Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 347 (2006) 347–357
BBRC
www.elsevier.com/locate/ybbrc

Vitronectin and collagen I differentially regulate osteogenesis in mesenchymal stem cells

Anup K. Kundu [a], Andrew J. Putnam [a,b,*]

[a] Department of Chemical Engineering and Materials Science, University of California, Irvine, Irvine, CA, USA
[b] Department of Biomedical Engineering, University of California, Irvine, Irvine, CA, USA

Received 14 June 2006

Abstract

The roles of various soluble factors in promoting the osteogenic differentiation of adult mesenchymal stem cells (MSCs) have been widely studied, but little is known about how the extracellular matrix (ECM) instructs the phenotypic transition between growth and differentiation. To investigate this question, we cultured MSCs on purified vitronectin or type-I collagen, motivated by our earlier tissue engineering work demonstrating that MSC adhesion to polymer scaffolds is primarily mediated by the passive adsorption of these two ECM ligands from serum. Using alkaline phosphatase activity and matrix mineralization as indicators of the early and late stages of osteogenesis, respectively, we report here that both substrates supported differentiation, but the mechanism was substrate dependent. Specifically, osteogenesis on vitronectin correlated with enhanced focal adhesion formation, the activation of focal adhesion kinase (FAK) and paxillin, and the diminished activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI3K) pathways. By contrast, MSCs on type-I collagen exhibited reduced focal adhesion formation, reduced activation of FAK and paxillin, and increased activation of ERK and PI3K. Inhibition of ERK and FAK blocked mineral deposition on both substrates, suggesting that the observed differences in signaling pathways ultimately converge to the same cell fate. Understanding these mechanistic differences is essential to predictably control the osteogenic differentiation of MSCs and widen their use in regenerative medicine.

Keywords: Extracellular matrix; Integrins; Differentiation; Osteogenesis; Focal adhesions; Signal transduction

The use of stem cells to restore tissue function is a major focus of regenerative medicine. Given their pluripotency, there is a great deal of excitement associated with the use of embryonic stem cells [1]. However, bone marrow-derived mesenchymal stem cells (MSCs) remain a viable alternative, and are particularly attractive in the context of bone tissue engineering and other reconstructive applications given that autologous cells may be used for eventual therapy [2]. Prior studies using MSCs have demonstrated their capacity to differentiate into multiple lineages [3], including bone [4,5], cartilage [6], fat [7,8], tendon [9], muscle [10,11], and even nervous tissue [12,13]. Despite this potential, predictably controlling the differentiation of MSCs and understanding the signaling pathways committing them to different fates are critical to widen their use in clinical applications.

The roles of various soluble factors in promoting the osteogenic differentiation of MSCs have been extensively studied. Dexamethasone, bone morphogenetic protein-2 (BMP-2), β-glycerophosphate, ascorbic acid, and transforming growth factor-β (TGF-β) have all been identified as key soluble factors involved in regulating osteogenesis [3,4]. Media containing these osteoinductive supplements induce a series of molecular events, including activation of signal transduction pathways, that eventually lead to the expression of osteogenic genes including type-I collagen (Col I), osteocalcin, osteopontin, and alkaline phosphatase, and the eventual deposition of calcium phosphate mineral that is characteristic of bone [14]. While a number of studies have focused on the signaling pathways activated by

* Corresponding author. Fax: +1 949 824 1727.
E-mail address: aputnam@uci.edu (A.J. Putnam).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.06.110

these osteogenic supplements and the role of these pathways in MSC differentiation, comparatively little is known about the effects of extracellular matrix (ECM) on the signaling pathways governing osteogenesis.

The ECM, which surrounds most cells in the body in three dimensions, is a highly hydrated network of proteins and polysaccharides that provide a set of instructive molecular signals to cells that are attached to it. Much of the information content of the ECM resides in the insoluble collagenous and noncollagenous glycoproteins (e.g., elastin, laminin, vitronectin or fibronectin). Cellular interactions with these proteins are primarily mediated by integrins, a family of heterodimeric cell adhesion receptors that provide both a biophysical bridge and a biochemical link integrating the outside and inside of the cell [15,16]. Prior studies with committed osteoblasts show that integrin-mediated adhesion and signaling regulate osteoblastic differentiation [17–22]. However, the role of integrins and their downstream effectors on MSC differentiation remains unclear.

One likely molecular target of ECM-initiated signals is the mitogen-activated protein kinase (MAPK) cascade. Activation of the extracellular signal-regulated kinase (ERK1/2, the most widely studied of the MAPK enzymes) by soluble growth factors is mediated by a Ras-dependent signal transduction pathway, and is a key event in the regulation of growth, differentiation, survival, and migration. Cell adhesion to Col I, fibronectin, and vitronectin (VN) has been shown to induce the activity of ERK in many cell systems [23,24] and is also critical to sustain growth-factor-induced ERK activation [25–27]. Furthermore, ERK signaling in response to soluble agonists and ECM ligands appears to play a critical role in the osteogenic differentiation of pre-osteoblastic cells [28] by activating the Runx2/CBFA-1 transcriptional activator [29]. However, there are conflicting reports with respect to ERK's role in the osteogenic differentiation of stromal cells [30,31].

Recently, we documented that the adhesion of MSCs to two polymer scaffolds widely used in tissue engineering applications is primarily mediated by the passive adsorption of vitronectin (VN) and type-I collagen (Col I) [32]. (Some fibronectin and laminin were also deposited, but the MSCs adhered to the polymers primarily via VN and Col I.) We hypothesized that this differential adhesion mechanism controls the osteogenic differentiation of MSCs seeded on these polymers. Accordingly, here we have explored the influence of integrin expression and downstream signaling on the osteogenic differentiation of MSCs on substrates coated with purified VN or Col I. Our results show that MSCs differentially initiate integrin-mediated signaling events depending on whether they are cultured on VN- or Col I-coated substrates. While our results confirm previous reports that ERK is critical for osteogenesis, we show here for the first time that focal adhesion kinase (FAK) is required too, regardless of which adhesion ligand is used by the cells. Finally, our data also reveal that ECM identity influences the kinetics of integrin-mediated signaling, which appears critical to control the switch between mitogenesis and osteogenesis in MSCs.

Materials and methods

Materials. Purified type-I collagen and human vitronectin were purchased from Cohesion (Palo Alto, CA) and Chemicon International (Temecula, CA), respectively. Phospho-p44/42 MAPK antibody (anti-phospho-ERK1/2, monoclonal) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies to ERK1, paxillin, and p-FAK (Y397) were purchased from BD Transduction Laboratories (Lexington, KY). Anti-p^{ser473}-Akt (rabbit polyclonal) and anti-pY^{31}-paxillin (rabbit polyclonal) antibodies were from Biosource International, Inc. (Camarillo, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. Mouse monoclonal anti-$\alpha_2\beta_1$, anti-$\alpha_5\beta_1$, and anti-$\alpha_v\beta_3$ integrin antibodies were from Chemicon. Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were all purchased from Jackson Immunoresearch Laboratories, Inc. (Baltimore, PA). Monoclonal anti-human vinculin and monoclonal anti-FAK antibodies were obtained from Sigma (St. Louis, MO). MEK-1 inhibitor PD98059 was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Kaleidoscope pre-stained standards and pre-stained SDS–PAGE standards (low range) were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). FAK siRNA, control siRNA, and siRNA transfection reagents were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other routine tissue culture supplies and reagents were purchased from either Fisher Scientific or Invitrogen (Carlsbad, CA).

Routine cell culture. Cryopreserved human bone marrow-derived mesenchymal stem cells (MSCs) were purchased from Cambrex-Bio-whittaker (Walkersville, MD) at passage 2. According to the manufacturer, these cells are tested for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. Cells are positive for the cell surface markers CD105, CD166, CD29 (integrin β_1), and CD44, and negative for CD14, CD34, and CD45. In our hands, the MSCs were routinely cultured and expanded in a non-differentiating growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown in a 5% CO_2-atmosphere at 37 °C and the medium was renewed every two to three days. Upon nearing confluence, cells were detached using trypsin–EDTA (Invitrogen) and passaged 1:3 into fresh culture flasks. All experiments were conducted using cells below passage 8.

