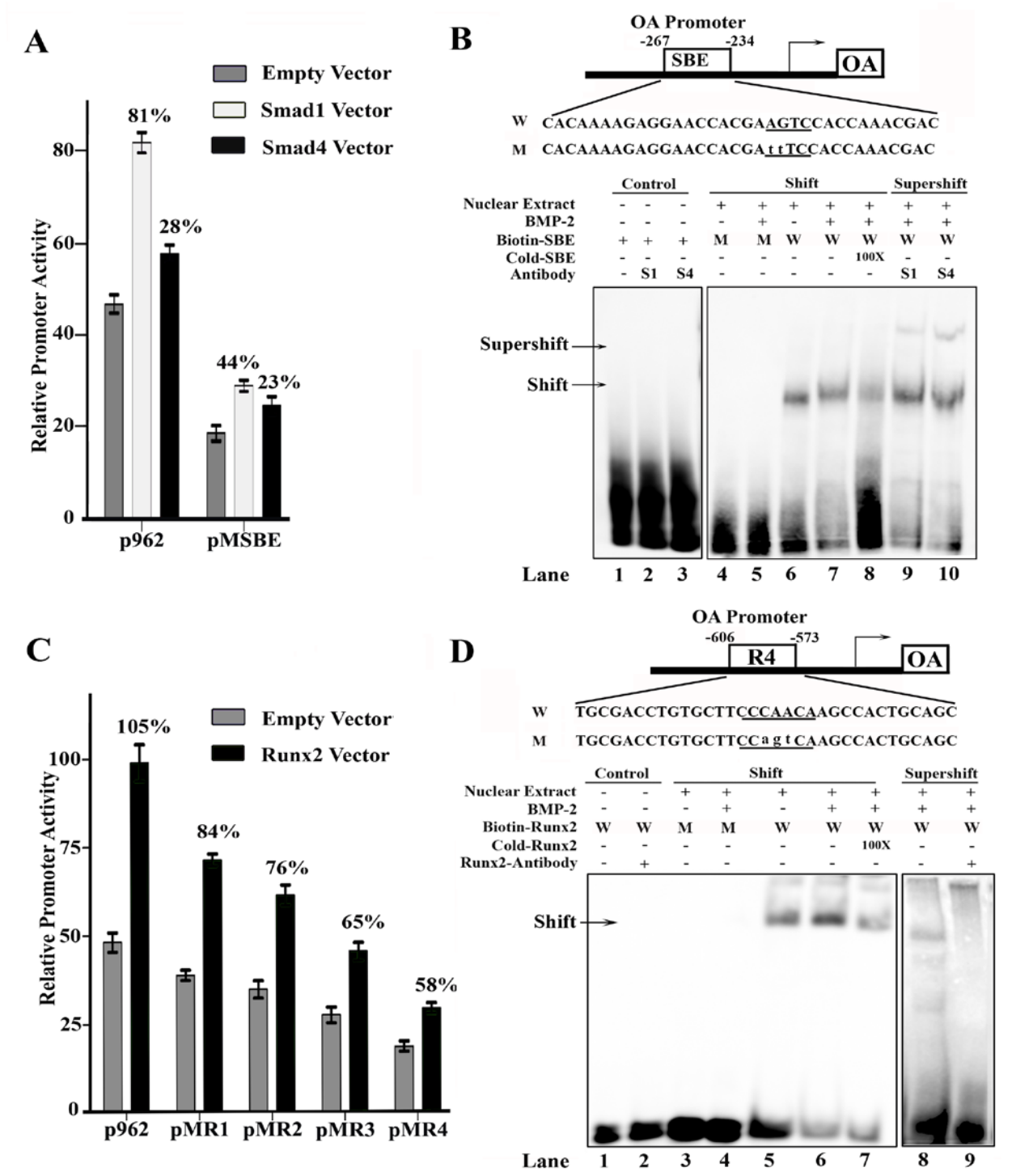


Figure 4. Effect of BMP-2 on binding of Smads1/4 and Runx2 to SBE and Runx2 binding site on the OA promoter. A. p962 (W) or pMSBE was co-transfected with Smad1 vector (1µg) or EV (1µg).

