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Journal of Cellular Biochemistry 115:1243–1253 (2014)

Journal of Cellular Biochemistry

Osteoactivin Promotes Osteoblast Adhesion Through HSPG and αvβ1 Integrin

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ABSTRACT

Osteoactivin (OA), also known as glycoprotein nmb (gpnmb) plays an important role in the regulation of osteoblast differentiation and function. OA induced osteoblast differentiation and function in vitro by stimulating alkaline phosphatase (ALP) activity, osteocalcin production, nodule formation, and matrix mineralization. Recent studies reported a role for OA in cell adhesion and integrin binding. In this study, we demonstrate that recombinant osteoactivin (rOA) as a matricellular protein stimulated adhesion, spreading and differentiation of MC3T3-E1 osteoblast-like cells through binding to $\alpha_v\beta_1$ integrin and heparan sulfated proteoglycans (HSPGs). MC3T3-E1 cell adhesion to rOA was blocked by neutralizing anti-OA or anti-α_v and β_1 integrin antibodies. rOA stimulated-osteoblast adhesion was also inhibited by soluble heparin and sodium chlorate. Interestingly, rOA stimulated-osteoblast adhesion promoted an increase in FAK and ERK activation, resulting in the formation of focal adhesions, cell spreading and enhanced actin cytoskeleton organization. In addition, differentiation of primary osteoblasts was augmented on rOA coated-wells marked by increased alkaline phosphatase staining and activity. Taken together, these data implicate OA as a matricellular protein that stimulates osteoblast adhesion through binding to $\alpha_v\beta_1$ integrin and cell surface HSPGs, resulting in increased cell spreading, actin reorganization, and osteoblast differentiation with emphasis on the positive role of OA in osteogenesis. J. Cell. Biochem. 115: 1243–1253, 2014.

KEY WORDS: OSTEOACTIVIN; OSTEOBLAST; ADHESION; INTEGRIN; OSTEOBLAST DIFFERENTIATION

Extracellular matrix (ECM) is the materials that are secreted by cells and form the cells environment, which is very important for cells survival, proliferation, differentiation, and function [Frantz et al., 2010]. ECM consists of three main components, they are: collagen, glycoprotein, and proteoglycans [Ruoslahti et al., 1985]. ECM also contains growth factors that are essential for cell adhesion, which is one of the most important requirements for cells differentiation and function [Ruoslahti et al., 1985; Frantz et al., 2010]. Different cell types have its own specific ECM composition that is important for tissue and cell homeostasis. Any alteration in the unique composition of tissue ECM can lead to a possible severe defect in cells or organ function [Frantz et al., 2010; Xiao et al., 2002]. Cells bind to proteins in ECM through their transmembrane glycoproteins, called integrins. Each integrin consists of two subunits α and β. Fibronectin and laminin are the two major ECM proteins that bind integrins within cell membrane, and cause cell adhesion to ECM [Ruoslahti et al., 1985; Johansson et al., 1997].

Previous studies have established that integrin-mediated cell–matrix interactions are essential for cell adhesion, migration, proliferation, differentiation, and survival [Damsky and Werb, 1992; Meredith et al., 1996; Giancotti and Ruoslahti, 1999]. Also, it has been well established that integrin–matrix interactions play an important role in osteoblast function [Lai and Cheng, 2005]. Integrins are structured as a heterodimeric transmembrane protein, formed by non-covalent association of alpha and beta subunits. Both subunits are type 1 transmembrane proteins with large extracellular ectodomains and short cytoplamic tails [Hynes, 1992]. Integrins work as ECM receptors that transduce signals from the environment into the cell interior. Signals from integrin receptors regulate

Grant sponsor: National Institute of Arthritis and Musculoskeletal and Skin Diseases; Grant number: R01AR048892; Grant sponsor: Ohio Department of Development.
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Manuscript Received: 1 January 2014; Manuscript Accepted: 7 January 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 10 January 2014
DOI 10.1002/jcb.24760 • © 2014 Wiley Periodicals, Inc.

multiple cellular functions such as cytoskeletal organization and cell morphology, tissue-specific differentiation, cell proliferation, migration, and survival [Damsky and Werb, 1992; Giancotti and Ruoslahti, 1999; Raisz, 1999; Zimmerman et al., 2000]. The importance of integrins in osteoblast function has been well recognized. Administration of RGD peptide, a non-specific integrin inhibitor reduced bone formation [Gronowicz and Derome, 1994]. It has been reported that osteoblasts express numerous integrins on various substrates that function as binding receptors for different proteins [Castoldi et al., 1997]. For attachment on collagen type I, osteoblasts express $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, for fibronectin, they express $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$; and for vitronectin, they express $\alpha_v\beta_3$, $\alpha_v\beta_5$ [Clover et al., 1992; Hughes et al., 1993; Pistone et al., 1996; Gronthos et al., 1997; Cheng et al., 2000; Nakayamada et al., 2003; Lai and Cheng, 2005].

The initial discovery of osteoactivin (OA) emerged from studies using an animal rat model of osteopetrosis. The OA gene is a homolog of human glycoprotein nmb (GPNMB), mouse dendritic cell-associated heparan sulfate proteoglycan dependent-integrin ligand (DC-HIL) and human melanocyte protein Pmel 17 (Pmel17) [Kawakami et al., 1994; Shikano et al., 2001]. The OA gene 1,716 bp that encodes a protein of 572 amino acids with a predicted molecular weight of 65 kDa. OA also exists in another form, as a secreted glycoprotein with a molecular weight of 115 kDa [Safadi et al., 2001]. OA has 13 predicted N-linked glycosylation sites and an RGD domain which is potentially an integrin recognition site at position 556. Our previous studies have shown that OA mRNA is temporally expressed by differentiated human and mouse primary osteoblasts reaching maximum in terminally differentiated osteoblasts [Safadi et al., 2001]. Recent studies demonstrated that an OA antibody significantly decreased osteoblast differentiation, alkaline phosphatase (ALP) activity, osteocalcin production, nodule formation, and calcium deposition [Selim et al., 2003]. Recent results demonstrated that OA had no significant effects on osteoblast proliferation or viability. However, OA significantly induced osteoblast differentiation and function in vitro by stimulating ALP activity, osteocalcin production, nodule formation, and matrix mineralization [Selim et al., 2007].

In this study, we examined the role of rOA as an ECM protein in MC3T3-E1 osteoblast-like cell-adhesion and spreading. We also investigated the OA matrix-modulated regulation of osteoblast differentiation. We demonstrated that OA binds to osteoblasts through $\alpha_v\beta_1$ integrin and heparan sulfated proteoglycans (HSPGs). This binding resulted in the activation of the tyrosine phosphorylation cascade through FAK and ERK, which further resulted in the formation of focal adhesion and actin cytoskeleton reorganization and function.

MATERIALS AND METHODS

RECOMBINANT OA

Recombinant Human Osteoactivitn/GPNMB Fc Chimera is a disulfide-linked homodimeric protein. Based on the N-terminal sequencing, the mature protein starts at Lys 23 and has a calculated molecular mass of 78.6 kDa synthesized by R&D Systems.

