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Activation of FAK Is Necessary for the Osteogenic Differentiation of Human Mesenchymal Stem Cells on Laminin-5

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Abstract Human mesenchymal stem cell (hMSC) differentiation into osteoblasts and the signaling events involved are poorly understood. We recently established that contact with specific extracellular matrix (ECM) proteins, in particular laminin-5, is sufficient to induce an osteogenic phenotype in hMSC through an extracellular signal-related kinase (ERK)-dependent pathway. Activation of ERK 1/2 by laminin-5 induces phosphorylation of the runx2/cbfa-1 transcription factor that controls osteogenic gene expression. We hypothesized that focal adhesion kinase (FAK) mediated signaling pathways supply a link between cell surface integrin-ECM binding and activation of ERK 1/2, and that laminin-5 promotes its osteogenic effects through this pathway. To test this hypothesis, we plated hMSC on a laminin-5 matrix in the presence or absence of FAK-specific small inhibitory RNAs (siRNA), and assayed for phosphorylation of runx2/cbfa-1 as well as expression of established osteogenic differentiation markers (bone sialoprotein, osteocalcin, alkaline phosphatase, calcium deposition, and mineral:matrix ratio). We found that siRNA treatment reduced total endogenous FAK protein by ~40%, and reduced FAK phosphorylation on Y397 by ~33% in cells plated on laminin-5 for 30 min. SiRNA treated cells exhibited a decrease in ERK 1/2 phosphorylation after 1 h, and reduced serine/threonine phosphorylation of Runx2/Cbfa-1 after 8 days. Finally, FAK inhibition blocked osteogenic differentiation of hMSC, as assessed by lowered expression of osteogenic genes (RT-PCR), decreased alkaline phosphatase activity, greatly reduced calcium deposition, and a lower mineral:matrix ratio after 28 days in culture. These results establish FAK as an important mediator of laminin-5-induced osteogenic differentiation of hMSC. J. Cell. Biochem. 100: 499–514, 2007.

Key words: focal adhesion kinase; osteogenesis; laminin-5; mesenchymal stem cell; osterix; extracellular matrix

Human mesenchymal stem cells (hMSC) are multipotent bone marrow progenitor cells that have the potential to differentiate into lineages of mesenchymal tissues including bone [Owen and Friedenstein, 1988; Jaiswal et al., 1997; Pittenger et al., 1999; Salasznyk et al., 2004b], cartilage [Mackay et al., 1998], fat [Jaiswal et al., 2000], and cardiomyocytes [Toma et al., 2002]. In vivo application of hMSC to cell-based therapies has been significantly impeded because the signaling mechanisms controlling hMSC commitment along these differentiation lineages are not fully understood. Recent evidence suggests that signals generated by integrin ligation to bone marrow extracellular matrix (ECM) proteins regulate hMSC commitment along the osteogenic lineage [Salasznyk et al., 2004a; Klees et al., 2005]. In particular, laminin-5 and $\alpha 3\beta 1$ integrin-induced signaling increase osteogenic gene expression and matrix mineralization in hMSC [Klees et al., 2005]. However, the molecular mechanisms underlying laminin-5-induced osteogenic differentiation of hMSC are totally unknown.

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is activated upon integrin ligation to the ECM at sites of focal adhesions [Mitra et al., 2005]. FAK also serves as a scaffold for assembling complexes of several classes of

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signaling molecules that initiate downstream signaling events, including activation of mitogen activated protein kinases (MAPKs) leading to mesodermal tissue maturation and stem cell differentiation. FAK knockout mice exhibit early embryonic lethality and experience abnormal mesodermal differentiation, including improper vasculogenesis and angiogenesis [Furuta et al., 1995]. In addition, differentiation of C(2)C(12) myoblasts into myotubes requires FAK activation [Clemente et al., 2005]. In mouse embryonic stem cells, FAK is a key regulator of cardiogenesis and plays and integral role in directing stem cell lineage commitment [Hakuno et al., 2005]. Thus, FAK plays a critical role in the development of the vascular system [Shen et al., 2005].

FAK also appears to be important for osteogenesis. Most of the FAK studies published thus far have focused on cells already committed to this differentiation state [Perinpanayagam et al., 2001; Manduca et al., 2005], and suggest that FAK is part of the signal transduction machinery for promoting maturation of osteoblasts or committed, "osteoblastic" cell lines [Takeuchi et al., 1997]. In hMSC, inhibition of FAK signaling by modeled microgravity inhibits collagen-I directed osteogenic differentiation [Meyers et al., 2004]. Here, we focus on an earlier step in osteogenesis, when undifferentiated hMSC commit to the osteogenic phenotype, using Ln-5 as a novel osteogenic stimulus.