Transfected osteoblasts were treated with BMP-2 and relative promoter activity was quantified as described in **3D**. Percentage increase in promoter activity with over-expression relative to EV transfected osteoblasts with BMP-2 treatment is shown. B. Upper panel, location and sequence of W oligonucleotide used for Smads1/4 shift assay, consensus -248 SBE is underlined. Substituted nucleotides in Moligonucleotide are in small case. Lower panel is mobility shift assay, **Controls:** Lane 1, Biotin-W-SBE without nuclear extract, Lane 2 and 3, Biotin-W-SBE with no nuclear extracts incubated with Smad1 or Smad4 antibody, respectively. **Gel-Shift:** Lanes 4 and 5, M-SBE incubated with BMP-2 untreated and treated nuclear extracts, respectively. Lane 6, untreated nuclear extract with Biotin-W-SBE. Lane 7, BMP-2 treated nuclear extracts with Biotin-W-SBE. Lane 8, competition with cold-W-SBE (100X) added to nuclear extracts before addition on Biotin-W-SBE. **Super-shift**: Lane 9 and 10 contained BMP-2 treated nuclear extracts pre-incubated with Smad1 and Smad4 antibodies, respectively for 20 min and then incubated with Biotin-W-SBE for another 15 min. Shift and supershift shown with arrow. **C.** W or OA promoter LUC vector with mutations in Runx2 binding sites was co-transfected with Runx2 vector (1µg) or EV (1µg) in MC3T3-E1 osteoblasts. Relative promoter activity in BMP-2 treated cells was determined. **D**. Position and sequence of Runx2 oligonucleotide (W) on OA promoter is shown. The consensus Runx2 binding site 4 (R4) is underlined and mutated nucleotides in mutant Runx2 (M) are in small case. The MC3T3-E1 osteoblasts over-expressing Runx2-isoform II as described in **4C** were treated with BMP-2 and gel shift was performed. **Controls:** Lane 1, Biotin-W-Runx2 without nuclear extracts and lane 2, Biotin-W-Runx2 incubated with Runx2 antibody without nuclear extracts. **Shift:** Lane 3 and 4, Biotin-M-Runx2 was incubated for 20 min with untreated or BMP-2 treated nuclear extracts, respectively. Lane 5 and 6, Biotin-W-Runx2 incubated with untreated or BMP-2 treated nuclear extracts, respectively. Arrow shows the observed gel-shift. Lane 7, 100X-Cold-W-Runx2 was pre-incubated with nuclear extracts prior to addition of Biotin-W-Runx2. Lane 8 contains BMP-2 treated nuclear extracts incubated with Biotin-W-Runx2 as described for Lane 6. Lane 9 was treated similar to Lane 6 and 8; the only difference was the pre-incubation of Runx2 antibody to the BMP-2 treated extracts 20 min before addition of Biotin-W-Runx2 probe.