Preparation of Col I- and VN-coated dishes. Substrates were coated with VN and Col I according to the manufacturers' recommendations that accompanied each product. In the case of Col I, a 100 μg/ml solution was prepared in acidic H_2O (pH 3.0), while a 2 μg/ml VN solution was prepared in phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+} supplements (denoted as PBS(−)). (Although these solutions were of different concentrations, we chose to use these recommended concentrations since both exceed the theoretical 1 μg/cm^2 saturation density of ECM ligands on 1 cm^2 24-well dishes [33].) Tissue culture dishes were then incubated with one of these solutions overnight at 4 °C. The plates were washed 2× with PBS(−) and then blocked with PBS(−) containing 1% bovine serum albumin (BSA) for 30 min at 37 °C. After washing with PBS(−) again, plates were ready for cell seeding.

Flow cytometric analysis of integrin expression. The cell surface expression of $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrins was quantified using quantitative flow cytometry. Briefly, MSCs were harvested from their culture environment via routine techniques, counted, and resuspended in PBS + 0.l% BSA to yield 5×10^5 cells/ml. Suspensions were incubated with anti-$\alpha_2\beta_1$, anti-$\alpha_5\beta_1$ or anti-$\alpha_v\beta_3$ antibodies (diluted 1:200) and rotated at 4 °C for 45 min. Cells were collected via centrifugation and then washed 3–4× with PBS. Bound primary antibodies were then detected by incubating cells with a FITC-conjugated anti-mouse secondary antibody (diluted 1:100) and rotated at 4 °C for another 45 min. Following

additional centrifugation and washing steps to remove unbound antibodies, MSCs were fixed in PBS + 1% paraformaldehyde and washed 1–2 more times. Non-specific background staining was determined by staining cells with secondary antibody only in parallel (negative control). Stained cells were then analyzed using a BD FACSCalibur instrument (Becton–Dickinson, San Jose, CA) equipped with CellQuest Pro software. A minimum of 10,000 cells were counted in each sample. All experimental data were analyzed offline using FlowJo software (Tree Star, Inc., Ashland, OR), and the mean fluorescent intensity for each sample was used to determine the relative expression of each of the three integrin heterodimers.

Immunofluorescence staining experiments. MSCs were plated on Lab-Tek Chamber Slides (Nalge Nunc International, Naperville, IL) in osteoinductive media and incubated at 37 °C for 7 days. The media were replenished every two days. To examine the localization of vinculin, the chamber slides were washed twice with PBS(−), and the cells subsequently fixed with PBS + 4% formaldehyde for 15 min. MSCs were permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 5 min and then incubated with anti-vinculin antibody (diluted 1:400 in PBS-T) for 2 h. Subsequently, cells were washed 3× with PBS(−). Slides were then incubated in the presence of a TRITC-conjugated secondary antibody for another hour. F-actin was visualized by staining the cells with Alexa Fluor 488-conjugated phalloidin for 40 min. Nuclei were also stained with DAPI (1 μg/ml) to triple-label the cells. Stained cells were visualized on a Nikon E800 microscope equipped for epifluorescence with a charge-coupled device (CCD) camera (Optronics, Goleta, California). Images were compiled and merged with PictureFrame software (Optronics).

Proliferation assays. MSCs were seeded at a density of 2.5×10^4 cells/cm^2 on Col I- or VN-coated 24-well plates in osteoinductive media [DMEM + 10% FBS and osteoinductive supplements (0.1 μm dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid)]. In experiments involving the MEK inhibitor, cells were initially seeded in the absence of drug. After 24 h, fresh media containing PD98059 at a final concentration of 50 μM were added. Drug-free controls were exposed to media containing equal volumes of DMSO (vehicle control), the solvent used to solubilize PD98059. Fresh inhibitor was added with each media change. At each time point (day 3 and 7) cells were harvested from triplicate wells by incubating with trypsin–EDTA at 37 °C for prolonged periods of time to ensure all cells detached. After neutralizing trypsin–EDTA with an equal volume of serum-containing medium, cell suspensions were diluted in isotonic buffer and subsequently counted using a Coulter counter Model ZM (Beckman-Coulter, Fullerton, CA).

Adhesion blocking studies. Standard adhesion blocking studies were performed to determine which integrins MSCs use to adhere to VN and Col I. Cell suspensions in osteoinductive media were pre-incubated with or without anti-$\alpha_2\beta_1$, anti-$\alpha_5\beta_1$, and/or anti-$\alpha_v\beta_3$ integrin antibodies for 25 min at 37 °C prior to seeding onto 24-well tissue culture dishes pre-coated with Col I or VN. Suspensions contained a defined concentration of cells (2.5×10^4 cells/ml) that would yield a theoretical seeding density of 1.25×10^4 cells/cm^2 (0.5 ml/well) in the absence of antibody. (Note this cell seeding density was 50% of that used for the proliferation and differentiation assays in order to minimize any undesired effects of cell–cell interactions on cell–ECM adhesion.) After a 40 min incubation at 37 °C, non-adherent cells were removed by gently rinsing with PBS(−) several times. Adherent cells from triplicate wells were then removed by trypsin–EDTA, neutralized, and counted in a Coulter counter.

Electrophoresis and Western blot analyses. Detection of activated or total levels of ERK, Akt/PKB, paxillin, and FAK in MSCs was performed by electrophoresis and Western blot analyses. Briefly, MSCs were seeded on Col I- or VN-coated plates at the same density as for the proliferation assays in either DMEM only, DMEM + 10% FBS (DM + FBS), or osteoinductive media [Ost(+FBS)]. At two different time points post-seeding (4 h and 7 days), cells were lysed in modified RIPA lysis buffer [150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40 including 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride (PMSF) plus 10 μM sodium orthovanadate]. In one set of parallel control experiments, harvested MSCs were suspended in the same three respective media for 15 min and then lysed (to generate the $t = 0$ data characterized by the absence of cell adhesion). In all cases, lysates were then cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. The total protein concentration of the resulting supernatant was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Equal protein amounts were then separated by gel electrophoresis using pre-cast 10% Tris–glycine gels (Invitrogen) under reducing conditions. The separated proteins were then electrophoretically transferred to a PVDF membrane by applying 125 mA current for 3 h. The resulting PVDF membrane was then washed 2× with PBS(−) containing 0.1% Tween 20 (PBS-T) and subsequently blocked overnight with PBS-T containing 2% BSA. Membranes were then incubated for 2 h at room temperature with one of the following primary antibodies at the indicated dilutions: anti-pY397-FAK (1:1000), anti-p-paxillin (1:2500), anti-p-ERK1/2 (1:2000), and anti-p-Akt (1:1000). Blots were then washed 3–4× with PBS-T and incubated with the appropriate HRP-conjugated secondary antibody for >1 h. After the incubation period, the membranes were washed 3–4× with PBS-T, incubated with an enhanced chemiluminescent substrate, and then exposed to autoradiography film (X-Omat LS, Kodak; Rochester, NY). After the detection of the phosphorylated forms of these proteins, membranes were stripped and probed again for the total levels of these proteins to confirm equal expression and loading.

Osteogenic differentiation assays. The early and late stages of osteogenic differentiation were monitored using standard alkaline phosphatase (ALP) and matrix mineralization assays, respectively. For both assays, MSCs were initially seeded at a density of 2.5×10^4 cells/cm^2 on Col I- or VN-coated 24-well culture plates in osteoinductive media and then cultured for up to 7 days. In those studies utilizing the MEK inhibitor, MSCs were incubated in the presence of PD98059 (50 μM) over the 7-day time course. Fresh inhibitor was added each time the media were changed (every other day). For ALP assays, cell lysates were generated at day 3 and day 7 as previously described [34]. Briefly, MSCs were lysed in passive lysis buffer (Promega, Madison, WI) for 15 min at room temperature. Lysates were scraped and collected from the wells and then incubated with 50 mM *p*-nitrophenylphosphate (PNPP) in assay buffer (containing 100 mM glycine, 1 mM $MgCl_2$, pH 10.5) at 37 °C for 25 min. The reaction was stopped with 500 μl of 0.1 N NaOH and the absorbance read at 405 nm. Specific ALP activity was determined using PNPP's extinction coefficient (1.85×10^4 M^{-1} cm^{-1}) and was then expressed in units of ALP activity per milligram of protein.