MATERIALS AND METHODS

Tissue culture media (DMEM) and penicillin G-streptomycin sulfate were purchased from Mediatech (Cellgro, VA). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA). Trypsin-EDTA and poly-L-lysine (cat. # P4832) were obtained from Sigma Chemical Co. (St. Louis, MO). Purified human plasma fibronectin (cat. # FC010) was from Chemicon International (Temecula, CA). Rabbit polyclonal IgG phospho-specific antibodies against anti-FAK (pY^{397}) (cat. # 44-624G), ERK 1/2 ($pTpY^{185/187}$) (cat. #44-680G), and Pyk2 (pY^{402}) (cat. #44-618G) were purchased from Biosource International (Camarillo, CA). Rabbit polyclonal IgG antibodies against anti-human FAK (cat. #AHO0502) were obtained from Biosource International. Mouse monoclonal IgG antibodies against anti-Pyk2/CAKβ (cat. #05-488) and the Catch and Release® v2.0 Reversible Immunoprecipitation System (cat. #17-500) were from Upstate Cell Signaling Solutions (Lake Placid, NY). Rabbit polyclonal IgG antibodies against anti-ERK 1/2 (cat. #AB3053), phospho-serine (cat. #AB1603), and phospho-threonine (cat. #AB1607) were purchased from Chemicon International. Mouse monoclonal IgG antibodies against anti-Runx2/Cbfa-1 were obtained from MBL International (Watertown, MA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG secondary antibodies were obtained from Jackson Immuno Research (West Grove, PA). Reverse transcriptase-polymerase chain reaction (RT-PCR) primers listed in Table I were purchased from IDT Technologies (Coralville, Iowa). The protein assay kit was purchased from Pierce (Rockford, IL). Unless otherwise specified, the other standard reagents were

TABLE I. Primers for RT-PCR

Gene name	Primer sequences	Product size (bp)
IBSP (bone sialoprotein 2) NM_004967	Forward 5′-ACAACACTGGGCTATGGAGAGGAC-3′ Reverse 5′-ACTTGTGGTGGGGTTGTAGGTTCA-3′	401
BGLAP (osteocalcin) NM_199173	Forward 5′-CGCAGCCACCGAGACACCAT-3′ Reverse 5′-AGGGCAAGGGGAAGAGGAAAGAA-3′	400
SP7 (osterix) NM_152860	Forward 5′-AACCCCCAGCTGCCCACCTACC-3′ Reverse 5′-GACGCTCCAGCTCATCCGAACG-3′	457
CBFA1 (core binding factor alpha 1) NM_004348	Forward 5′-ATGGCGGGTAACGATGAAAAT-3′ Reverse 5′-ACGGCGGGGAAGACTGTGC-3′	421
GAPDH (glyceraldehyde-3-phosphate dehydrogenase) NM_002046	Forward 5′-ATGGAAATCCCATCACCATCT-3′ Reverse 5′-GGTTGAGCACAGGGTACTTTATT-3′	1,000
MEPE (extracellular matrix glycoprotein) NM_020203	Forward 5′-TTGGGCAAGAGAATAAATCA-3′ Reverse 5′-TCTGGGCTTGGGAATCTCTT-3′	466
DSPP (dentin sialophospho protein) NM_014208	Forward 5′-GGAGAAGATGCTGGCCTGGATAA-3′ Reverse 5′-CACTGCTGGGACCCTTGATTTCTA-3′	451
DMP-1 (dentin matrix protein 1) NM_004407	Forward 5′-CAGGGCCCAAAGACAGACAA-3′ Reverse 5′-TGAGATGCGAGACTTCCTAAAAAT-3′	461

obtained from Fisher Scientific (Fair Lawn, NJ).

Cell Culture

Cryopreserved hMSC were purchased from Cambrex, Inc. (Walkersville, MD) and were grown according to the manufacturers' instructions. Briefly, cells were plated at 5×10^3 cells/cm^2 in a T75 flask (75 cm^2) for continuous passaging in DMEM medium supplemented with 10% FBS, 1% L-glutamine (29.2 mg/ml), penicillin G (10,000 units/ml), and streptomycin sulfate (10,000 μg/ml). Medium was changed twice weekly and cells were detached by trypsin-EDTA and passaged into fresh culture flasks at a ratio of 1:3 upon reaching confluence. For in vitro osteogenic assays, hMSC were passaged three times before they were inducted and plated at densities of 3.1×10^3 cells/cm^2 in 0.2 ml/cm^2 of medium on 100 mm Falcon dishes (78.5 cm^2). The following day (day 0), we replaced the culture medium with fresh control medium in the absence or presence of osteogenic supplements (OS) containing 0.1 μM dexamethasone, 0.05 mM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate (Cambrex, Inc.). In each experiment, control and OS-treated cells were processed in parallel. Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

Laminin-5 matrix was isolated from rat bladder carcinoma 804G cells as described previously [Plopper et al., 1996].