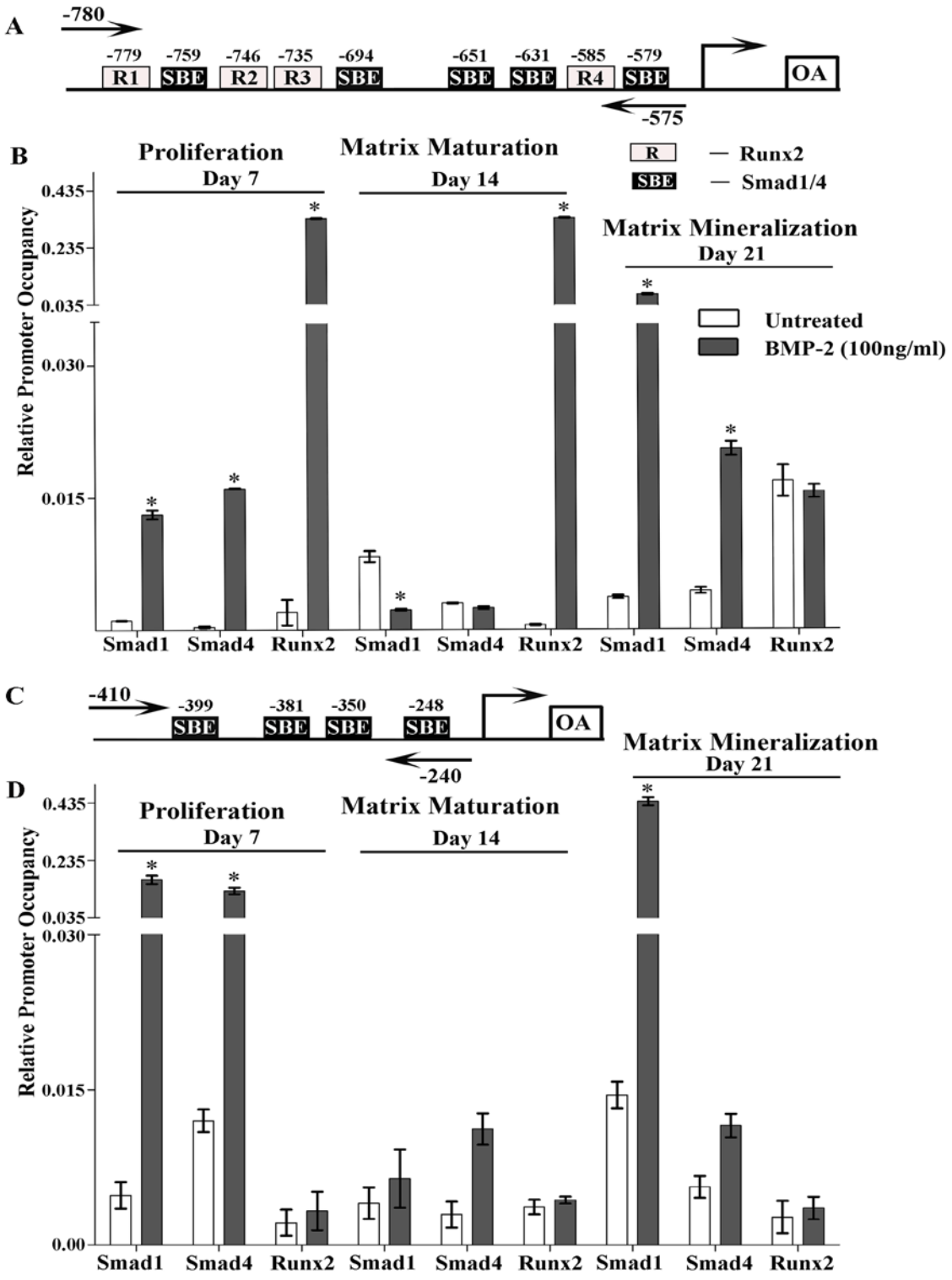


Figure 5. Occupancy of Smad1, Smad4 and Runx2 proteins on the OA promoter during osteoblast differentiation. A. Schematic representation of amplified region of the OA promoter that contains five Smad1/4 binding sites (SBE) and four Runx2 binding sites (R). Arrows show the position of primers used

to amplify the OA promoter region. **B & D.** Rat primary osteoblasts in differentiation media at day 7, 14 and 21 were untreated or BMP-2 (100ng/ml) treated for 2 hours, and harvested for chromatin immunoprecipitation assay. *p-value < 0.001 represents relative promoter occupancy with BMP-2 (100ng/ml) treatment in comparison to untreated osteoblast cultures. **C**. Schematics of the amplified region of OA promoter that contains four Smads1/4 binding sites (SBE).