CELLULAR LABELING REAGENTS

An OA antibody was raised against the peptide corresponding to the C-terminal domain of the OA protein. This peptide was selected on the basis of its potential antigenicity and screened against a protein sequence database to assure a lack of cross-reactivity to other proteins (CRB, Billingham, UK). Mouse monoclonal anti-vinculin, (H + L) FITC-conjugated goat anti-mouse IgG, mouse monoclonal anti-phospho-FAK (y397), mouse monoclonal anti-integrin $\alpha_V\beta_5$, TRITC-conjugated phalloidin and DAPI were purchased from Chemicon Int. (Temecula, CA); rabbit polyclonal anti-FAK was purchased from Abcam (Cambridge, MA); rabbit polyclonal anti-integrin α_V was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and hamster monoclonal anti-integrin β_1 was purchased from BD Biosciences (Franklin Lakes, NJ). HRP-conjugated goat anti-Mouse IgG, HRP-conjugated donkey anti-Rabbit IgG, and Cy2 conjugated affinity purified goat anti-Armenian hamster IgG were purchase from Jackson Immunoresearch (West Grove, PA).

CELL ADHESION ASSAY

MC3T3-E1 subclone-4 cells (ATCC, Manassas, VA) were detached using 0.25% Trypsin EDTA, washed twice within Hanks balanced salt solution, and resuspended in serum-free α-MEM medium containing 0.5% bovine serum albumin (BSA). rOA or control cell adhesion molecules were diluted to the desired concentration in phosphate buffered saline (PBS) and used at 100 μl/well to pre-coat non-tissue culture 96-well plates (BD Biosciences). rOA-coated plates were incubated at 4°C for 12 h; control wells were incubated with poly-L-lysine for 5 min at room temperature, washed, and dried for 2 h at room temperature. The wells were then blocked for 1 h with PBS containing 1% BSA prior to adding 100 μl of an MC3T3-E1 suspension (2×10^6 cells/ml) for 2 h at 37°C in 5% CO_2. The wells were washed three times with 1× PBS, and adherent cells were fixed with 4% paraformaldehyde for 10 min and stained with 100 μl/well methylene blue in borate buffer, pH 8.5, for 30 min at room temperature. Dye extraction was performed using 100 μl/well 100% ethanol (EtOH): 0.1% HCL (1:1 v/v), and absorbance was measured at 620 nm using a microplate reader. To determine the dose response curve, wells were pre-coated with rOA at different concentrations: 0.1, 0.2, 0.5, 1, and 2 μg/ml with final volumes of 100 μl/well. To fulfill divalent cation requirements, cells were resuspended in α-MEM medium containing 0.5% BSA and EDTA (5 mM), Ca^{2+} (10 mM), Mg^{2+} (10 mM), Mg^{2+} EDTA, or Ca^{2+} EDTA. To block osteoblast adhesion to rOA, wells were coated with rOA and incubated with anti-OA antibody (40 μg/ml) for 30 min at 37°C prior of cell plating. For heparan sulfate proteoglycans cell surface requirements, cells were incubated with heparin (40 μg/ml) for 30 min at 37°C prior to plating. To inhibit the glycosamine-glycan sulfation, cells were cultured in 0.5% BSA α-MEM medium containing sodium chlorate (80 mM) and sodium sulfate (20 mM) for 24 h at 37°C in 5% CO_2 prior to trypsinizing cells for the assay. For cell surface integrin receptor, cells were incubated with either anti-integrin α_V (30 μg/ml), anti-integrin α_V β_1 (10 μg/ml), or anti-integrin β_1 (10 μg/ml) for 30 min at 37°C prior to plating.

IMMUNOFLUORESCENT STAINING

For immunofluorescent staining of actin cytoskeleton and focal adhesion, MC3T3-E1 cells were cultured (in a density of 5000 cells) on glass slides precoated with 0.1%BSA as a negative control, 2 μg/ml fibronectin as a positive control or 0.2 μg/ml OA for 6 h at 37°C. Cells were immunostained for: nucleus using VECTASHIELD Mountining Medium with DAPI; actin cytoskeleton using TRITC-Conjugated Phalloidin; and focal adhesion using MsX Vinculin. At the end of incubation time, cells were fixed with 4% paraformaldehyde in 1× PBS for 15–20 min at room temperature. Fixative was washed twice with 1× wash buffer (1% PBS containing 0.05 % Tween-20). Cells were permeabilized with 0.1% Triton X-100 in 1× PBS for 1–5 min at room temperature then washed twice with 1× wash buffer. Blocking solution (2.5% BSA in 1× PBS) was applied for 30 min at room temperature. Primary antibody (Anti-Vinculin) was diluted to a concentration of 1:200 in blocking solution, and incubated overnight at 4°C. The following day, wells were washed three times (5–10 min each) with wash buffer. Secondary antibody was diluted in a concentration of 1:500 in blocking solution and incubated for 1 h at room temperature. For the double labeling, TRITC-conjugated Phalloidin was incubated simultaneously with the secondary antibody for 1 h at room temperature followed by washing three times (5–10 min each) with 1× wash buffer. Mounting medium containing DAPI was applied on slides followed by cover slip. Fluorescent images were visualized with fluorescence microscope.

PROTEIN ISOLATION AND IMMUNOBLOTTING

MC3T3-E1 osteoblast-like cells were cultured for 4 days, trypsinized and re-suspended in α-MEM media containing 0.5% BSA; cells were then plated at a density of 2×10^6 cells/dish on 65 mm non-tissue culture dishes pre-coated with rOA (0.2 μg/ml). Cells were incubated at 37°C in 5% CO_2 for different time points: 15 min, 30 min, 1 h, or 2 h. Cells were then lysed in ice-cold RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 5,000 U/I apoprotinin, 200 mM sodium orthovanadate, 100 mM sodium fluoride, and 10% glycerol). Samples were incubated for 1 h at 4°C. The supernatants containing total protein were collected for Western blot analysis. Total protein concentration was measured using bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Twenty micrograms of protein isolate was mixed with 2× sample buffer and heated at 100°C for 5 min to denature the proteins. Samples were separated using 10% SDS–PAGE in 1× TGS (0.25 M Tris, 1.92 M glycine and 1.0% SDS in distilled, deionized H_2O, pH 8.6) (Biorad, Hercules, CA) at 100 mV for 1 h. Gels were then transferred to PVDF membranes by using a mini trans-blot electrophoretic transfer cell (Biorad) at 30 mV overnight at 4°C. The blot was incubated in blocking buffer and blocked with 5% BSA for 1 h at room temperature. Primary antibodies were added to 5% BSA and the membranes were incubated in the resulting solution overnight at 4°C. The following day, the blot was washed three times in 1× TBST (Tris buffered saline + 0.1% Tween-20) for 10 min per wash on an orbital shaker. The blot was then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The blot was washed three times in TBST for 10 min per wash on an orbital shaker. Protein was visualized using Super Signal Chemiluminescent substrate (Pierce), and signals were detected using XL-exposure films.