FAK Small Interference RNA Transfection

To determine the contribution of FAK signaling in the osteogenic differentiation of hMSC, cells were trypsinized and grown overnight to approximately 75% confluence on 100 mm Falcon dishes (78.5 cm^2) and subsequently transfected with Silencer™ validated siRNAs targeting FAK (sense seq: GGAGUGGAAAUAUGAAUUGTT; anti-sense seq: CAAUUCAUAUUUCCACUCCTC) (cat. #51323) purchased from Ambion, Inc. (Austin, TX), using siPORT™ Amine transfection agent (Ambion, Inc), according to the manufacturers' instructions. Briefly, the siPORT™ Amine was incubated in serum-deprived DMEM for 10 min and subsequently mixed with siRNA diluted in serum-deprived DMEM for an additional 10 min to allow transfection complexes to form. Transfection complexes at a final siRNA concentration of 50 or 100 μM were dispensed onto the cells and assayed 72 h after transfection. For in vitro osteogenic assays, transfected cells were replated 72 h after transfection on tissue culture plastic (±OS), FN, or LN-5 in fresh DMEM and assayed at 8, 14, 21, or 28 days as described below. The final concentration of transfection agent never exceeded 0.3%, and the same amount of siPORT™ Amine vehicle was added to control conditions. Where appropriate, hMSC were also transfected with Ambions's validated Silencer™ negative control #1 siRNA (cat. #4611) and validated Silencer™ GAPDH siRNA control (cat. #4605) to demonstrate non-specific effects of gene silencing and to confirmed the transfection procedure, respectively.

Real Time RT-PCR

Quantitative RT-PCR analysis was performed with the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) using the GeneAmp 5700 sequence detection system (Applied Biosystems), according to the manufacturers' instructions. Briefly, the quantitative RT-PCR was performed using 25 μl of 2× QuantiTect SYBR Green Mix, 0.5 μl of QuantiTect RT Mix, 1 μl of FAK primers (forward 5′-GCGCTGGCTGGAAAAAGAGGAA-3′; reverse 5′-TCGGTGGGTGCTGGCTGGTAGG-3′) or GAPDH primers (forward 5′-ATGGAAATCCCATCACCATCT-3′; reverse 5′-GGTTGAGCACAGGGTACTTTATT-3′), RNAse-free water, and 0.5 μg of template RNA in a final volume of 50 μl. The reverse transcription step ran for 30 min at 50°C, followed by PCR activation for 15 min at 95°C. Thirty-five amplification cycles were run, consisting of 15 s denaturation at 94°C, 30 s of annealing at 58°C, and one 30 s of extension at 72°C. Samples were assayed in triplicate, and the values were normalized to the relative amounts of β-Tubulin (forward 5′-GGATCGACCCCACAGGCAGTTA-3′; reverse 5′-GGCGTTGTAGGGCTCCACC-3′).

Adhesion Assays

Cell adhesion assays were performed as previously described using Sarstedt 96-well suspension cell culture plates [Plopper et al., 1998]. Tissue culture plates were coated with purified ECM proteins (FN or LN-5) at a concentration of 20 μg/ml and poly-L-lysine for 1 h at room temperature. Wells were washed twice with

PBS and incubated with nd-blotto (5% non-diary creamer in PBS + 0.2% Tween 20) for 30 min prior to the assay. Control cells and those containing FAK siRNA were allowed to attach for 30 min at 37°C and were subsequently fixed with 3% paraformaldehyde, washed twice in PBS, and incubated in crystal violet dye for 15 min. Wells were washed thoroughly with water and the violet dye was extracted with 10% SDS solution. Absorbance was measured using a TECAN SPECTAFluor spectrophotometer at 595 nm and relative adhesion was compared to cells attached to nd-blotto.