For mineralization assays, calcium phosphate hydroxyapatite was detected using a commercial von Kossa staining kit (American MasterTech Scientific Inc., Lodi, CA) according to the manufacturer's instructions. Briefly, MSCs were cultured on VN- or Col I-coated substrates in growth medium containing osteoinductive supplements. After 7 days, cells were washed 3× with PBS and fixed in 4% paraformaldehyde solution for 40 min. After extensive washing in water, the fixed cells were incubated with 5% silver nitrate solution and exposed to UV light at 365 nm for 40 min. Again after washing 5× with water, cells were placed in 5% sodium thiosulfate solution for 2–3 min. The cells were again washed with water and placed in Nuclear Fast Red for 5 min to stain the nucleus. Finally, after additional extensive washing, the stained samples were visualized on a Nikon E800 microscope using a 4× objective, and the images compiled with Picture Frame software.

FAK silencing via RNA interference. The influence of focal adhesion kinase (FAK) on the osteogenic differentiation of MSCs was determined by silencing FAK using commercially available small-interfering RNA reagents (Santa Cruz Biotech) according to the manufacturer's instructions. MSCs were initially seeded on VN- or Col I-coated substrates in serum-containing medium. Four hours after seeding, cells were transfected either with a siRNA oligonucleotide sequence targeting FAK or a non-targeted siRNA sequence (negative control) and incubated for 12 h. After that time period, medium containing 20% FBS was added and the cultures were incubated for an additional 6 h. Finally, these media were changed to osteoinductive media and subsequently cultured with two additional media changes throughout the remaining duration of the experiments (up to 7 days).

Results

Integrin expression and utilization in MSCs

Based in part on our recent report that MSCs undergo osteogenesis to differing degrees when cultured on thin polymer films due to the differential deposition of serum-derived ECM proteins [32], we hypothesized that MSC differentiation must be influenced by integrins. Our first goal in testing this hypothesis was to use flow cytometry to quantify the integrin expression repertoire and adhesion blocking to determine which integrins are actually used to adhere to Col I and VN. Flow cytometric analysis revealed that the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ are expressed at considerably higher levels than the $\alpha_v\beta_3$ integrin (Fig. 1A). Nevertheless, adhesion-blocking studies revealed that $\alpha_v\beta_3$ integrin plays a prominent role in MSC adhesion to both VN and Col I (Fig. 1B). On Col I, blocking $\alpha_v\beta_3$ integrins reduced adhesion by nearly 85%, while blocking $\alpha_2\beta_1$ integrin prevented nearly 65% of cell binding. By contrast, on VN, blocking either the $\alpha_v\beta_3$ or the $\alpha_2\beta_1$ integrins inhibited more than 80% of MSC adhesion. On either matrix, blocking $\alpha_5\beta_1$ inhibition did not significantly affect the binding of MSCs (Fig. 1B).

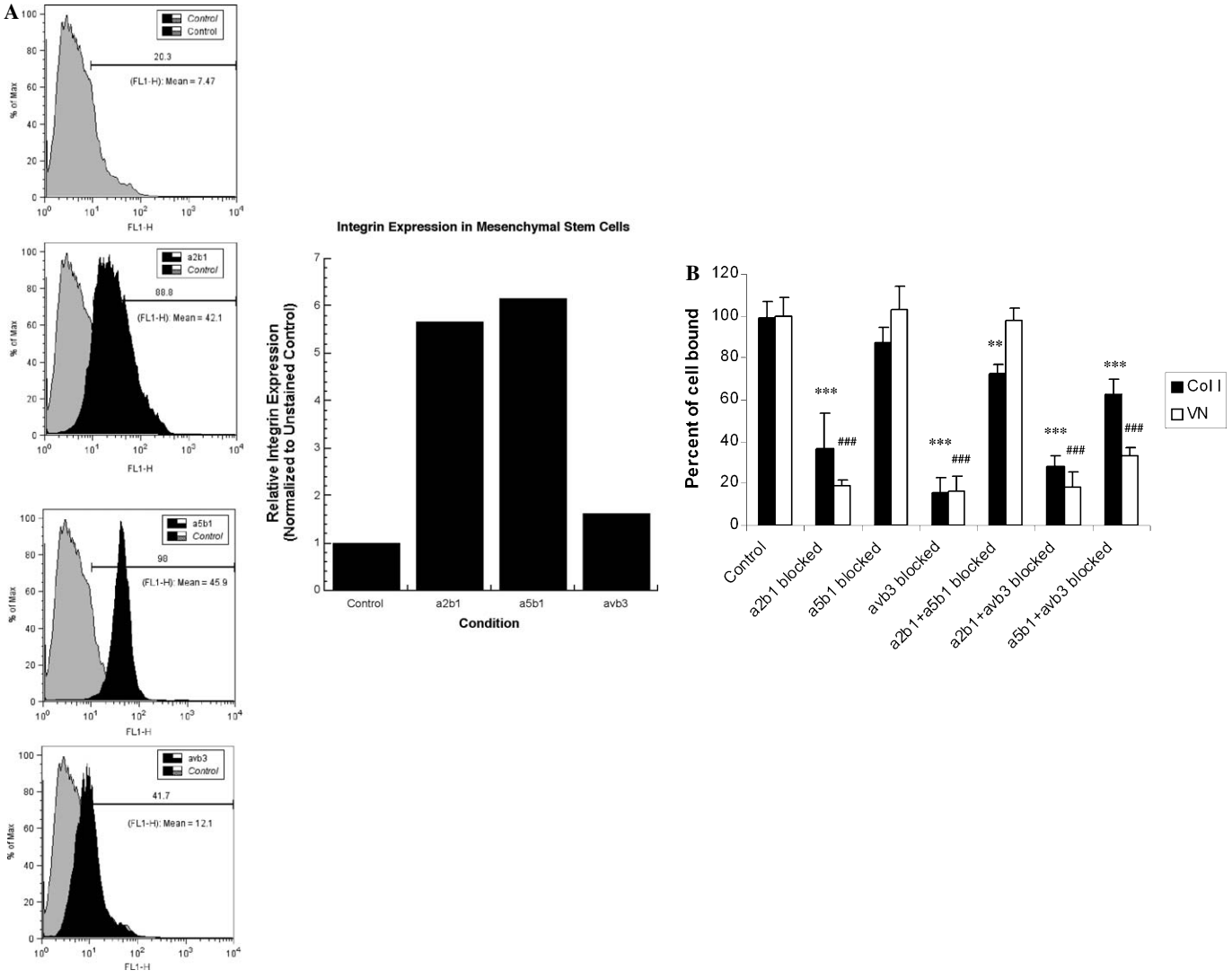

Fig. 1. Integrin expression and their utilization in MSC adhesion to VN and Col I. (A) Quantitative flow cytometry was used to assess the relative expression of the $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrins in MSCs. Histograms presenting the number of cells as a function of fluorescent intensity are shown (left) with the expression of $\alpha_2\beta_1$ (second histogram), $\alpha_5\beta_1$ (third histogram) and $\alpha_v\beta_3$ (fourth histogram) displayed relative to control cells labeled with 2° antibody only (top histogram, gray trace in each additional graph). Mean fluorescent intensities noted on each histogram are graphed (right) to yield the relative expression of each integrin. (B) Adhesion blocking studies were performed to determine which of these integrins are actually used by the MSCs to adhere to VN and Col I. Cells were harvested from culture flasks and incubated in suspension in the presence of antibodies to $\alpha_2\beta_1$, $\alpha_5\beta_1$, or $\alpha_v\beta_3$ integrin (or the indicated combinations) for 25 min at 37 °C. Suspension was then seeded onto either Col I- or VN-coated substrates and allowed to incubate at 37 °C for another 40 min. Adherent cells were trypsinized and counted, and the percentage of cell adhesion was compared to unblocked controls for each surface. Black bars denote Col I-coated surfaces, while the white bars refer to VN. Values represent mean adhesion ± SD ($n = 3$). (***$p < 0.001$ and **$p < 0.01$ with respect to unblocked Col I; $^{\#\#\#}p < 0.001$ with respect to unblocked VN.)