IMMUNOPRECIPITATION

MC3T3-E1 osteoblast-like cells were cultured for 4 days and immmunoprecipitation was performed with an Immunoprecipitation Kit (Protein G) (Roche Diagnostics Corporation, Indianapolis, IN). Immunoprecipitated proteins were mixed with 2× sample buffer and heated at 100°C for 5 min to denature the proteins. Samples were separated using 10% SDS–PAGE in 1× TGS at 100 mV for 1 h. Gels were then transferred to PVDF membranes by a mini trans-blot electrophoretic transfer cell at 30 mV overnight at 4°C. The blots were incubated in blocking buffer and blocked with 5% BSA for 1 h at room temperature. Primary antibodies were added to 5% BSA and the membranes were incubated in the antibody solution overnight at 4°C. The following day, the blots were washed three times in 1× TBST for 10 min per wash on an orbital shaker. The blots were then incubated with HRP-conjugated secondary antibody, for 1 h at room temperature. The blots were washed in TBST 10 times, for 3 min per wash, on an orbital shaker. Protein was visualized using SuperSignal Chemiluminescent substrate and signals were detected using XL-exposure films.

PRIMARY OSTEOBLAST ISOLATION AND CELL CULTURE

C57Balck6 adult mice were housed in cages containing white pine bedding and covered with polyester filters. The environment was kept at 21°C with a 12-h light and 12-h dark cycle. Rats colonies were maintained at Temple University School of Medicine in an AAALAC-accredited facility under veterinary supervision and according to the guidelines of the Temple University Institutional Animal Care and Use Committee (IACUC). Primary osteoblast cells were isolated from the calvaria of newborn pups (4–5 days old) following approved protocol (ACUP-3457) of Temple University School of Medicine IACUC committee. In order to isolate neonatal calvaria, pups were decapitated, and heads were swabbed with ethanol. The calvarias were isolated and placed in Petri dishes containing isolation media (PBS, 1% penicillin/streptomycin, Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO)). After removal of the dura, each calverium was cut along the sagittal and coronal sutures, and all pieces were transferred to another Petri dish. The pieces were then transferred into a 50 ml siliconized Erlenmeyer flask with digest media (PBS, 0.1% collagenase P, 0.25% trypsin). The flask was placed in a shaker bath at 37°C for 5 min and, the supernatant was discarded following the first digestion. The same procedure was repeated again for second and third digestions, and the resulting supernatants were pooled together and centrifuged for 5 min at 1,200 rpm at 4°C. The cell pellets were re-suspended in 5 ml of fresh washing media. Fifty microliters of the resulting solution was added to 50 μl of Trypan blue in order to count the cells using a hemacytometer. Cells were then plated in 100 mm cell culture dishes at a density of 8.6×10^3 cells/cm^2 with 12 ml of plating medium EMEM (Mediatech-Cellgro, Kansas City, MO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were then incubated in a humidified incubator at 37°C and 5% CO_2.

ALKALINE PHOSPHATASE STAINING AND ACTIVITY

Primary mouse osteoblasts were plated at a density of 2.3×10^3 cells/well, in a 12-well non-tissue culture plate (BD Biosciences)

pre-coated with either poly-L-lysine or rOA (20 μg/ml). On day 3 of culture, the media was replaced with EMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml ascorbic acid. On day 7 of culture, the media was replaced with EMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 50 mM β-glycerophosphate. Cultures were terminated at day 14, and staining was performed using an ALP staining kit (Sigma–Aldrich) as follows. Cells were fixed with citrate-acetone-formaldehyde for 1 min and then rinsed with distilled H_2O. Alkaline dye mixture was added to the cells and incubated at room temperature for 15 min, protected from light. For the measurement of ALP activity, cultures were terminated at day 14, and the cell layers were scraped and digested for 12 h in digestion buffer. Ten microliters of the digestion product was added to 90 μl of p140 p-nitrophenol substrate; samples were then incubated at 37°C, and colorimetric kinetic determination of ALP activity was measured at an absorbance of 405 nm from 1 to 6 min.

STATISTICAL ANALYSIS

For all quantitative generated data, differences between individual groups were analyzed for statistical significance using Prism 5 software (GraphPad, La Jolla, CA). In most cases, when the data follow a normal distribution, one-factor or two-factor analysis of variance (ANOVA) was employed, followed by a Bonferroni post hoc-test. For comparisons between two group means, an unpaired-*t*-test was performed. Any difference with a probability value <0.05 were considered statistically significant. Group means $\pm$ standard errors of the mean ($\pm$SEM) were plotted in graphs. All in vitro experiments were repeated ($n \geq 3$) with three replicates per experiment.

RESULTS

OSTEOBLAST ATTACHMENT TO OA MATRIX IS SPECIFIC

Previous studies have shown the significant role of OA in osteoblast differentiation and function [Selim et al., 2003; Abdelmagid et al., 2008]. In this study, we examined the role of OA in stimulating cell adhesion of osteoblasts. MC3T3-E1 osteoblast-like cells were seeded on different concentrations of recombinant OA (rOA) coating matrix. To further support the adhesive properties of OA, we compared it to a well-known cell adhesive molecule; fibronectin (FN)