Immunoprecipitation of Runx2 and Western Blotting

Whole cell extracts were prepared by harvesting serum-deprived cells overnight (DMEM + 0.1% FBS) in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, 1% Triton-X, 0.3 mM sodium vanadate, 1% deoxycholic acid, 0.2% SDS, pH 7.4). For immunoprecipitation of Runx2, the Catch and Release® v2.0 Reversible Immunoprecipitation System (Upstate) was employed. Briefly, 1× wash buffer, 500 μg of cell lysate, 4 μg of Runx2 antibody, and antibody capture affinity ligand were added to a spin column at a final volume of 500 μl. Following overnight incubation on a rotator at 4°C, the column was centrifuged at 5,000 rpm for 30 s to discard all the non-labeled proteins. Denatured Runx2 protein was eluted after the addition of 70 μl of 1× denaturing elution buffer containing β-ME and centrifugation. Proteins were diluted in 5× Laemmli's sample buffer, denatured at 100°C for 5 min, resolved by 8% SDS–PAGE, and electrophoretically transblotted to Trans-Blot® nitrocellulose membranes (0.2 μm) (Bio-Rad, Hercules, CA). The membranes were incubated with blocking solution (5% non-fat dried milk in 1× PBS + 0.2% Tween-20 (PBST) or 5% BSA in PBST for pY^{397} FAK and total FAK blots) for 1 h, then probed with various primary antibodies (1:500) overnight at 4°C. After three washes with PBST, membranes were incubated with HRP-conjugated secondary IgG (1:25,000) for 1 h, followed by another three washes with PBST. Immunoreactive bands were detected using the SuperSignal® Chemiluminescent reagent (Pierce) and quantitatively analyzed in triplicate by normalizing band intensities to the controls on scanned films by IMAGEJ software.

Reverse Transcriptase PCR (RT-PCR)

For in vitro osteogenic assays, RNA was isolated from 10×10^8 hMSC cultured in the presence or absence of FAK siRNA on tissue culture plastic (±OS), FN, or LN-5 in control media for 14 days. Total RNA was isolated using the RNeasy mini kit (Qiagen). RT-PCR was performed with the OneStep RT-PCR Kit (Qiagen) and a 96-well thermal cycler (MJ Research, Waltham, MA) using the primers listed in Table I, which were designed by the Lasergene v5.0 program (DNASTAR, Madison, WI). Template RNA (0.5 μg) was used per reaction. The reverse transcription step ran for 30 min at 50°C, followed by PCR activation for 15 min at 95°C. Thirty amplification cycles were run, consisting of 1 min denaturation at 94°C, 1 min of annealing at 58°C, and 1 min of extension at 72°C. Final extension was allowed to run 10 min at 72°C. Reaction products were separated by gel electrophoresis using a 1% agarose gel. Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a ChemiImager 4400 Gel imaging system (Alpha Innotech, San Leandro, CA). Band intensity was quantitatively analyzed in triplicate by IMAGEJ software for each gene and was normalized to corresponding GAPDH values.

Histological Staining of Alkaline Phosphatase Activity and Calcium Precipitation

For detection of alkaline phosphatase activity, a solution of naphthol AS-MX phosphate and fast blue RR dissolved in $d\mathrm{H_2O}$ was poured into 100 mm dishes on day 14 according to the manufacturer's instructions contained in Sigma Kit #85 (Sigma). Cellular specimens were scored according to the quantity and intensity of the precipitated dye as determined by IMAGEJ software and was normalized to control values. For the detection of a calcium-phosphate containing matrix after 21 days, cell layers were stained by the alizarin red-S method. Specimens were washed three times with PBS and fixed in ice-cold 70% ethanol for 1 h. Afterwards, cells were incubated with a 0.4% alizarin red-S solution in water (pH 4.2) for 15 min at room temperature. Cells layers were washed thoroughly with $d\mathrm{H_2O}$ five times and left in PBS for 15 min. Cellular specimens were scored according to the quantity and size of precipitated granules.

Calcium Assay

Specimens were washed twice with PBS and extracted off the dishes in 0.5 N HCl. Accumulated calcium was removed from the cellular component by shaking for 5 h at 4°C, followed by centrifugation at 2,000*g* for 10 min. The consequent supernatant was utilized for calcium determination according to the manufacturer's instructions contained in Sigma Kit #587 (Sigma). Total calcium was calculated from standard solutions prepared in parallel and expressed as ng/dish after absorbance at 575 nm was measured.

Fourier Transform Infrared (FTIR) Analysis

The presence of apatite in the cell matrix was detected by FTIR of ground powders following 28 days of cell culture. Cell layers were collected in 50 mM ammonium bicarbonate (pH 8.0), lyophilized, and analyzed as potassium bromide (KBr) pellets on a Thermo-Nicolet Spectrometer 4700 (Madison, WI). The data was collected under nitrogen purge, and the spectral baseline was corrected and analyzed using GRAMS/386 software (Galactic Industries, Salem, NH) as previously described [Kato et al., 2001]. The mineral content was calculated based on the spectrally derived mineral-to-matrix ratio (the integrated areas of the phosphate absorbance (900–1,200/cm) and protein amide I band (1,585–1,720/cm)).

Statistical Analysis

All experiments were repeated a minimum of two times and the representative data were presented as mean ± SE. Statistical analyses were preformed using Student's unpaired *t*-test, and a *P*-value less than 0.05 was considered significant.