Adhesion to VN and Col I differentially regulates cytoskeletal assembly and signaling in MSCs

Our second goal was to assess the downstream consequences of the observed differences in integrin-mediated adhesion of MSCs to Col I and VN. As part of this effort, MSCs were cultured for 7 days on polystyrene dishes coated with either Col I or VN in osteoinductive media and stained to visualize their focal adhesions and actin stress fibers. We consistently observed an increase in the number of focal adhesions in MSCs cultured on VN versus Col I, as shown by the punctate localization of vinculin at the ends of actin stress fibers (Fig. 2). We also compared the signal transduction profiles of MSCs cultured on VN and Col I by focusing on several signals known to be regulated by adhesion (Fig. 3). As shown in Fig. 3A, MAPK was activated in MSCs suspended for 15 min in DMEM only, but not Akt, FAK or paxillin. The presence of serum and osteoinductive supplements induced significant activation of Akt in suspended cells, but only very minor activation of paxillin and no activation of FAK. On the other hand, when cells were cultured on VN for two different time points, it was observed that both FAK and ERK were activated 4 h after adhesion to VN. FAK remained active after 7 days in culture, paralleled by an increase in paxillin phosphorylation (Fig. 3B). However, the levels of ERK activity were dramatically reduced by 7 days in MSCs cultured on VN. By contrast, on Col I-coated substrates, neither FAK nor ERK was significantly activated after 4 h (Fig. 3C). However, by 7 days, ERK activity was notably increased while FAK activity remained suppressed. Paxillin phosphorylation again paralleled the FAK profile for cells on Col I. Distinct differences in Akt regulation were also observed between MSCs grown on VN versus Col I, with little or no evidence for phospho-Akt in MSCs grown on VN and elevated levels observed in MSCs cultured in osteoinductive media on Col I. In all cases, the presence or absence of soluble osteogenic supplements had no additional effects on the activities of these proteins beyond those induced by serum.

ERK plays a critical role in both mitogenesis and osteogenesis of MSCs on both VN and Col I

Given these observed differences in signaling, next we focused on the role of ERK. There are contradictory reports regarding ERK's role in osteogenic differentiation [30,31,35,36], and we hypothesized that its differential regulation in MSCs cultured on Col I versus VN may play a critical role in directing their phenotype. This possibility was addressed by monitoring both cell proliferation and osteogenic differentiation. With regard to proliferation, MSCs cultured in osteoinductive media proliferated to a significantly greater extent ($p < 0.01$) on Col I than on VN after 3 days in culture. However, the effects of substrate identity were eliminated by day 7 (Fig. 4). Inhibition of ERK signaling via PD98059 retarded MSC proliferation on both substrates, with a reduction of ~70% on Col I at day 3 and of ~60% at day 7, and a reduction of ~20% on VN at day 3 and of ~50% at day 7.

Next, alkaline phosphatase activity and mineral deposition assays were used as metrics of early and late stage osteogenesis, respectively, to test the impact of substrate identity and ERK signaling on osteogenesis. Western

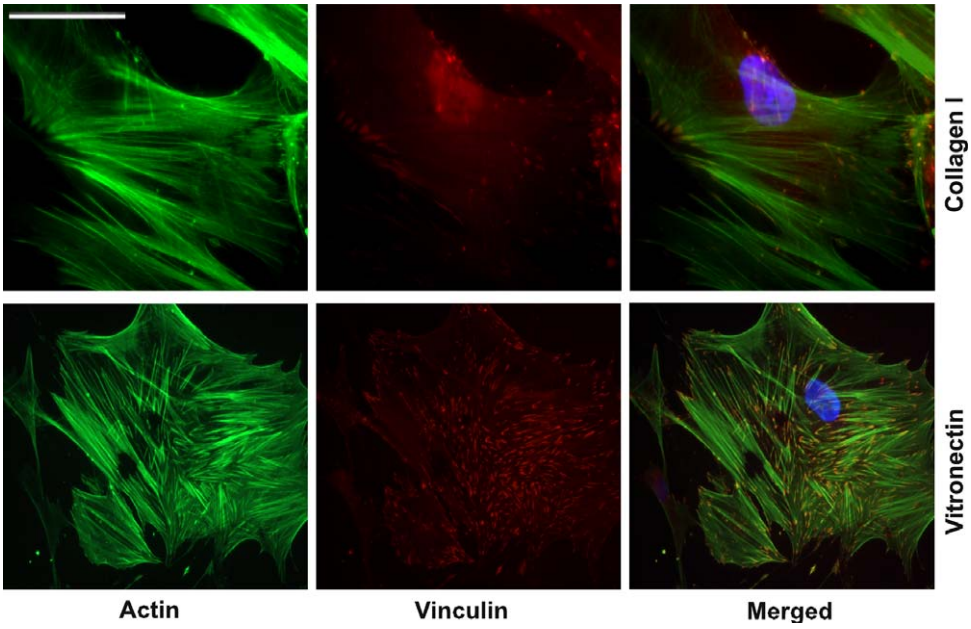

Fig. 2. MSCs cultured on VN display increased levels of focal adhesion-associated vinculin. MSCs cultured in osteoinductive media on either Col I or VN for 7 days were fixed and stained for vinculin (red), F-actin (green), and nuclei (blue), and visualized via fluorescent microscopy. Note the increased presence of punctate focal adhesions in cells cultured on VN compared to Col I (middle panel) in these representative images. Scale bar represents 50 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

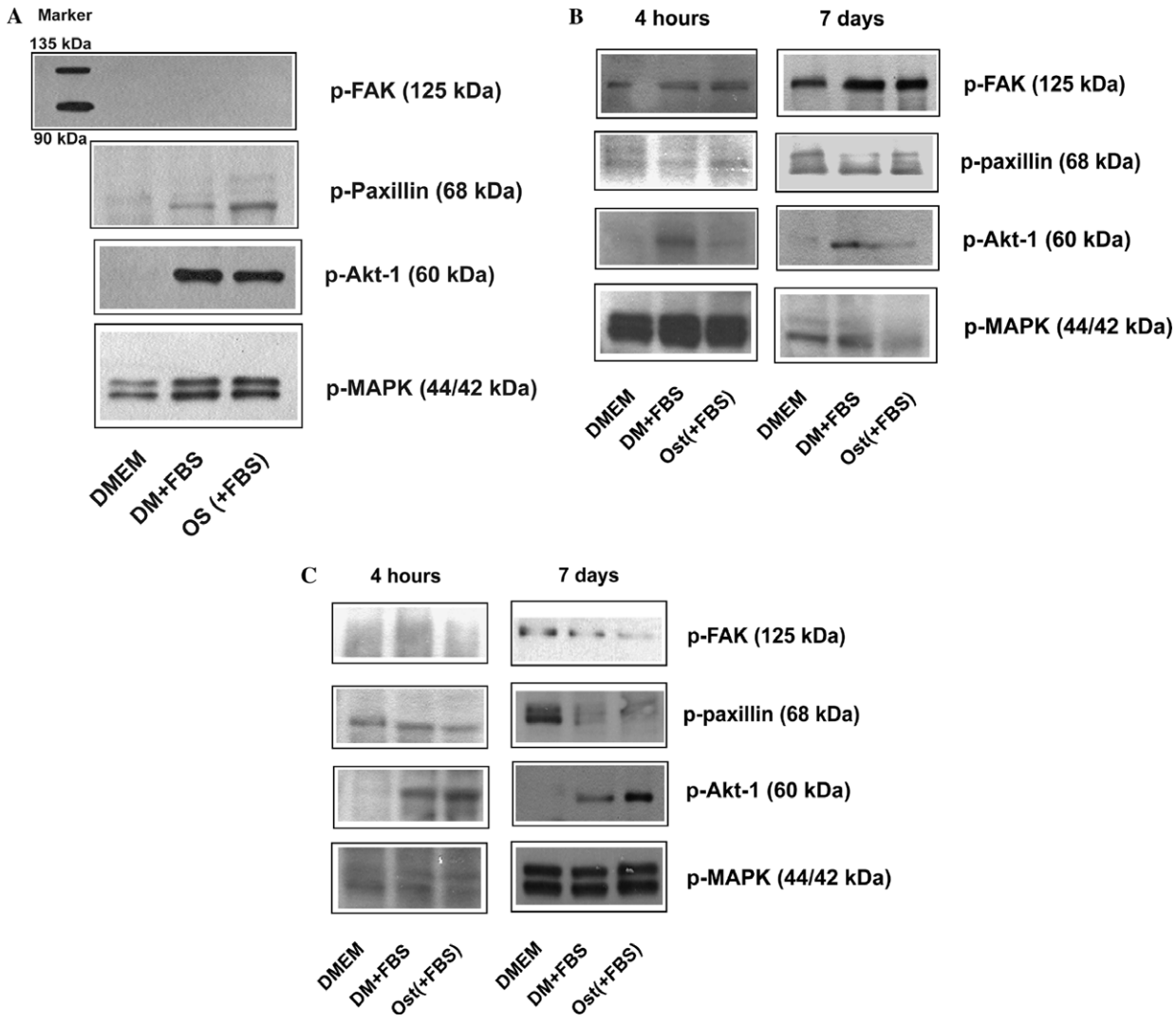

Fig. 3. MSC adhesion to Col I and VN differentially regulates signaling pathways. (A) MSCs were held in suspension in either basal media (DMEM), serum-containing media (DMEM + FBS), or osteoinductive media [Ost(+FBS)] and lysed after 15 min. MSCs cultured on substrates coated with either VN (B) or Col I (C) were lysed after both 4 h and 7 days. Equal protein amounts were then subjected to electrophoresis and Western blots were probed for the indicated proteins using phospho-specific antibodies. Blots representative of multiple repeated experiments show that the activation patterns of FAK, paxillin, Akt, and ERK (MAPK) are differentially regulated by adhesion to VN and Col I. Blots reprobed for the total levels of these proteins confirm equal protein loading (not shown).