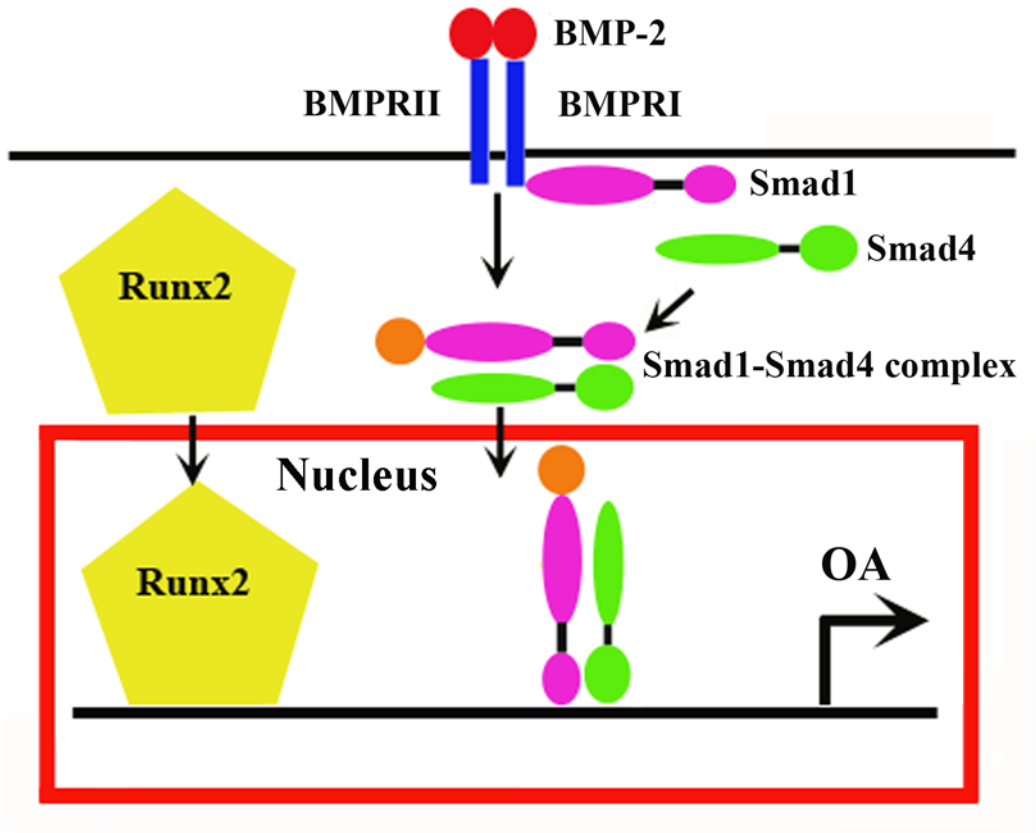


Figure 6. Proposed mechanism for BMP-2 induced recruitment of Smads1/4 and Runx2 transcription factors to the OA promoter. BMP-2 signals through transmembrane serine/threonine kinase BMP receptors (BMPRs), type I (BMPRI) and type II (BMPRII). Binding of BMP-2 to the BMPRII brings it in close proximity to the BMPRI that result in phosphorylation of the BMPRI. The activated BMPRI then phosphorylates downstream effector Smad1. Phosphorylated Smad1 associates with Smad4 and this complex of Smad1-Smad4 is translocated into the nucleus, where it functions as a transcription factor. Smad1 and Smad4 through their DNA binding domain (MH1 domain) interact with Smad binding elements (SBE) on the OA promoter region. Inside the nucleus, BMP-2 activated Smad1/4 complex and Runx2 transcription factors independently up-regulate OA transcription.

Table 1. Primers Used for this Study

A. OA promoter Deletion constructs

Construct	Forward Primer	Reverse Primer
p962	5'-GGGGTACCCCTGAGTGTCCGTTTTTA-3'	5'- AAGATCTTCCTGCGATGCCTCTCTGT-3'
p835	5'-GGGGTACCCCTACTTTGTGGTTGTACA-3'	5'- AAGATCTTCCTGCGATGCCTCTCTGT-3'
p685	5'-GGGTACCCCAGCATTCCACACTTGCT-3'	5'- AAGATCTTCCTGCGATGCCTCTCTGT-3'
p285	5'-GGGGTACCCCAGGTTTCACTTGGTGAA-3'	5'- AAGATCTTCCTGCGATGCCTCTCTGT-3'
p105	5'-GGGGTACCCAGATGCCAGGAGCAAGT-3'	5'- AAGATCTTCCTGCGATGCCTCTCTGT-3'