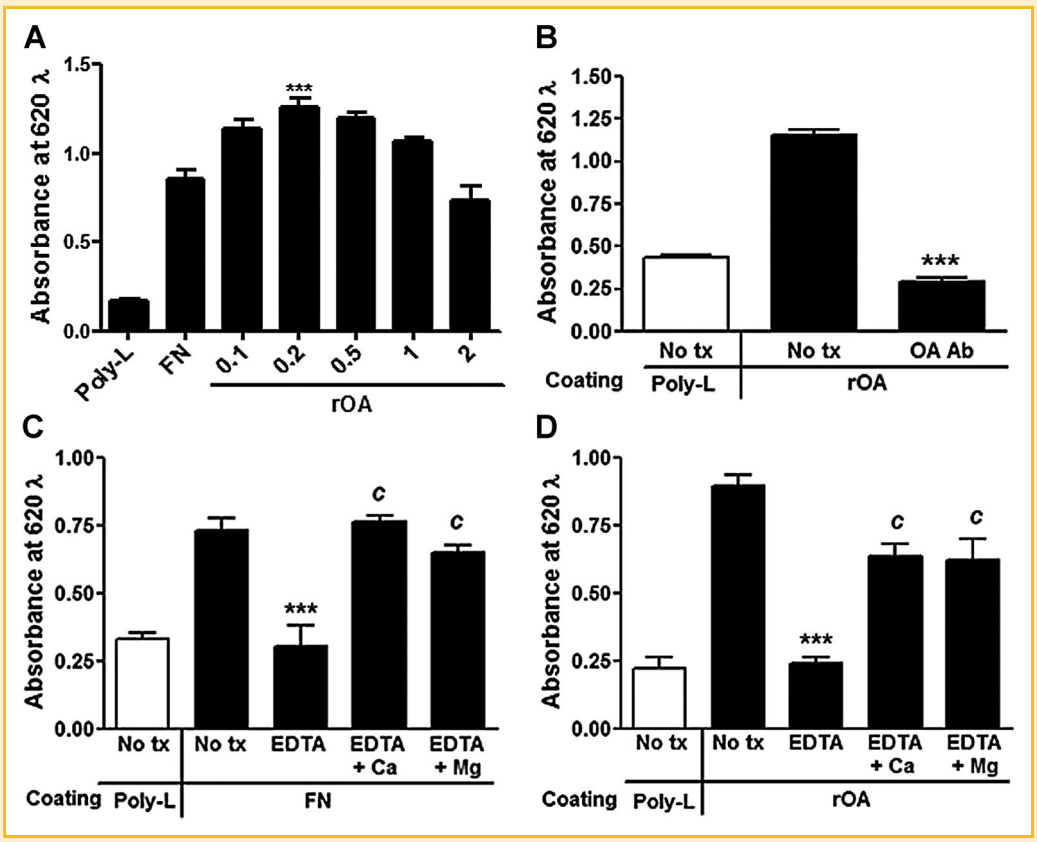

Fig. 1. OA as an extracellular matrix protein stimulates osteoblasts adhesion. *(A)* Adhesion of MC3T3-E1 cells on recombinant osteoactivin (rOA), recombinant fibronectin (FN), or Poly-L (negative control) pre-coated plates. MC3T3-E1 cells were plated on different concentrations of rOA protein or poly-L-lyisne (poly-L, negative control) coated plate and incubated for 2 hours. *(B)* Adhesion of MC3T3-E1cells in the presence of OA Ab (40 μg/mL) added to rOA for 30 minutes at 37 °C, then use to coat the plates. No tx (untreated cultures) *(C-D)* MC3T3-E1 cells adhesion to FN or rOA with addition of EDTA and cations. MC3T3-E1 cells were plated on pre-coated plates with poly-L-lysine or FN or rOA; EDTA, Mg^{++} EDTA or Ca^{++} EDTA were added alone or in combination. Experiment was repeated three to five times with six replicates per experiment. Data are presented as the Mean + SEM. *** $P < 0.001$ when compared to poly-L in A. SEM. *** $P < 0.001$ when compared to No tx over rOA in B. *** $P < 0.001$ when compared to No tx over FN in C and *c* $P < 0.01$ when compared to EDTA over FN in C. *** $P < 0.001$ when compared to No tx over rOA in D and *c* $P < 0.01$ when compared to EDTA over rOA in D.

[Johansson et al., 1997]. Adhesion of MC3T3-E1 cells to rOA reached the highest point at 0.2 μg/ml of rOA that was significantly higher than adhesion to fibronectin (Fig. 1A). The specificity of MC3T3-E1 cell-binding to rOA was supported by the presence of OA antibody that blocked MC3T3-E1 cell-adhesion to rOA (Fig. 1B). The role of calcium (Ca^{2+}) and magnesium (Mg^{2+}) divalent cations in integrin function and cell to substrate adhesion is well established [Takeichi and Okada, 1972]. To further specify the adhesion of MC3T3-E1 cells to OA matrix, we examined the adverse effect of EDTA as a chelator for Ca^{2+} and Mg^{2+} on osteoblasts adhesion to FN and rOA coating matrices [Hynes, 2002]. Adhesion of MC3T3-E1 to FN was significantly reduced ($P < 0.001$; Fig. 1C) with a similar results for rOA ($P < 0.001$; Fig. 1D). Moreover, individual addition of Ca^{2+} or Mg^{2+} fully restored EDTA-inhibited cell binding to fibronectin compared to EDTA treated condition ($P < 0.001$; Fig. 1C). Whereas addition of Ca^{2+} or Mg^{2+} partially restored cell binding to rOA compared to EDTA treated condition ($P < 0.001$; Fig. 1D). Taken together, these data support the adhesive function of rOA on osteoblasts.

OSTEOBLAST ADHESION TO OA IS MEDIATED BY $A_V\beta_1$ INTEGRINS

The specific attachment of MC3T3-E1 cells to OA coated matrix suggests that it is integrin mediated binding. It was previously reported that osteoblasts express numerous types of integrins that function as binding receptors for different matricellular proteins [Clover et al., 1992; Hughes et al., 1993; Pistone et al., 1996; Gronthos et al., 1997; Cheng et al., 2000; Nakayamada et al., 2003; Lai and Cheng, 2005]. To investigate which integrin mediates osteoblast adhesion to rOA, MC3T3-E1 cells were incubated with neutralizing antibodies against various integrin subunits and then plated over rOA-coated matrix. Antibodies against the β_5 subunit had no effect on cell adhesion to OA, whereas cells incubated with antibodies against the, β_1, or β_2 subunits effectively blocked ($P < 0.001$) MC3T3-E1 adhesion to rOA by ~75% compared to no treated control (Fig. 2A). Moreover, cells incubated with antibodies against the α_v subunit (from several vendors) effectively blocked ($P < 0.001$) cell binding to rOA, whereas cells incubated with antibodies against the $\alpha_v\beta_1$ subunits blocked adhesion to OA by 85% compared to rOA treated control (data not shown). Next, to determine the specific binding of rOA to $\alpha_v\beta_1$ integrin on MC3T3-E1 osteoblast-like cells, we performed immunoprecipitation of β_1 integrin using anti-β_1 integrin antibody and mouse non-immune IgG (control) antibodies then probed with anti-OA antibody, and vice versa. Interestingly, the glycosylated OA isoform at 115 kDa and the native transmembrane OA isoform at 65 kDa protein were detected after β_1 integrin immunoprecipitation (Fig. 2B). On the other hand, β_1 integrins were detected after OA immunoprecipitation (Fig. 2C). Collectively, these data support the adhesion of MC3T3-E1 osteoblasts to rOA via $\alpha_v\beta_1$ integrins on the cell surface.

ADHESION OF OSTEOBLASTS TO OA REQUIRES CELL SURFACE HSPGS

As anti-$\alpha_v\beta_1$ antibodies did not completely block osteoblast adhesion to rOA coated matrix, this will suggest other possibilities of binding between OA and osteoblasts. OA/GPNMB and its homologs DC-HIL have a highly preserved heparin-binding motif (BBXB, where B represents a basic amino acid residue, Fig. 3A) at the amino acid sequence 23–26 that is important for cell adhesion by controlling integrin receptor sensitivity [Proudfoot et al., 2001; Safadi et al., 2001; Shikano et al., 2001]. To determine whether osteoblast binding to rOA requires cell surface heparan sulfate proteoglycans (HSPGs), heparin was incubated with rFN or rOA protein prior plating of MC3T3-E1. Heparin incubation did not affect MC3T3-E1 cell binding to FN (Fig. 3B), whereas cell adhesion to rOA coating matrix was inhibited by ~30% due to heparin treatment ($P < 0.01$; Fig. 3C).

Since glycosaminoglycans (GAG) represents the core disaccharide structure of the Heparin/heparan sulfate GAG and that is important