blotting confirmed that 50 μM PD98059 effectively inhibited ERK activity in media containing osteogenic supplements at both early ($t \leqslant 3$ days) and later ($t = 7$ days) time points on both VN and Col I substrates (Fig. 5A). The levels of ALP activity were slightly (but insignificantly) higher in MSCs cultured on VN versus Col I after 3 days (Fig. 5B). The trend was reversed by day 7 as significantly higher levels were observed in MSCs cultured on Col I versus VN. Inhibition of ERK signaling via PD98059 reduced the levels of ALP activity at day 7 by 75% and 50% on Col I and VN, respectively, indicating the dependence of the MAPK pathway for osteogenesis on both substrates. Von Kossa staining of deposited mineral confirmed this conclusion, with the high levels of mineral deposition observed on both substrates after 7 days in osteoinductive media qualitatively reduced by treatment with PD98059 (Fig. 5C).

FAK is also essential for the osteogenic differentiation of MSCs on both VN and Col I

Finally, our observations with regard to adhesion-mediated signaling (Fig. 3) led us to test the hypothesis that FAK plays a role in differentiating the osteogenic response of MSCs cultured on Col I versus VN. Here we utilized a small interfering RNA (siRNA) approach to knock down FAK expression (and thereby activity). Osteogenesis was again monitored using ALP and mineral deposition. Initially we confirmed that MSCs were receptive to siRNA approaches by transfecting a fluorescein-conjugated control siRNA and visualizing via fluorescence microscopy (not shown). Then, we assessed the effectiveness of FAK silencing via Western blotting and revealed that silencing was both substrate- and time-dependent (Fig. 6A). FAK

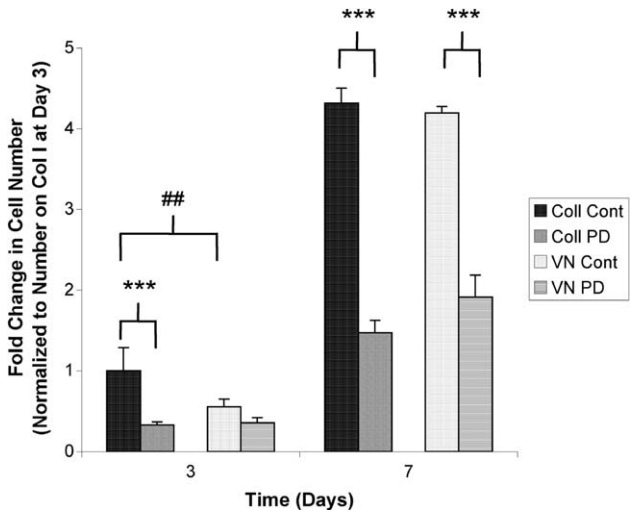

Fig. 4. MSC proliferation is ERK-dependent on both VN and Col I, but is enhanced on Col I at earlier time points. MSCs were cultured on either Col I or VN in osteoinductive media ± PD98059 (50 μM) for up to 7 days. Media containing fresh inhibitors were replenished every other day. At the indicated time points, cells were trypsinized and counted via a Coulter counter. Data shown are normalized to the uninhibited cell numbers on Col I-coated substrates at the day 3 time point (arbitrarily assigned a value of 1) and represent means ± SD ($n = 4$). ($^{***}p < 0.001$ with respect to uninhibited cells at the same time point and on the same coating; $^{\#\#}p < 0.01$ between cell numbers on Col I versus VN at $t = 3$ days.)

downregulation was highly effective in MSCs cultured on Col I at 12 h after transfection (Fig. 6A, top panel). The silencing remained effective throughout the 7-day duration of the experiments on Col I (Fig. 6A, bottom panel). By contrast, on VN-coated surfaces, FAK silencing was less effective after 12 h (Fig. 6A, top panel), but was significantly more effective by day 7 (Fig. 6A, bottom panel). Functionally, FAK silencing significantly reduced ALP activities by 40% (Fig. 6B) in MSCs on VN at day 3, but only by 10% in MSCs cultured on Col I at the same time point. Nevertheless, despite this more substantial impact on the early stages of osteogenesis on VN-coated surfaces versus Col I, FAK silencing qualitatively reduced mineralization on both substrates after 7 days (Fig. 6C).

Discussion

It is widely recognized that cell–ECM interactions play an important role in tissue development [37,38], and that signaling pathways initiated by integrin binding are involved in regulating nearly every aspect of cell function, including cell cycle progression, migration, apoptosis, and differentiation [16,39,40]. In this study, we investigated the impact of integrin-mediated adhesion to vitronectin and type-I collagen on the osteogenic differentiation of MSCs. Our exclusive focus on these two ECM ligands was motivated in part by our recent study in which we monitored the osteogenic differentiation of MSCs attached to thin films of PCL and PLGA, two biodegradable polymers widely used in the tissue engineering literature [32]. In that study, we found that the extent of osteogenesis depended on the polymer identity. Because cell attachment to these and other polymers is widely believed to occur via indirect means (i.e., via serum-derived ECM proteins), we demonstrated that inhibiting the adsorption of Col I, VN, fibronectin, and laminin via antibodies reduced MSC adhesion to thin PCL films by 5%, 47%, 20%, and 13%, respectively. Similar antibody-blocking studies on thin PLGA films reduced MSC attachment by 40%, 5%, 25%, and 14%, respectively. Because these data suggested that MSCs adhere to PCL primarily via VN and to PLGA via Col I, we hypothesized that the differential adsorption of these two proteins governs the osteogenic differentiation of MSCs on these polymers. By restricting our focus to Col I and VN here, we have attempted to address the mechanistic underpinnings behind this hypothesis.

Using quantitative flow cytometry, first we determined that MSCs express the $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrin heterodimers, the predominant pairs responsible for cell adhesion to Col I, fibronectin, and VN, respectively. The expression levels of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ were significantly higher than that of $\alpha_v\beta_3$, although adhesion blocking studies revealed that $\alpha_v\beta_3$ is involved in MSC adhesion to both VN and Col I. Prior studies with committed osteoblasts have shown that inhibition of α_5 or β_1 integrin subunits using isotype-specific antibodies reduces mineralization by approximately 20% and 45%, respectively, while antibodies to $\alpha_v\beta_3$ and $\alpha_2\beta_1$ reduced mineralization by 65% and 95%, respectively [17]. These results suggest that preferential adhesion and signaling via $\alpha_v\beta_3$ and $\alpha_2\beta_1$, which are primarily responsible for adhesion to VN and Col I, may be required for osteogenesis. In our examination of the consequences of MSC adhesion to VN and Col I, we found that both substrates supported the osteogenic differentiation of MSCs, in agreement with another recent report [41]. However, importantly, our data also show that ECM identity influences the switch between growth and differentiation in bone marrow-derived MSCs via distinct mechanisms. These findings are particularly striking given the expectation that the composition and spatial orientation of the adhesion substrate likely change significantly with time due to additional serum-derived ECM proteins and cell-mediated remodeling. Our results illustrate that the identity of the initial cell–ECM interaction is an important regulator of MSC fate.