RESULTS

Transfection of hMSC With FAK siRNA Suppresses FAK Expression In Vitro

To understand the mechanisms and potential role of FAK-induced signal transduction, small interfering RNAs (siRNA) were used to examine the effects of endogenous FAK reduction on laminin-5-induced hMSC differentiation. To validate FAK expression knockdown in our studies, hMSC were first transfected with Silencer™ FAK siRNA using siPORT™ Amine transfection agent at a final concentration of 50 or 100 nM for 72 h. Results show that these cells exhibited a greater than 40% reduction in endogenous FAK mRNA as compared to cells transfected with negative control siRNA, siPORT™ Amine alone, or untreated cells as determined by quantitative real-time RT-PCR (Fig. 1A). Incubation with FAK siRNA for 48 h resulted in slightly less inhibition of FAK expression than 72 h (data not shown). Interestingly, there was no significant decrease in FAK expression in cells transfected with a higher concentration of FAK siRNA. To further confirm our transfection procedure, hMSC transfected with validated Silencer™ GAPDH siRNA exhibited an approximately 50% decrease in endogenous GAPDH mRNA expression compared to cells transfected with the negative control siRNA, transfection agent alone, or untreated cells (Fig. 1B). These data clearly indicate that siRNA-induced knockdown is an effective method for partially inhibiting endogenous FAK expression.

FAK Knockdown by siRNA Transfection Inhibits Laminin-5-Induced FAK Phosphorylation

FAK phosphorylation of Y397 occurred in hMSC plated on laminin-5 in 30 min as evidenced by reactivity with an anti-FAK (pY397) antibody in Western blot analyses (Fig. 2A). Specifically, cells plated on laminin-5 had a 3.1-fold greater phosphorylation of FAK than cells plated on the non-specific substrate poly-L-lysine (Fig. 2C). Furthermore, hMSC adhesion to laminin-5 induced a 1.3-fold greater phosphorylation of FAK than cells plated on a purified fibronectin matrix. These results suggest that activation of FAK may be regulated by integrin-dependent adhesion and signaling events on these proteins.

The inhibition of FAK expression in hMSC by siRNA oligonucleotides did not induce cellular apoptosis in our MTT viability assays (data not shown) or inhibit cell-ECM adhesion (Fig. 1C). As a result, we next examined the effects of loss of FAK expression on integrin-dependent signaling. hMSC were transfected with 50 nM FAK siRNA for 72 h and plated on the indicated substrates in serum-deprive basal or OS medium for 30 min. Our data indicate that transfection with FAK-specific siRNA effectively decreased FAK protein levels in our cells (Fig. 2A). Furthermore, FAK knockdown cells exhibited significantly reduced FAK phosphorylation on Y397 in all experimental conditions