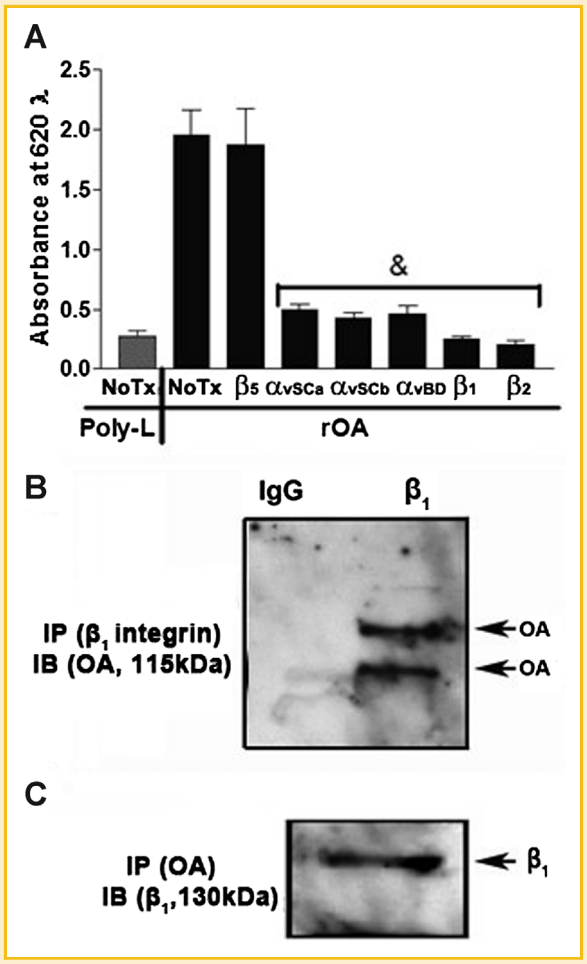

Fig. 2. OA binds to osteoblast through integrin $\alpha_v\beta_1$. MC3T3-E1 cells were either left undertreated (No Tx) or incubated with anti-integrins antibodies for 30 minutes prior plating on Poly-L or rOA pre-coated plates. (A) MC3T3-E1cells adhesion to rOA pre-coated plates after cells incubation with β_5, α_{vSCa}, α_{vSCb}, α_{vBD}, $\alpha_v\beta_1$, β_1 anti-integrins antibodies. MC3T3-E1 cells were pre-incubated with anti-integrin (β_5, α_{vSCa}, α_{vSCb}, α_{vBD}, $\alpha_v\beta_1$ or β_1). *(B)* Immunoprecipitation of OA and β_1 integrin. IgG or anti-integrin β_1 immunoprecipitate (IP) lysates from MC3T3-E1 cells were subjected to SDS-PAGE and western blot (IB) analysis using either anti-OA or β_1 antibodies. Experiment was repeated three to five times with six replicates per experiment. Data are presented as the Mean + SEM. & $P < 0.001$ when compared No tx over rOA.

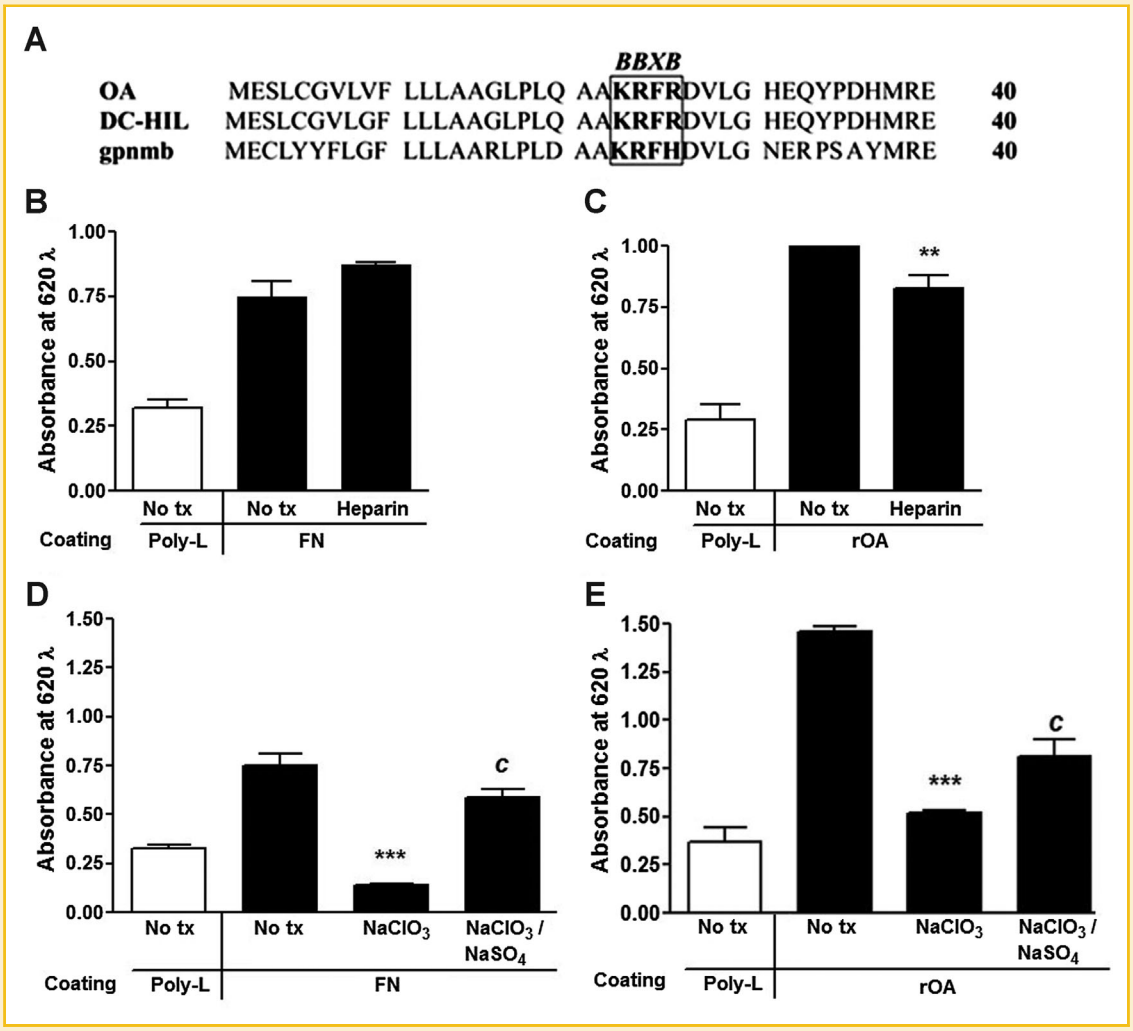

Fig. 3. Osteoblast adhesion to rOA requires cell surface HSPG. *(A)* Comparison of the predicted amino acid sequences of heparin-binding motif (BBXB, shown in the boxed sequence) in rat, mouse and human osteoactivin protein. *(B - C)* Osteoblast adhesion to FN or rOA after treatment with heparin. Cells were incubated with heparin for 30 min prior plating on pre-coated plates with either Poly-L-lysine (control), FN, or rOA. *(D –E)* Cells adhesion to FN or rOA after treatment with $NaClO_3$ or $NaClO_3/NaSO_4^{-2}$. MC3T3-E1 cells were treated with $NaClO_3$ or $NaSO_4^{-2}/NaClO_3$ for 24 h prior plating on pre-coated plates with either Poly-L-lysine, FN or rOA. Experiment was repeated three to five times with six replicates per experiment. Data are presented as the Mean + SEM. ** $P < 0.01$ when compared to heparin treatment to No Tx over rOA in C. *** $P < 0.001$ when compared to $NaClO_3$ treatment to No Tx over FN and rOA in D and E. *c* $P < 0.001$ when compared $NaSO_4^{-2}/NaClO_3$ to $NaClO_3$ over FN or rOA in D and E.

in the complex function in cell adhesion [Rabenstein, 2002], we investigated if cell surface GAG are necessary for osteoblast adhesion to rOA matrix. MC3T3-E1 cells were incubated with sodium chlorate; an inhibitor of 3-phosphoadenosin 5′- phosphosulfate synthesis, to block proteoglycan sulfation [Todorovic et al., 2005]. MC3T3-E1 cell-binding to rFN was reduced by ~80% in the presence of sodium chlorate ($P < 0.001$; Fig. 3D). Similarly, sodium chlorate inhibited ~65% of cell-adhesion to rOA ($P < 0.001$; Fig. 3E). However, adding sodium sulfate reversed ~80% of the inhibitory effect of sodium chlorate in MC3T3-E1 binding to rFN (Fig. 3D) and ~50% of cell-adhesion to rOA ($P < 0.01$; Fig. 3E); thus, providing evidence that blocking GAG sulfation inhibited cell adhesion (Fig. 3D,E). Taken together, these data show the critical role of heparin sulfates and GAGs in osteoblast adhesion to rOA coating matrix.