B. Runx2 mutant OA promoter constructs

Construct	Forward Primer	Reverse Primer
pMR1 (-779 Runx2)	5'-TGAACTAGAGACTTTTGCTACTTTG**CATG** TGTACAGAAGCCTCCAGTTCTCCTC-3'	5'GAGGAGAACTGGAGGCTTCTGTACA**CATG**CAA AGTAGCAAAAGTCTCTAGTTCA-3'
pMR2 (-746 Runx2)	5'-CCTCCAGTTCTCCTC**CATG**CTAGTTTCCC CCACACACAAGCTC-3'	5'- GAGCTTGTGTGTGGGGGAAACTAG**CATG**GAG GAGAACTGGAGGC-3'
pMR3 (-735 Runx2)	5'-CAGTTCTCCTCCATGTGGTTTTCCC**TAGT**A CACAAGCTCTATTCCCTGAATTAC-3'	5'-GTAATTCAGGGAATAGAGCTTGTGT**ACTA**GGG AAAACCACATGGAGGAGAACTG-3'
pMR4 (-585Runx2)	5'-GGCAATGCGACCTGTGCTTCC**TGAG**AGCC ACTGCAGCTATTCTGAG-3'	5'-CTCAGAATAGCTGCAGTGGCT**CTCA**GGAAGCA CAGGTCGCATTGCC-3'

C. SBE mutant OA promoter construct

Construct	Forward Primer	Reverse Primer
pMSBE (-248 SBE)	5'-GGGACACAAAAGAGGAACCACGATTTCCA CCAACGACCAGGTTTCACTTGGTG-3'	5'- CACCAAGTGAAACCTGGTCGTTGGTGGAAATC GTGGTTCCTCTTTTGTGTCCC-3'

D. Chromatin Immunoprecipitation

OA Promoter Region	Forward Primer	Reverse Primer
-780 to -575	5'-GGCTGGGCTTGAACTTAGAGACAT-3'	5'-TCTGTCTGCTTGTTTACACACATG-3'
-410 to -240	5'-TCACAACCACTCGTACGTGTGTGT-3'	5'-CGTTGGTGGACTTCGTGGTTCCT-3'

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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, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

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.Homeodomain Transcription Factors Regulate BMP-2-Induced Osteoactivin Transcription in Osteoblasts	M Singh, F Carpio-Cano, M Monroy, S Popoff, F Safadi	Journal of Cellular Physiology	March 2011	☐Submitted ☐Accepted ☒Published

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

Yes____X_____ No_____

If yes, please describe your plans:

Findings are being submitted to PLOSONE.

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.

Understanding the regulation of OA expression in bone will lead to the development of diagnostic tools for treating bone loss and osteoporosis.

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.

None

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_____X____ No_____

If yes, please describe your plans:

Additional new data required before submitting a patent

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.*

PI is no longer at Temple University.

PI biosketch is below:

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Fayez F. Safadi	Professor of Anatomy and Neurobiology

eRA COMMONS USER NAME safadi

EDUCATION/TRAINING

	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Southampton, Southampton, UK	B.Sc.	1992	Biochemistry, Physiology
University of Southampton, Southampton, UK	Ph.D.	1996	Endocrinology and Cancer Cell Biology

A. Personal Statement – The focus of my research program is on the regulation of bone cell development and function. Many of my studies have involved cellular, molecular, and bone histomorphometric measurements in animal models characterized by abnormal excess or loss of bone tissue. My laboratory utilizes genetically engineered mouse models of genes that are important for skeletal development and function. My laboratory also discovered Osteoactivin (OA) as a novel bone anabolic factor that regulates bone cell differentiation and function. My current position as the Professor and the Ohio Research Scholar at Northeast Ohio Medical University (NEOMED) focuses on continued my research program and building a network collaborative research programs in Northeast Ohio with the consortia of clinical and academic enterprise. I served on multiple study sections for NIH/NIAMS, NIA, NIDCR, DoD, USDA and different foundations. Currently, I serve on many steering committees such as the FDA and ABIA collaborative research program, SUMMA Orthopaedic Research Steering Committee and Board member of the Biomedical Engineering Program at the University of Akron. Taken together, I believe that my background and experiences will justify my interests in serving on study sections for NIDCR.