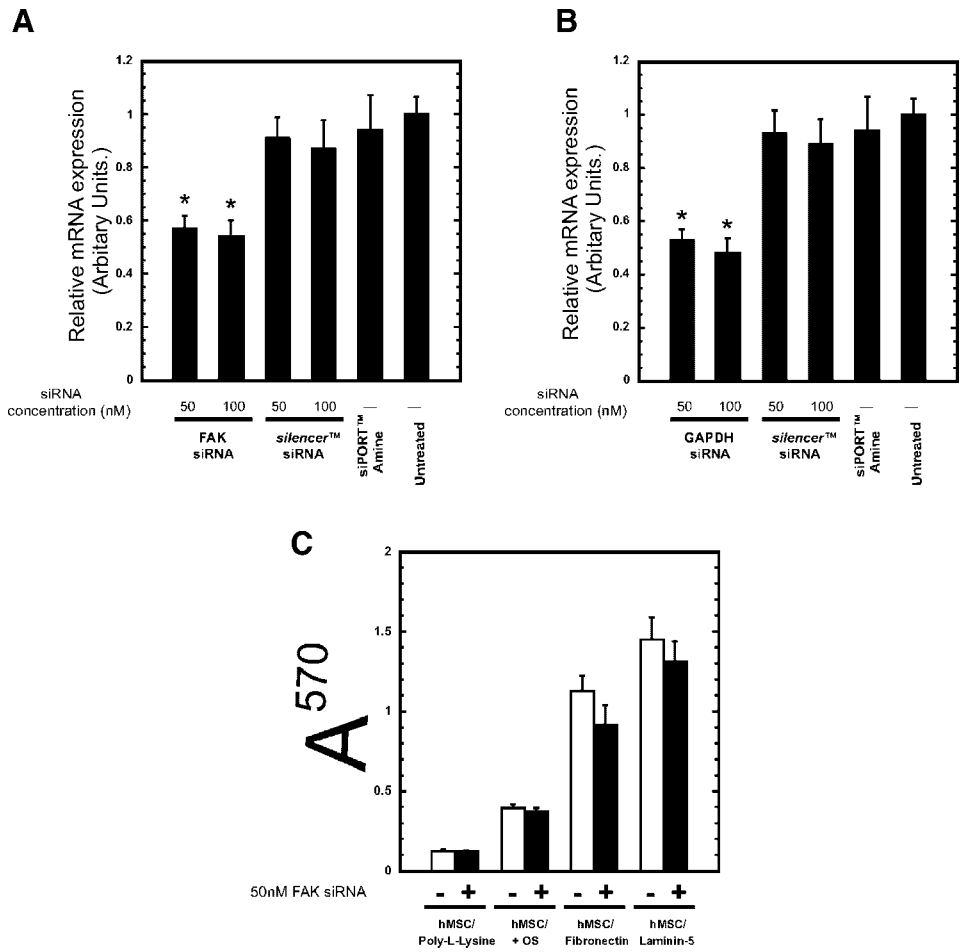

Fig. 1. siRNA-mediated knockdown in hMSC decreases FAK mRNA expression without affecting adhesion. hMSC were transiently transfected in triplicate with 50 and 100 nM of Silencer™ validated siRNA against FAK (**A**) and GAPDH (positive control) (**B**), and their respective mRNA levels assessed after 72 h using real-time RT-PCR. FAK mRNA expression is reduced more than 40% compared to cells transfected with negative control siRNA, siPORT™ Amine alone, or untreated cells. **C**: hMSC were transfected with 50 nM Silencer™ validated siRNA against FAK for 72 h then plated on poly-L-lysine in the presence or absence of OS medium, 20 μg/ml FN, or Ln-5 and allowed to adhere for 30 min. As a control, cells were allowed to adhere to poly-L-lysine with nd-blotto. Non-adherent cells were removed by washing and adherent cells were stained with crystal violet, then solubilized in SDS and absorbance determined at 570 nm. OS medium was used as a positive control for an osteogenic stimulus in this and all subsequent figures. Asterisks indicate significant ($P < 0.05$) reductions in FAK siRNA treated samples, relative to control samples, using Student' s unpaired *t*-test.

as shown in Western blot analyses (Fig. 2A). Specifically, FAK activity decreased approximately 35% on laminin-5 compared to its non-transfected counterpart (Fig. 2C). Moreover, FAK Y397 phosphorylation in hMSC plated on FN or cultured on poly-L-lysine in OS medium also exhibited a 46% and 26% decrease in activation, respectively. Several studies suggest that FAK and protein tyrosine kinase 2 (PYK2) may be in direct competition by association with limited sites in focal complexes. However, decreased levels of FAK expression did not induce a compensatory response by PYK2 signaling as evidence by a lack of increased phosphorylation on PYK2 Y402 in transfected cells plated on fibronectin or laminin-5 (Fig. 2B,D).

Inhibition of FAK Expression by siRNA Decreases Laminin-5-Induced Activation of MAPK and Runx2/Cbfa-1 Signaling Pathways

To investigate whether the inhibitory effect of FAK knockdown affects downstream components of the osteogenic signaling pathway, hMSC were transfected with 50 nM FAK siRNA and assayed as above. Knockdown of FAK expression with siRNA inhibited the activation of the MAPK pathway, a downstream target of FAK, as assessed by a Western blot against