Recent studies from Plopper and colleagues showed that VN, Col I, and laminin-5 all permit osteogenesis in MSCs, and may even be capable of inducing differentiation in the absence of soluble osteoinductive factors [35,41]. Our pharmacologic inhibition studies support their finding that ERK is required for osteogenesis, likely by activating the osteogenic transcription factor Runx2/CBFA-1 [35,36]. However, our results provide some new mechanistic insights as well. First, we found that the kinetics of osteogenic differentiation depend on ECM identity. The fact that MSCs cultured on Col I-coated substrates proliferated to a significantly greater extent after 3 days in culture than did those cells grown on VN, even in the presence of soluble

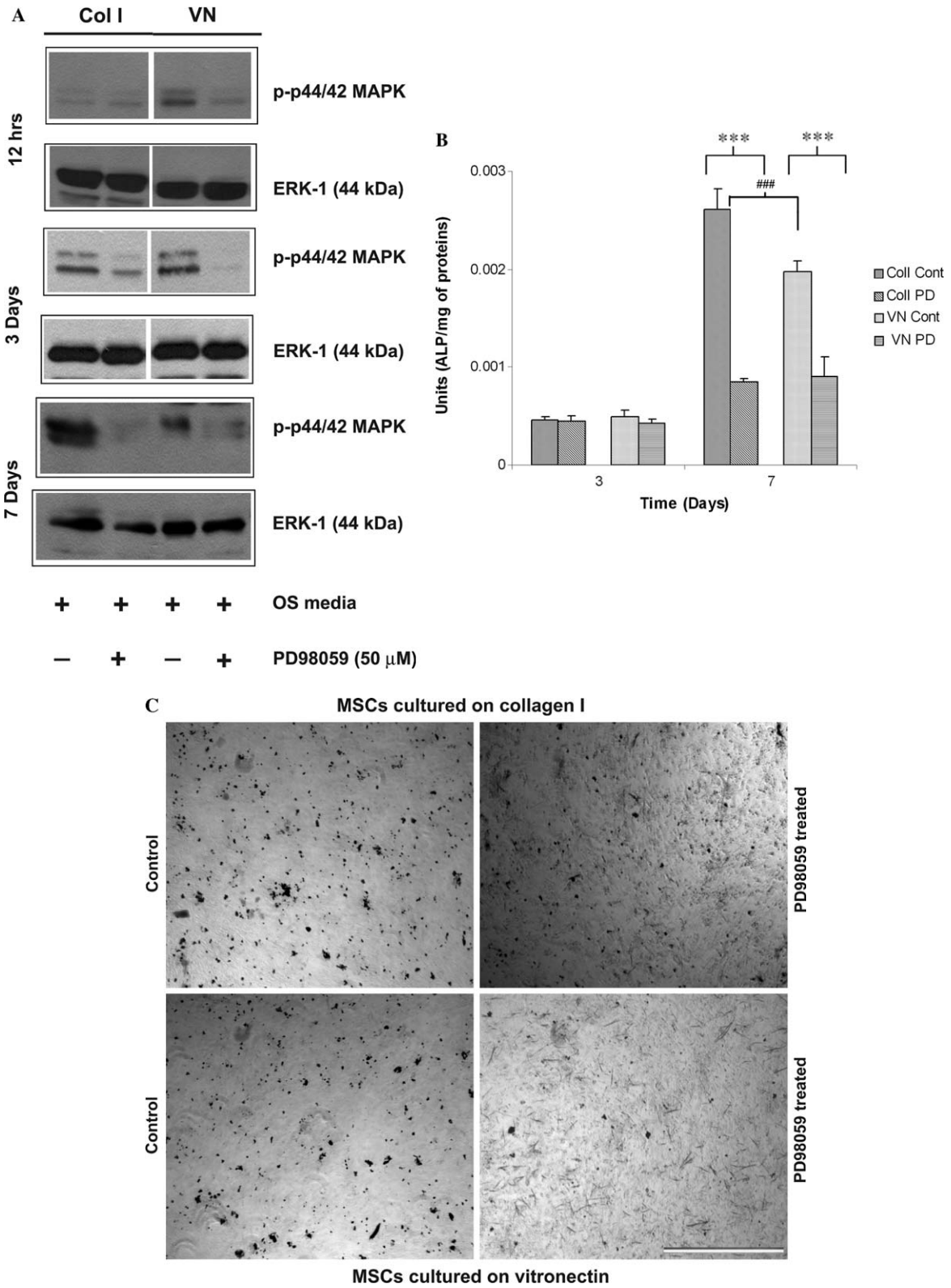

Fig. 5. Blocking ERK signaling inhibits the osteogenic differentiation of MSCs on both Col I and VN. (A) MSCs were cultured on Col I or VN in osteoinductive media (OS media) ± 50 μM PD98059 with fresh inhibitor added with every media change. Cell lysates were generated after 12 h, 3 days, and 7 days. Total protein levels were determined by BCA assay, and the levels of phosphorylated ERK (phospho-p44/42 MAPK) and total ERK1 were then assayed by Western blotting to confirm the efficacy of PD98059 inhibition. (B) The levels of ALP activity were significantly reduced ($^{***}p < 0.001$, $n = 3$) by ERK inhibition via PD98059 in MSCs cultured on both Col I and VN. In addition, the levels of ALP activity were insignificantly higher on VN versus Col I after 3 days, but significantly higher on Col I versus VN after 7 days ($^{\#\#\#}p < 0.001$, $n = 3$). (C) von Kossa staining of MSC cultures grown on VN or Col I ± PD98059 after 7 days revealed that mineralized matrix deposition (denoted by the black focal deposits) was ERK-dependent on both substrates. However, MAPK inhibition failed to completely inhibit mineral deposition on both matrices. Scale bar represents 50 μm.