B. Positions and Honors.
Employment/Experience:

1996-1998 Postdoctoral Fellowship, Endocrinology and Genetics, University of Pennsylvania Medical School
1998-1999 Assistant Scientist of Anatomy and Cell Biology, Temple University Medical School
1999-2004 Assistant Professor of Anatomy and Cell Biology, Temple University School of Medicine
2000-2011 Director of Molecular Histology Core Facility, Temple University School of Medicine
2004-2011 Associate Professor of Anatomy and Cell Biology, Temple University School of Medicine
2005-2011 Vice Chairperson for Research, Anatomy and Cell Biology, Temple University S School of Medicine
2006-2011 Associate Professor of Orthopaedic and Sport Medicine, Temple University School of Medicine
2011-Present Professor of Anatomy and Neurobiology, Northeast Ohio Medical University
2011-Present Ohio Research Scholar, Northeast Ohio Medical University
2011-present Adjunct Professor of Anatomy and Cell Biology, Temple University School of Medicine

Awards and Honors:

B.Sc. Honors, University of Southampton 1992
Ph.D. Scholarship, University of Southampton 1994-1995
Endocrine Society Travel Award 1998
NIH Grant Travel Award 1999
ASBMR Travel Award 1999AIMM-ASBMR J. Haddad Young Investigator Award 2000
Alice Jee Memorial Young Investigator Award 2001
Bone Biology Workshop Travel Award, Davos 2002
Arthritis Foundation Travel Award 2003
1 Million Dollar Research Club Award (Temple University) 2004, 2005, 2006, 2007
USDA study section 2004
NIH/NIAMS Special Panel for R21 2004
NIH/NIA Special Panel RO1 2005
DoD study section for Bone-related Diseases 2006
DoD study section for osteoporosis 2006
NIH/NIAMS MSA review committee special panel 2006, 2007
NIH/NIAMS RO3 review committee 2006
DoD study section special panel 2007
NIH/NIAMS RO3 review committee 2007
NIH/NIAMS RO3 review committee 2008
ASBMR Bridge Grant (reviewer) 2008
NIDCR Special Study Section 2009
Weill Cornell Medical College in Qatar Teaching Award 2009, 2011
Qatar University Teaching Award 2009
NIH/NIAMS RO3 review committee 2010
NIH/NIAMS RO3 review committee 3/2011, 4/2011, 9/2011
NIH/NIAMS RO3 review committee 3/2012, 7/2012, 10/2012, 3/2013

Editorial Board:

Bone and Tissue Regeneration Insights-member 2012-present
New Journal of Science-member 2013-present

Professional and Academic Organizations

American Association for the Advancement of Science
The Endocrine Society
American Society for Bone and Mineral Research
International Society of Bone and Minerals
British Endocrine Society
Sigma Xi, The Scientific Research Society
Orthopedic Research Society
New York Academy of Sciences
American Society for Cell Biology
Society for Histochemistry
American Society for Matrix Biology
The CCN International Society
European Calcified Tissue Society

C. Peer-Reviewed Publications:

1. Abdelmagid S.M., Barbe M.F., Arango-Hisijara I., Owen T.A., Popoff S.N., **Safadi F.F.** (2007) Osteoactivin acts as downstream mediator of BMP-2 effects on osteoblast function. *J. Cell. Physiol.* 210(1):26-37.