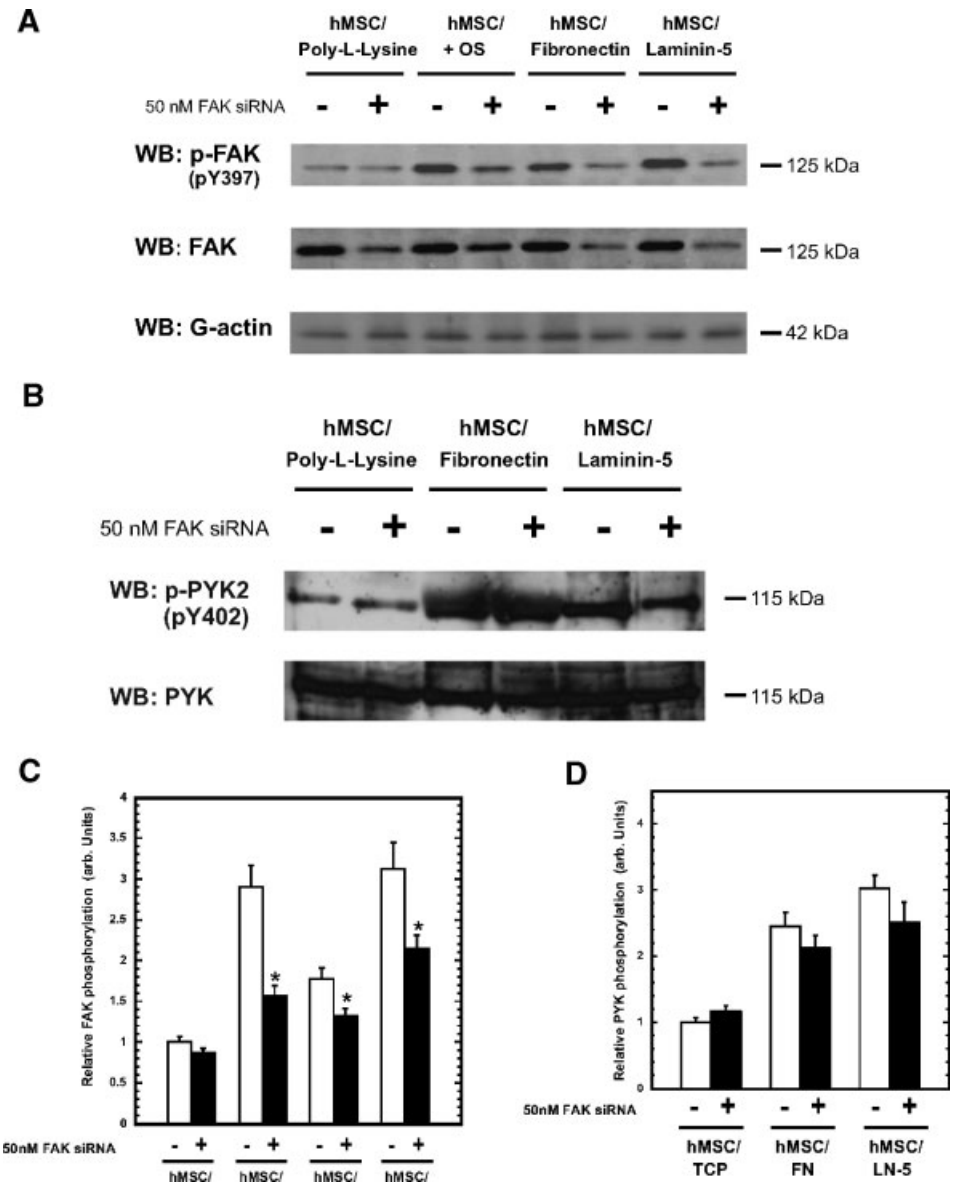

Fig. 2. Adhesion to insoluble Ln-5 induces FAK Y397 phosphorylation in hMSC and is attenuated following FAK siRNA transfection. **A**: hMSC were transfected with either 50 nM Silencer™ validated siRNA against FAK or siPORT™ Amine alone for 72 h and subsequently cultured in serum-free medium overnight. Cells were lysed and probed for activated FAK by Western blot with phospho-Y397 FAK-specific antibodies following 30 min adhesion to indicated substrates. Ratios of the densitometry of phospho-Y397 FAK to total FAK are shown in (**C**). Decreased levels of FAK expression caused by siRNA knockdown results in no compensatory response by PYK2. **B**: Cells were plated for 30 min on the indicted substrates and assayed for phosphorylated pY402 PYK as above. Densitometric measure of band intensity for PYK is shown in (**D**). All gels shown are representative of at least three experiments. Asterisks indicate significant ($P < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired *t*-test.

anti-ERK (pTpY185/197) antibody (Fig. 3A). Specifically, untransfected hMSC plated on laminin-5 for 1 h had an approximately 3.9-fold greater phosphorylation of ERK 1 and ERK 2 than cells plated on poly-L-lysine (Fig. 3B,C). However, addition of FAK siRNA oligonucleotides reduced ERK 1 and ERK 2 phosphoorylation in hMSC, demonstrating the laminin-5-induced ERK1/2 phosphorylation occurred through a FAK-dependent mechanism. FAK knockdown cells plated on laminin-5 exhibited an approximately 2.9-fold and 2.6-fold reduction of ERK 1 and ERK 2 phosphorylation, respectively, as compared to untreated cells (Fig. 3B,C). As expected, hMSC plated on poly-L-lysine and stimulated with OS medium also