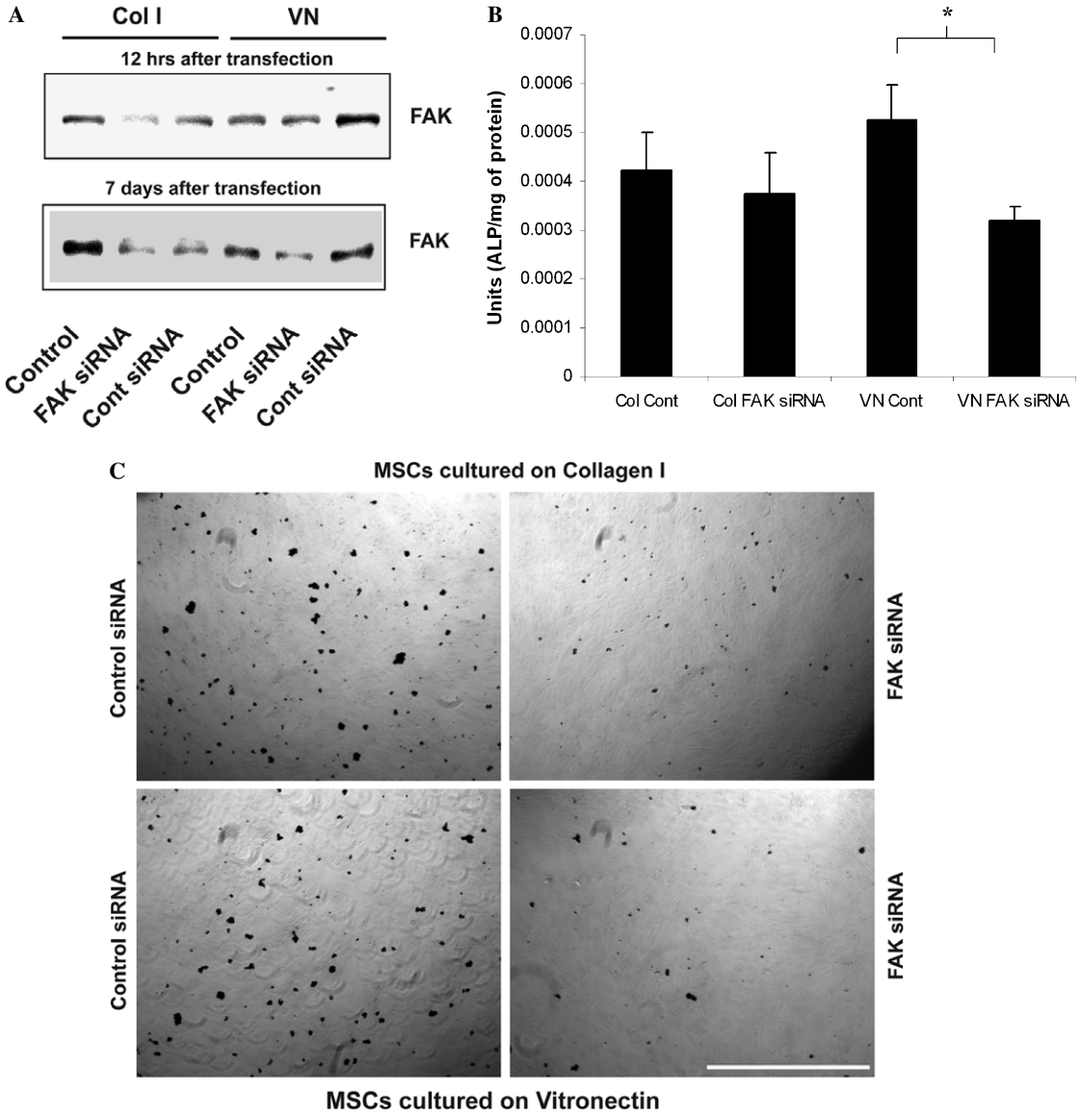

Fig. 6. Inhibition of FAK expression in MSCs via siRNA reduces osteogenesis on both VN and Col I. (A) MSCs cultured on Col I- or VN-coated substrates in growth media were transfected with FAK siRNA or a control siRNA according to the manufacturer's instructions. Cell lysates were prepared 12 h and 7 days after transfection, and equal amounts of proteins were subjected to SDS–PAGE and Western blotting. Evidence for reduced FAK levels was observed at both time points, indicating the silencing was sustained for up to a week. Similar results were obtained from multiple experiments. (B) After 3 days of culture, FAK silencing significantly inhibited ALP activity relative to unsilenced controls in MSCs seeded on VN ($*p < 0.05$, $n = 3$). The trend was similar but not significant in MSCs cultured on Col I. (C) FAK silencing also inhibited mineral deposition on both Col I and VN, as revealed in these representative 10× images of von Kossa stained cultures. Scale bar represents 50μm.

osteoinductive supplements, suggests that Col I favors mitogenesis at earlier time points. Second, MSC adhesion to VN for 4 h stimulated the activation of FAK and ERK, neither of which was activated at the same time point following adhesion to Col I. However, after 7 days, ERK activity was reduced in MSCs cultured on VN and enhanced in MSCs cultured on Col I.

The differential kinetics of ERK activation could perhaps explain the discrepancy in the literature for a role for MAPK signaling in osteogenesis. Higuchi, et al. suggested that continuous inhibition of MAPK signaling actually favors early osteogenic differentiation [31], albeit in different cell types than those studied here. Our results imply that it is not a simple matter of turning ERK on or off that regulates osteogenesis, but rather the mechanism by which it is activated, the level to which it is activated, or perhaps the kinetics of its activation. There is clear precedence for this idea in other cell types [42]. In our system, ERK activity was initially low in MSCs cultured on Col I and then ramped up coincident with mitogenesis. Inhibiting ERK in this phase of the cell cycle likely favors differentiation by turning down proliferation, consistent with the results of Higuchi et al. [31]. However, once confluence has been achieved, ERK shifts its focus towards differentiation, and becomes required for both ALP activity and mineral deposition. The fact that ALP levels are more sensitive to

PD98059 at day 7 versus day 3 supports this idea. By contrast, ERK activity is initially high in MSCs cultured on VN, even at subconfluency, but this does not translate into increased proliferation. Future studies will focus on addressing these kinetic differences in greater detail, and deciphering cross-talk mechanisms between integrin-mediated signaling and signaling induced by soluble osteoinductive factors.

Besides differentially activating ERK, MSCs cultured on VN-coated substrates exhibited an increased assembly of focal adhesions and stress fibers relative to Col I-coated substrates after 7 days in culture. This correlated with increased phosphorylation of FAK (Y397) and paxillin in MSCs grown on VN. Silencing FAK via a small interfering RNA approach had a more significant effect on ALP activity in MSCs cultured on VN, which supported higher levels of FAK activity and focal adhesion assembly in the first place. Nevertheless, an essential role for FAK in osteogenesis was found on both substrates, a finding previously unknown in the context of MSC differentiation. Combining these results with the fact that increased stress fibers and focal adhesions are hallmarks of increased RhoA activity, it is tempting to infer that VN-stimulated FAK activation may influence the activity of RhoA in MSCs as it does in other cell types [43]. This is particularly relevant given RhoA's known capacity to induce the osteogenic phenotype of MSCs [7]. Furthermore, previous reports suggest that β1 and β3 integrins differentially regulate RhoA [44,45], raising the possibility that restricting MSC adhesion to Col I and VN via β3 integrins may stimulate osteogenesis. However, because we have not investigated RhoA activity in these studies, the question of whether or not adhesion to VN or Col I differentially signals through the FAK-RhoA axis to ERK to ultimately drive osteogenesis remains to be answered. Further clouding the picture is the fact that fibronectin is amongst the strongest stimulators of RhoA activity in other cell types [46], but there is at least one report suggesting that fibronectin does not support the osteogenic differentiation of MSCs to the same degree as do VN and Col I [41]. Future work to explicitly test RhoA's role in osteogenesis will require a broader set of ECM ligands. Regardless, the observed differences in cytoskeletal architecture and signaling in MSCs cultured on VN versus Col I suggest that distinct upstream events induced by differential integrin binding eventually converge on one or more common downstream mediators to ultimately govern cell fate.

In summary, the results presented here show that both VN and Col I permit the osteogenic differentiation of MSCs, but do so via unique integrin-mediated signaling mechanisms. Osteogenesis on VN substrates correlated with enhanced focal adhesion formation, the activation of FAK and paxillin, and the diminished activation of MAPK and PI3K pathways. By contrast, MSCs cultured on Col I exhibited reduced focal adhesion formation, reduced activation of FAK and paxillin, and the increased activation of MAPK and PI3K signaling pathways. Despite these differential signaling cascades, our data clearly show that FAK and ERK are both involved in osteogenesis on these two substrates. Collectively, these results strengthen the need for an improved understanding of the ECM's role on the differentiation of osteoprogenitor cells. Such an improved understanding is essential to foster the development of novel biomimetic materials, particularly those that preferentially engage specific integrins [47], in order to reproducibly instruct the transition from mitogenesis to osteogenesis both *in vitro* and *in vivo*.

Acknowledgments

This work was partially supported by a grant from the NIH/NIDCR (1R03-DE016117) awarded to A.J.P. and by internal set-up funds provided by UC Irvine. In addition, we acknowledge Aswathi Sreedharan for cell culture assistance, Cyrus Ghajar for critically reading the manuscript, and the UC Irvine Center for Immunology for assistance with flow cytometry.

References

[1] J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, J.M. Jones, Embryonic stem cell lines derived from human blastocysts, Science 282 (1998) 1145–1147.
[2] A.I. Caplan, S.P. Bruder, Mesenchymal stem cells: building blocks for molecular medicine in the 21st century, Trends Mol. Med. 7 (2001) 259–264.
[3] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[4] P.J. Ter Brugge, J.A. Jansen, In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone, Tissue Eng. 8 (2002) 321–331.
[5] S.M. Warren, R.K. Nacamuli, H.M. Song, M.T. Longaker, Tissue-engineered bone using mesenchymal stem cells and a biodegradable scaffold, J. Craniofac. Surg. 15 (2004) 34–37.
[6] B. Johnstone, T.M. Hering, A.I. Caplan, V.M. Goldberg, J.U. Yoo, In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells, Exp. Cell Res. 238 (1998) 265–272.
[7] R. McBeath, D.M. Pirone, C.M. Nelson, K. Bhadriraju, C.S. Chen, Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment, Dev. Cell 6 (2004) 483–495.
[8] R. Sordella, W. Jiang, G.C. Chen, M. Curto, J. Settleman, Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis, Cell 113 (2003) 147–158.
[9] R.G. Young, D.L. Butler, W. Weber, A.I. Caplan, S.L. Gordon, D.J. Fink, Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair, J. Orthop. Res. 16 (1998) 406–413.
[10] G. Ferrari, G. Cusella-De Angelis, M. Coletta, E. Paolucci, A. Stornaiuolo, G. Cossu, F. Mavilio, Muscle regeneration by bone marrow-derived myogenic progenitors, Science 279 (1998) 1528–1530.
[11] M.A. LaBarge, H.M. Blau, Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury, Cell 111 (2002) 589–601.
[12] A. Hermann, R. Gastl, S. Liebau, M.O. Popa, J. Fiedler, B.O. Boehm, M. Maisel, H. Lerche, J. Schwarz, R. Brenner, A. Storch, Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells, J. Cell Sci. Pt. (2004).