OSTEOBLAST ADHESION TO OA ACTIVATES CYTOSKELETAL REORGANIZATION

Focal adhesions are large complexes involving integrins and are formed at the end of prominent actin stress fibers [Nobes and Hall, 1995; Chen et al., 2001]. To investigate the formation of focal adhesion sites on MC3T3-E1 cells adherent to rOA matrix protein, MC3T3-E1 cells were allowed to attach on coverslips coated with either rOA (0.2 μg/ml), rFN, or BSA for 12 h. Cells were then stained for actin cytoskeleton and Vinculin at focal adhesion sites. We next examined the morphological changes in MC3T3-E1 cells adherent to rOA, FN, or BSA by fluorescent microscopy. Our findings showed that MC3T3-E1 cells that adhered to rOA matrix have well organized and extended actin filaments. In addition, MC3T3 cells attached to rOA have numerous focal adhesion complexes marked by vinculin staining compared to cells adherent to rFN or BSA (Fig. 4A–C). Collectively, these data suggest that MC3T3-E1

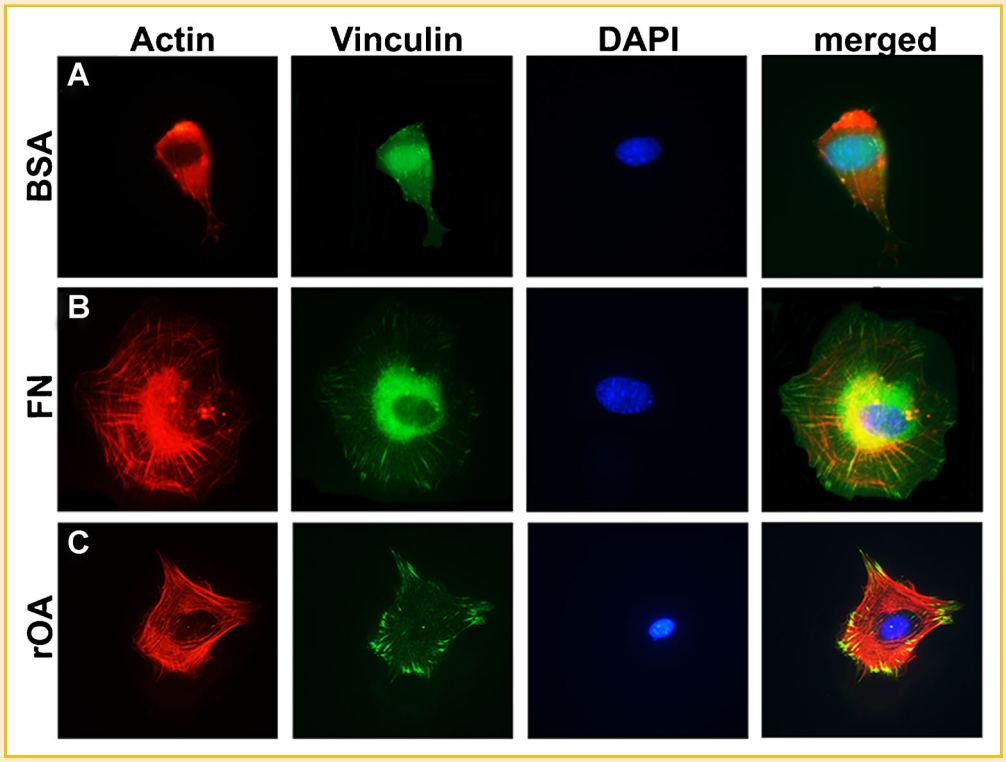

Fig. 4. Osteoblasts adhesion to rOA induces actin cytoskeletal reorganization and formation of focal adhesion complexes. MC3T3-E1 osteoblasts were plated glass slides precoated with either 0.1%, BSA (A), 2.0 μg/ml FN (B), or (0.2 μg/ml) rOA (C). Cells were incubated at 37 °C for 6 h, and adherent cells were fixed with 4% paraformaldehyde in PBS fllowed by permeablization with 0.1% TritonX-100 in PBS. Actin filaments were stained TRITC-Conjugated Phalloidin (red signal) or for focal adhesion using MsX Vinculin (green signal) or Dapi (blue signal). Data shown are typical of three experiments. Magnification (200x).

cell-binding to rOA results in enhanced cytoskeletal reorganization and improved cell spreading.

ADHESION OF OSTEOBLASTS TO OA ACTIVATES THE FAK AND ERK PATHWAYS

It has been reported that focal adhesion complex triggers many protein kinases such as; FAK [Brown et al., 2005]. To investigate whether cell-binding to rOA activates the FAK pathway in osteoblasts, Phospho-FAK (y397) and total FAK were examined in MC3T3-E1 cells attached to rOA for 15, 30, and 60 min. As shown in (Fig. 5A), FAK phosphorylation was stimulated as early as 15 min of cell adhesion to rOA. The histogram of Western blot analysis showed the highest FAK phosphorylation at 15 min then declined at 30- and 60 min of cell adhesion (Fig. 5B). We also showed similar results for extracellular signal-regulated kinase (ERK) phosphorylation, that was increased to the highest levels at 15 minutes of cell adhesion and decreased thereafter (Fig. 5C,D). These data suggest activation of FAK and ERK pathways upon cell adhesion to OA coating matrix.

ADHESION OF OSTEOBLASTS TO OA STIMULATES CELL DIFFERENTIATION

Previous studies have established the importance of integrin mediated cell–matrix interaction in osteoblast differentiation and function [Jikko et al., 1999; Meredith et al., 1996]. To investigate the effect of osteoblast adhesion to OA on cell differentiation, primary osteoblasts were differentiated over rOA or Poly-L-lysine coated matrix. Primary osteoblast adhered to rOA showed higher ALP staining compared to osteoblast differentiated over to poly-L-lysine (Fig. 6A). Moreover, primary osteoblasts adhered to rOA matrix showed increased ALP activity ($P < 0.05$) than osteoblasts adhered to poly-L-Lysine (Fig. 6B).

Therefore, the increased ALP staining and activity suggests that adhesion of osteoblasts to OA enhances osteoblast differentiation and function (Fig. 7).