2. Song J.J., Aswad R., Kanaan R.A., Rico M.C., Owen T.A., Barbe M.F., **Safadi F.F.,** Popoff

S.N. (2007) CTGF acts as a downstream mediator of TGF-□1 in promoting condens a tion and regula ting FN and S ox9 expression in mesenchymal stem cells. *J. Cell. Physiol.* 210:398-410.
3. Arnott J.A., Nuglozeh E., Rico M.C., Arango-Hisijara I., Odgren P.R., **Safadi F.F**., Popoff S.N. (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.
4. Rico M.C., Castaneda J.L., Manns J.M., Uknis A.B., Sainz I.M., **Safadi F. F.,** Popoff S.N., DeLa Cadena R.A. (2007) Amelioration of inflammation, angiogenesis and CTGF expression in an arthritis model by a TSP1-derived peptide. *J. Cell. Physiol*. 221: 504-512.
5. Sheibanie A.F., Khayrullina T,, **Safadi F.F.,** Ganea D. (2007) PGE2 exacerbates collagen-induced arthritis through the inflammatory IL-23-->IL-17 axis. *Arthritis Rheum* 56(8):2608-19
6. Lin Y.L., de Villiers W.J., Garvy B., Post S.R., Nagy T.R., **Safadi F.F.,** Faugere M.., Wang G., Malluche H.H., Williams, J.P. (2007) The effect of class a scavenger receptor deficiency in bone. *J Biol Chem*. 282(7):4653-60. Epub 2006 Dec 13.
7. Selim A.A., Castaneda J.L., Owen T.A., Popoff S.N., **Safadi F.F.** (2007) The role of osteoactivin-derived peptides in osteoblast differentiation. Med Sci Monit. 13(12):BR259-70.
8. Coq J.O., Strata F., Russier M., **Safadi F.F.**, Merzenich M.M., Byl N.N., Barbe M.F.. (2007) Impact of neonatal asphyxia and hind limb immobilization on musculoskeletal tissues and S1 map organization: Implications for cerebral palsy. Exp Neurol. Oct 24; [Epub ahead of print]
9. Zhu S., Barbe M.F., Amin N., Rani S., Popoff S.N., Safadi F.F., Litvin J. (2007) Immunolocalization of Periostin-Like-Factor and Periostin During Embryogenesis. J Histochem Cytochem. 2007 Nov 26; [Epub ahead of print].
10. Arnott J.A., Sanjay A., Owen T.A., Rehman S., DeLong W.G., **Safadi F.F.**, Popoff S.N. (2007) Molecular requirements for Smad and Src/Erk signaling in the induction of CTGF expression by TGF-□1 in osteoblasts. *Bone* 42(5): 871-885.
11. Abdelmagid S.M., Rico M.C., Barbe M.F., Salihoglu S., Arango-Hisijara I., Anderson, M.G., Simon WJ., Owen T.A., Popoff, S.N., **Safadi F.F.** (2008) Osteoactivin, an anabolic factor that regulates osteoblast differentiation and function Expr Cell Res. 314:2334-2351.
12. Abdelmagid S.M., Rico M.C., Barbe M.F., Salihoglu S., Arango-Hisijara I., Owen T.A., Popoff, S.N., **Safadi F.F.** (2009) Characterization and function of osteoacivin in osteoblasts. *J. Orthop. Surg. and Sports Med.* 3:40-56.
13. Zhu S., Barbe M.F., Amin N., Rani S., Popoff S.N., **Safadi F.F.,** Litvin J. (2008) Immunolocalization of Periostin-Like-Factor and Periostin During Embryogenesis. J Histochem Cytochem. 56(4):329-345 Nov 26; [Epub ahead of print].
14. Arnott J.A., Sanjay A., Owen T.A., Rehman S., DeLong W.G., **Safadi F.F.**, Popoff S.N. (2007) Molecular requirements for Smad and Src/Erk signaling in the induction of CTGF expression by TGF-□1 in osteoblasts. *Bone* 42(5): 871-885.
15. Abdelmagid S.M., Rico M.C., Barbe M.F., Salihoglu S., Arango-Hisijara I., Anderson, M.G., Simon WJ., Owen T.A., Popoff, S.N., **Safadi F.F.** (2008) Osteoactivin, an anabolic factor that regulates osteoblast differentiation and function Expr Cell Res. 314(13):2334-51. Mar 10 [Epub ahead of print]
16. Abdelmagid S.M., Rico M.C., Barbe M.F., Salihoglu S., Arango-Hisijara I., Owen T.A., Popoff, S.N., **Safadi F.F.** (2009) Characterization and function of osteoacivin in osteoblasts. *J. Orthop. Surg. and Sports Med.* 3:40-56.
17. Zhu S, Barbe MF, Liu C, Hadjiargyrou M, Popoff SN, Rani S, **Safadi FF**, Litvin J. (2009) 218(3):584-592.
18. Slupianek, A., Yerrum, S., **Safadi, F. F.**, Monroy, M. A., (2010) The Chromatin Remodeling Factor SRCAP Modulates Expression of Prostate Specific Antigen in Prostate Cancer Cells, J. Cell. Biochem. 224(2):369-75.