[13] J. Sanchez-Ramos, S. Song, F. Cardozo-Pelaez, C. Hazzi, T. Stedeford, A. Willing, T.B. Freeman, S. Saporta, W. Janssen, N. Patel, D.R. Cooper, P.R. Sanberg, Adult bone marrow stromal cells differentiate into neural cells in vitro, Exp. Neurol. 164 (2000) 247–256.
[14] J.E. Aubin, Advances in the osteoblast lineage, Biochem. Cell Biol. 76 (1998) 899–910.
[15] R.O. Hynes, Integrins: bidirectional, allosteric signaling machines, Cell 110 (2002) 673–687.
[16] F.G. Giancotti, E. Ruoslahti, Integrin signaling, Science 285 (1999) 1028–1032.
[17] G.B. Schneider, R. Zaharias, C. Stanford, Osteoblast integrin adhesion and signaling regulate mineralization, J. Dent. Res. 80 (2001) 1540–1544.
[18] S.L. Cheng, C.F. Lai, S.D. Blystone, L.V. Avioli, Bone mineralization and osteoblast differentiation are negatively modulated by integrin alpha(v)beta3, J. Bone Miner. Res. 16 (2001) 277–288.
[19] S.F. El-Amin, M. Attawia, H.H. Lu, A.K. Shah, R. Chang, N.J. Hickok, R.S. Tuan, C.T. Laurencin, Integrin expression by human osteoblasts cultured on degradable polymeric materials applicable for tissue engineered bone, J. Orthop. Res. 20 (2002) 20–28.
[20] S.F. El-Amin, H.H. Lu, Y. Khan, J. Burems, J. Mitchell, R.S. Tuan, C.T. Laurencin, Extracellular matrix production by human osteoblasts cultured on biodegradable polymers applicable for tissue engineering, Biomaterials 24 (2003) 1213–1221.
[21] R.S. Carvalho, P.J. Kostenuik, E. Salih, A. Bumann, L.C. Gerstenfeld, Selective adhesion of osteoblastic cells to different integrin ligands induces osteopontin gene expression, Matrix Biol. 22 (2003) 241–249.
[22] S.N. Stephansson, B.A. Byers, A.J. Garcia, Enhanced expression of the osteoblastic phenotype on substrates that modulate fibronectin conformation and integrin receptor binding, Biomaterials 23 (2002) 2527–2534.
[23] Q. Chen, M.S. Kinch, T.H. Lin, K. Burridge, R.L. Juliano, Integrin-mediated cell adhesion activates mitogen-activated protein kinases, J. Biol. Chem. 269 (1994) 26602–26605.
[24] X. Zhu, R.K. Assoian, Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation, Mol. Biol. Cell 6 (1995) 273–282.
[25] K. Roovers, G. Davey, X. Zhu, M.E. Bottazzi, R.K. Assoian, Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells, Mol. Biol. Cell 10 (1999) 3197–3204.
[26] M.W. Renshaw, X.D. Ren, M.A. Schwartz, Growth factor activation of MAP kinase requires cell adhesion, EMBO J. 16 (1997) 5592–5599.
[27] A.K. Howe, A.E. Aplin, R.L. Juliano, Anchorage-dependent ERK signaling—mechanisms and consequences, Curr. Opin. Genet. Dev. 12 (2002) 30–35.
[28] G. Xiao, D. Jiang, P. Thomas, M.D. Benson, K. Guan, G. Karsenty, R.T. Franceschi, MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1, J. Biol. Chem. 275 (2000) 4453–4459.
[29] R.T. Franceschi, G. Xiao, Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways, J. Cell. Biochem. 88 (2003) 446–454.
[30] R.K. Jaiswal, N. Jaiswal, S.P. Bruder, G. Mbalaviele, D.R. Marshak, M.F. Pittenger, Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase, J. Biol. Chem. 275 (2000) 9645–9652.
[31] C. Higuchi, A. Myoui, N. Hashimoto, K. Kuriyama, K. Yoshioka, H. Yoshikawa, K. Itoh, Continuous inhibition of MAPK signaling promotes the early osteoblastic differentiation and mineralization of the extracellular matrix, J. Bone Miner. Res. 17 (2002) 1785–1794.
[32] S.R. Chastain, A.K. Kundu, S. Dhar, J.W. Calvert, A.J. Putnam, Adhesion of mesenchymal stem cells to polymer scaffolds occurs via distinct ECM ligands and controls their osteogenic differentiation, J. Biomed. Mater. Res. A 78 (2006) 73–85.
[33] D.J. Mooney, R. Langer, D.E. Ingber, Cytoskeletal filament assembly and the control of cell spreading and function by extracellular matrix, J. Cell Sci. 108 (Pt 6) (1995) 2311–2320.
[34] W.L. Murphy, S. Hsiong, T.P. Richardson, C.A. Simmons, D.J. Mooney, Effects of a bone-like mineral film on phenotype of adult human mesenchymal stem cells in vitro, Biomaterials 26 (2005) 303–310.
[35] R.F. Klees, R.M. Salasznyk, K. Kingsley, W.A. Williams, A. Boskey, G.E. Plopper, Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway, Mol. Biol. Cell 16 (2005) 881–890.
[36] R.M. Salasznyk, R.F. Klees, M.K. Hughlock, G.E. Plopper, ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin, Cell Commun. Adhes. 11 (2004) 137–153.
[37] J.C. Adams, F.M. Watt, Regulation of development and differentiation by the extracellular matrix, Development 117 (1993) 1183–1198.
[38] N. Boudreau, M.J. Bissell, Extracellular matrix signaling: integration of form and function in normal and malignant cells, Curr. Opin. Cell Biol. 10 (1998) 640–646.
[39] M.A. Schwartz, M.D. Schaller, M.H. Ginsberg, Integrins: emerging paradigms of signal transduction, Annu. Rev. Cell Dev. Biol. 11 (1995) 549–599.
[40] C.K. Miranti, J.S. Brugge, Sensing the environment: a historical perspective on integrin signal transduction, Nat. Cell. Biol. 4 (2002) E83–E90.
[41] R.M. Salasznyk, W.A. Williams, A. Boskey, A. Batorsky, G.E. Plopper, Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells, J. Biomed. Biotechnol. 2004 (2004) 24–34.
[42] A.R. Asthagiri, C.M. Nelson, A.F. Horwitz, D.A. Lauffenburger, Quantitative relationship among integrin-ligand binding, adhesion, and signaling via focal adhesion kinase and extracellular signal-regulated kinase 2, J. Biol. Chem. 274 (1999) 27119–27127.
[43] X.D. Ren, W.B. Kiosses, D.J. Sieg, C.A. Otey, D.D. Schlaepfer, M.A. Schwartz, Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover, J. Cell Sci. 113 (Pt 20) (2000) 3673–3678.
[44] E.H. Danen, P. Sonneveld, C. Brakebusch, R. Fassler, A. Sonnenberg, The fibronectin-binding integrins alpha5beta1 and alphavbeta3 differentially modulate RhoA-GTP loading, organization of cell matrix adhesions, and fibronectin fibrillogenesis, J. Cell Biol. 159 (2002) 1071–1086.
[45] H. Miao, S. Li, Y.L. Hu, S. Yuan, Y. Zhao, B.P. Chen, W. Puzon-McLaughlin, T. Tarui, J.Y. Shyy, Y. Takada, S. Usami, S. Chien, Differential regulation of Rho GTPases by beta1 and beta3 integrins: the role of an extracellular domain of integrin in intracellular signaling, J. Cell Sci. 115 (2002) 2199–2206.
[46] J. Gu, Y. Sumida, N. Sanzen, K. Sekiguchi, Laminin-10/11 and fibronectin differentially regulate integrin-dependent Rho and Rac activation via p130(Cas)-CrkII-DOCK180 pathway, J. Biol. Chem. 276 (2001) 27090–27097.
[47] B.G. Keselowsky, D.M. Collard, A.J. Garcia, Integrin binding specificity regulates biomaterial surface chemistry effects on cell differentiation, Proc. Natl. Acad. Sci. USA 102 (2005) 5953–5957.