DISCUSSION

Our data demonstrated that osteoblasts attach to OA matrix, an effect mediated by integrin. Our results also show that osteoblast-like cells adhere specifically to OA mediated by an integrin. The adhesion property was reduced by neutralizing OA with antibodies, showing the binding specificity of osteoblasts to OA matrix. In addition, we demonstrated that OA can function as an adhesive molecule that can induce changes in cell signaling and function. We investigated the role of divalent cations (Mg^{2+}/Ca^{2+}) since they can affect cellular adhesion when such adhesion is mediated through integrin [Takeichi and Okada, 1972; Lange et al., 1994]. Previous studies also showed that extracellular Ca^{2+} plays an essential role in both integrin-

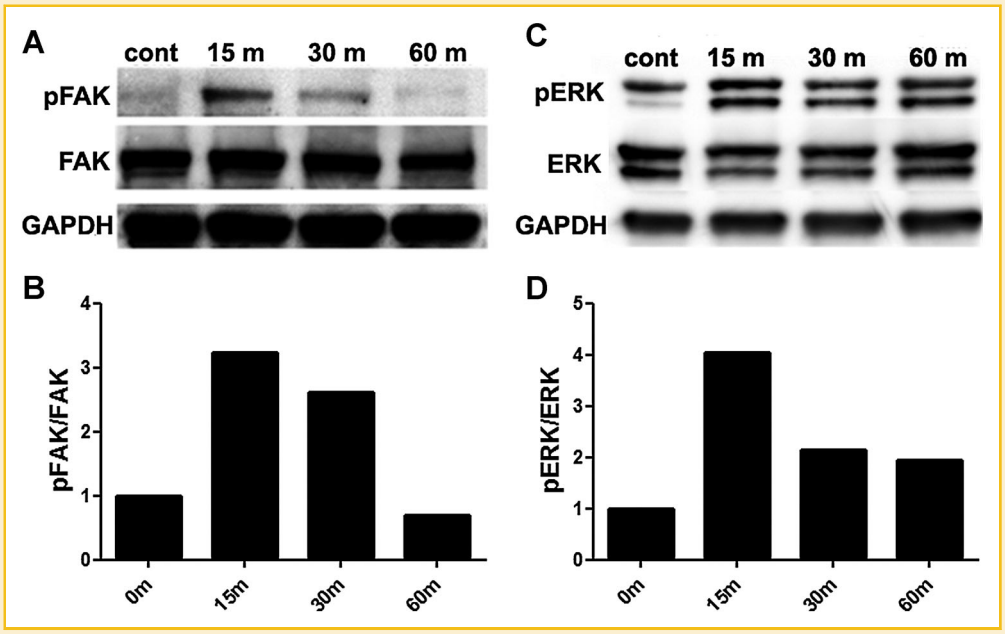

Fig. 5. Oseoblast adhesion to rOA activates FAK and ERK pathways. *(A – B)* MC3T3-E1 cells were plated on pre-coated plates with either poly-L-lysine (control) or rOA (0.2 μg/ml). Cells were incubated at different time points (15 min, 30 min, and 60 min) at 37 C. Cells were then harvested and lysed in fresh cold RIPA buffer; 20 μg of protein lysates were subjected to SDS-PAGE and western blot analysis using anti-p-FAK (A), anti-total FAK (A), anti-p-ERK (B), anti-total ERK (B) and anti-GAPDH (loading control) Upper panel. Densitometric analysis of presented Western blot. Data representative of at least three independent experiments.

adhesive functions and integrin-mediated signaling [Coppolino et al., 1997]. In addition, other reports showed the integral role of Mg^{2+} in platelet adhesion to ECM; laminin [Hindriks et al., 1992]. Our data demonstrate that EDTA; a chelator for Mg^{2+} and Ca^{2+}, has a negative effect on the cellular adhesion to rOA and both divalent cations partially restored cell adhesion suggesting the involvement of integrins in osteoblast adhesion to OA.

Little is known about integrin interaction with OA or its homologous DC-HIL. In osteoclasts, OA was reported to coimmunoprecipitate with integrin β_3 and β_1, indicating that OA co-localizes with these integrins in a hetero-polymeric complex [Sheng et al., 2008]. Our results demonstrate that adhesion of MC3T3-E1 osteoblast-like cell to OA was mediated by $\alpha_v\beta_1$ integrins. The binding specificity is supported by anti-α_v integrin antibodies that blocked ~55% of osteoblast adhesion to OA. Interestingly, neutralizing β_1 integrin with their antibodies blocked ~75% of osteoblast adhesion to OA. Moreover, it has been reported that overexpressing a dominant negative β_1 integrin subunit in the transgenic mouse also reduced bone formation. In addition, β_1 integrin is the major integrin expressed by osteoblasts when adhered to ECM substrates. Although β_1 integrin forms dimers with (α_1–α_5) and α_v integrins. β_1 integrin-mediated adhesion to bone matrix induces osteoblast proliferation, differentiation, and ECM synthesis and β_1 signaling has a major functional role in bone formation [Zimmerman et al., 2000]. OA protein isoforms were only identified in β_1 integrin immunoprecipitate lysates from osteoblasts, and not in the IgG immunoprecipitate lysates. These results demonstrate that β_1 integrin on osteoblast surface works as an adhesive receptor for OA. It would be interesting to study the adhesive response of osteoblasts, overexpressing dominant negative β1 integrins or osteoblasts null for β1 integrins, over OA coated matrix.

As a result of integrins binding to the ECM, they form clusters at the end of actin fibers that allow for focal adhesion complex formation so integrin serves as an integrator of the cell cytoskeleton to the ECM [Huttenlocher and Horwitz, 2011]. Moreover, previous studies showed that interaction between integrins and their adhesive ligands leads to intracellular signaling, which is essential for cellular responses such as cytoskeletal reorganization, cell spreading, and migration [Jockusch et al., 1995; Giancotti and Ruoslahti, 1999; Chen et al., 2001]. Numerous structural proteins, including paxillin and vinculin, are associated with focal adhesion complex [Giancotti and Ruoslahti, 1999]. Immunofluorescent staining of osteoblasts adhered to OA coated matrix revealed the presence of vinculin at the focal adhesion sites. The importance of vinculin came from vinculin null cells or cells expressing vinculin mutant that demonstrated its importance in contractility-dependent adhesion strength [Dumbauld et al., 2013]. Moreover, paxillin was reported to modulate focal adhesion dynamics and organization of membrane cytoskeletal structures responsible for cell migration and spreading [Hagel et al., 2002]. Here, we showed that upon integrin engagement to rOA matrix, osteoblasts exhibit changes in cytoskeletal organization and more cell spreading.