19. Abdelmagid SM, Barbe MF, Hadjiargyrou M, Owen TA, Razmpour R, Rehman S, Popoff SN, **Safadi FF**. (2010) Temporal and spatial expression of osteoactivin during fracture repair. J Cell Biochem. 2 [Epub ahead of print]
20. Zhang X, Arnott JA, Rehman S, Delong WG Jr, Sanjay A, **Safadi FF**, Popoff SN. (2010) Src is a major signaling component for CTGF induction by TGF-beta1 in osteoblasts. J Cell Physiol. 2010 Apr 28. [Epub ahead of print]
21. Raynaud CM, Maleki M, Lis R, Ahmed B, Al-Azwani I, Malek J, **Safadi FF**, Rafii A (2012) Comprehensive characterization of mesenchymal stem cells from human placenta and fetal membrane and their response to osteoactivin stimulation. Stem Cells Int. 2012;2012:658356.
22. Abdelmagid SM, Barr AE, Rico M, Amin M, Litvin J, Popoff SN, **Safadi FF**, Barbe MF. Performance of repetitive tasks induces decreased grip strength and increased fibrogenic proteins in skeletal muscle: role of force and inflammation. PLoS One. 2012;7(5):e38359. Epub 2012 May 31.
23. Exploratory study on the effect of osteoactivin on bone formation in the rat critical-size calvarial defect model. Bateman JP, **Safadi FF**, Susin C, Wikesjö UM. J Periodontal Res. 2012 Apr;47(2):243-247.

A. Research Support
Research Projects Ongoing or Completed in the Last Three Years
Ongoing:
Source of Support and Title: NIH-NIAMS R01AR048892-06
The Role of Osteoactivin in Osteoblast Development and Function
Role in the Project: Principle Investigator
Percentage of Time on the Project: 45%
Entire Project Period: 07/09-06/14

Source of Support and Title: Qatar National Research Fund, NPRP (Abnormalities in Bone Development Associated with Pre-eclampsia)
Role in the Project: Co-Principal Investigator
Percentage of Time on the Project: 10%
Entire Project Period: 12/10-11/14

Source of Support and Title: NIH-NIAMS AR05121-01, “Functional Notochordal Cell Interactions to Prevent and Repair Disc Degeneration”
Role in the Project: Collaborator (P.I. James Iatridis, Ph.D.)
Percentage of Time on the Project: 2%
Entire Project Period: 07/09-06/12

Source of Support and Title: R21 CA167126-0A1 Role of Osteoactivin in Invasion of Oral Cancers”
Role in the Project: Co-investigator, (P.I. Oneida Aroserena, M.D.)
Percentage of Time on the Project: 3%
Entire Project Period: 07/13-06/15

Source of Support and Title: ABIA, “Effects of Electromagnetic Stimulation on Growth Plate Development”
Role in the Project: (P.I. Fayez F. Safadi, Ph.D.)
Percentage of Time on the Project: 10%
Entire Project Period: 04/13-03/14

Completed:
Source of Support: ASBMR
Title: “The Role of Osteoactivin in Osteoblast Development and Function”
Role in the Project: Principal-Investigator
Entire Project Period: 08/08-07/09
The goal of this bridge funding mechanism is to generate preliminary data for the competing renewal for RO1 application
Overlap: There is no scientific or budgetary overlap with the present application.

Source of Support: NIH/NIAMS 1 R01 AR048892-05
Title “The Role of Osteoactivin in Osteoblast Development and Function”
Role in the Project: Principal Investigator
Entire Project Period: 8/02 - 6/08
The goal of this project is to demonstrate that osteoactivin acts as a novel anabolic bone growth factor by stimulating osteoblast differentiation and function.
Overlap: There is no scientific or budgetary overlap with the present application.

Source of Support: NIH/NIAMS R01 AR047432-05
Title: “The Role of CTGF in Osteoblast Development and Function”
Role in the Project: Co-Investigator (P.I. Steven N. Popoff)
Entire Project Period: 7/08-6/13
The goal of this project is to demonstrate that connective tissue growth factor (CTGF) is synthesized and secreted by osteoblasts, where it acts as an extracellular matrix-associated signaling molecule and/or soluble factor to promote osteoblast differentiation and function in an autocrine fashion.
Overlap: There is no scientific or budgetary overlap