Previous studies have indicated that HSPGs can act as co-receptors with integrins in cell-matrix interactions [Bernfield et al., 1999]. While cell surface HSPGs contribute to adhesion in most cases involving heparin-binding ECM molecules, they are not considered essential for cell attachment to substrates [Bernfield et al., 1992]. Previous studies have demonstrated that the DC-HIL

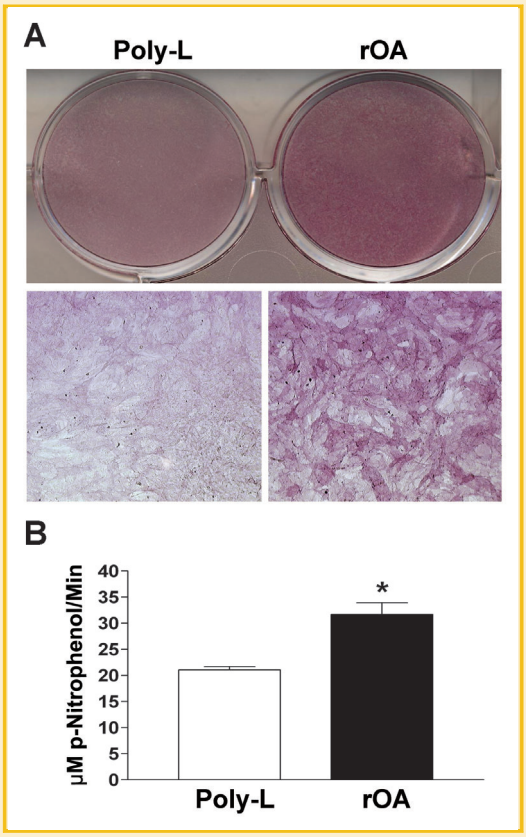

Fig. 6. Osteoblast adhesion to rOA induces osteoblast differentiation. *(A)* Primary Osteoblasts were plated on 12 well non-tissue culture plate coated with either Poly-L-lysine or 0.2 μg/ml rOA. On day 14 of culture, ALP staining was performed by adding ALP dye mixture and incubated at room temperature for 15 min protected from direct light. Upper panel: photomicrograph of culture plates, lower panel: inverted microscopy images of culture plates, Magnification (200×). *(B)* In parallel cultures, cell layer was scraped and digested, then ALP activity was measured at absorbance 405 nm. *$P < 0.05$ when compared rOA to Poly-L.

heparin binding site interacts with cell surface HSPGs to support epithelial cell (SVEC) adhesion [Shikano et al., 2001]. However, the interaction of cell surface HSPGs with the ECM is different for each cell type [Bernfield et al., 1999]. Our results showed that adhesion of osteoblasts to rOA coated matrix was significantly inhibited by the presence of heparin, whereas the same concentration of heparin had no effect on cell attachment to FN, suggesting the significant involvement of HSPGs in osteoblast adhesion to OA matrix. In addition, we showed that cell surface GAGs are necessary for osteoblast adhesion to OA matrix. Cells were treated with sodium chlorate, an inhibitor of 3′-phosphoadenosin 5′-phosphosulfate synthesis, to block proteoglycan sulfation [Safaiyan et al., 1999]. Our data showed that culture treated with sodium chlorate reduced osteoblast adhesion to OA matrix probably due to damaged integrity of the cell surface GAGs. In contrast, culture treatment with sodium sulfate rescued the inhibitory effect of sodium chlorate on cell adhesion. Taken together, the inhibitory effects of sodium chlorate and the moderated blocking effect of heparin suggest the involvement of HSPGs in osteoblast attachment to rOA coated matrix. However, the contribution of HSPGs in cell adhesion process is not always essential, considering that in vivo ECM consists of large amalgam of multi-domain proteins which bind to both integrin and cell surface HSPGs. While HSPGs have relatively low affinity interactions, integrin has higher specificity for ECM proteins, suggesting that $\alpha_v\beta_1$ integrins are the main binding adhesion receptor for OA in osteoblasts. Recent studies have shown that GAGs such as dermatan sulfate, chondroitin-6-sulfate, heparin and hyaluronic acid play significant role in regulating osteoblast differentiation and function [Mathews et al., 2012].

Previous studies showed compelling evidence that ECM molecules can function as adhesive signaling molecules upon binding to their integrins [Shah et al., 1999; Nesti et al., 2002]. FAK is one of the most common protein-tyrosine kinases activated by integrin [Giancotti and Ruoslahti, 1999]. Previous studies demonstrated the increased levels of tyrosine phosphorylated FAK

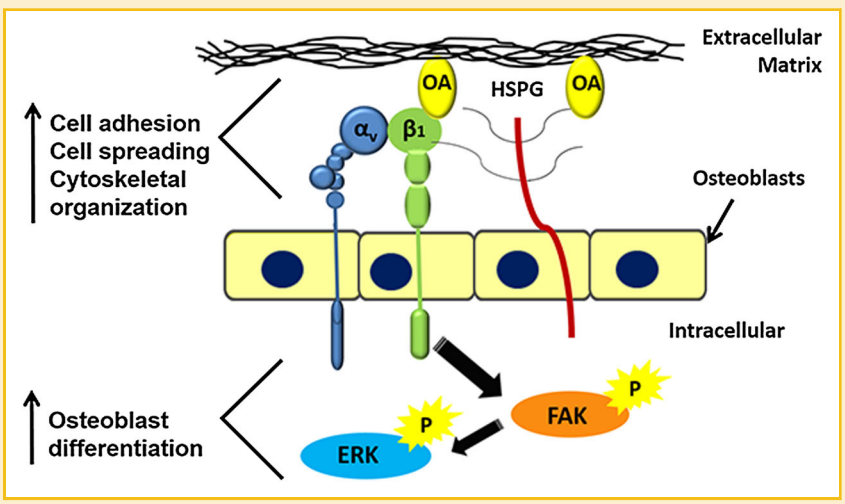

Fig. 7. Schematic diagram of the adhesive function of OA. OA binds to $\alpha_v\beta_1$ and HSPG that induces FAK and ERK activation and osteoblast differentiation.

as a result of β_1 integrin stimulation [Nakayamada et al., 2003]. FAK phosphorylation was abrogated by osteoblast transfection of dominant negative truncations of FAK [Nakayamada et al., 2003]. We showed that osteoblast adhesion to OA increased the phosphorylation of FAK at early time point that can be explained by the high affinity between $\alpha_v\beta_1$ integrin and OA coated matrix. We also found that increased FAK phosphorylation was correlated with the phosphorylation of ERK. This response may suggest that ERK is a downstream of FAK when β1 integrin is activated. Other studies showed that mice expressing dominant negative β_1 integrins have altered MAPK activity [Lee et al., 2006].

Together, our current data demonstrate that osteoblast adhesion to OA is mediated through $\alpha_v\beta_1$ integrins, resulting in the formation of focal adhesion complex with vinculin that is responsible for cytoskeletal reorganization. In addition our results show that osteoblast adhesion to OA through β_1 integrin stimulates phosphorylation of FAK and ERK signaling (Fig. 7).

In several studies, we demonstrated that the most important functions of OA in osteoblasts is the induction of osteoblast differentiation, as demonstrated by stimulation of ALP activity and matrix mineralization [Safadi et al., 2001; Owen et al., 2003; Selim et al., 2003; Abdelmagid et al., 2007, 2008, 2010; Singh et al., 2010; Bateman et al., 2012]. Moreover, β_1 integrin-mediated adhesion to the ECM induces osteoblast proliferation, differentiation and matrix mineralization [Brunner et al., 2011; Moursi and Globus, 1997; Yan et al., 2012]. In addition, focal adhesion kinase (FAK) plays a central role in integrin mediated cell signaling and differentiation [Kim et al., 2007; Salasznyk et al., 2007a; Salasznyk et al., 2007b] Our data showed that primary osteoblast differentiation was enhanced when attached to rOA matrix marked by ALP staining and activity suggesting that OA may stimulate osteoblast differentiation and matrix mineralization through binding to $\alpha_v\beta_1$ integrin.

Thus, we propose that OA functions as an adhesive molecule of osteoblasts and that adhesion is involved in osteoblast differentiation and signaling through the FAK/ERK pathways.

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