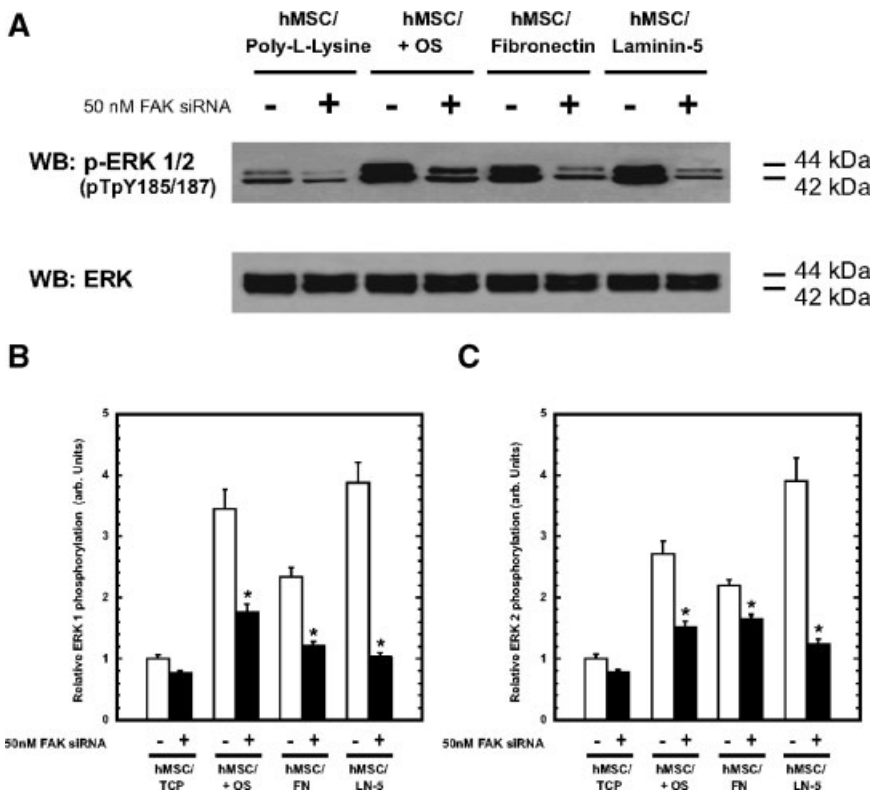

Fig. 3. Transient transfection with FAK siRNA inhibits ERK 1/2 phosphorylation in hMSC on Ln-5. **A**: hMSC were allowed to adhere for 1 h on the indicated substrates and conditions and were assayed for pTpY185/187 ERK 1/2 as per Figure 2A. Densitometric measurement of the ratios in band intensities for pTpY185/187 ERK 1/2 to ERK 1 and ERK 2 are shown in (**B**) and (**C**), respectively. ERK 1 and 2 bands were normalized to the intensity of ERK 1 and 2 in the 1 h poly-L-Lysine lane. Asterisks indicate a significant ($P < 0.05$) reduction in parallel untreated samples (white bars), using Student's unpaired *t*-test.

exhibited a high level of ERK 1 and ERK 2 activation after 1 h [Jaiswal et al., 2000]. However, siRNA transfection attenuated phosphorylation by approximately 1.7-fold and 1.2 fold, respectively. These data suggest that growth factor-induced ERK 1/2 activation in hMSC cultured in OS medium may be a FAK-dependent mediator of MAPK signaling.

Runx2/Cbfa-1 activity is regulated in part by the MAPK family in matrix-induced osteogenic differentiation of hMSC [Salasznyk et al., 2004a; Klees et al., 2005] and mature osteoblasts [Xiao et al., 2000]. To determine if upstream FAK activation plays a role in laminin-5-induced Runx2/Cbfa-1 activity, we assayed Runx2/Cbfa-1 serine and threonine phosphorylation by immunoprecipitation and Western blot analyses (Fig. 4A). Our studies indicated that laminin-5-induced a 5.3-fold and 3.7-fold increase in phospho-serine and threonine levels after 8 days, respectively, compared to cells plated on tissue culture plastic (Fig. 4B,C). Interestingly, the addition of FAK siRNA reduced this activity by 40% and 60%, respectively, compared to cells not transfected with FAK-specific siRNA. Cells plated on a purified fibronectin matrix did not induce significant serine and threonine phosphorylation of Runx2/Cbfa-1. However, OS-stimulated Runx2/Cbfa-1 activity was inhibited in FAK knockdown cells (Fig. 4). These results clearly indicate that the inhibition of FAK signaling by siRNA affects laminin-5-induced activation of ERK1/2 and Runx2/Cbfa-1 signaling pathways.

Inhibition of FAK Signaling by siRNA Knockdown Disrupts Laminin-5 Stimulated Osteogenic Gene Expression and Matrix Mineralization

To evaluate whether the induction of an osteogenic phenotype on laminin-5 was directly mediated through a FAK-dependent pathway in hMSC, we transfected cells with FAK siRNA and examined several hallmark markers defining this commitment step. In order to assess the effect of FAK signaling on osteogenic gene expression, we preformed RT-PCR analysis on

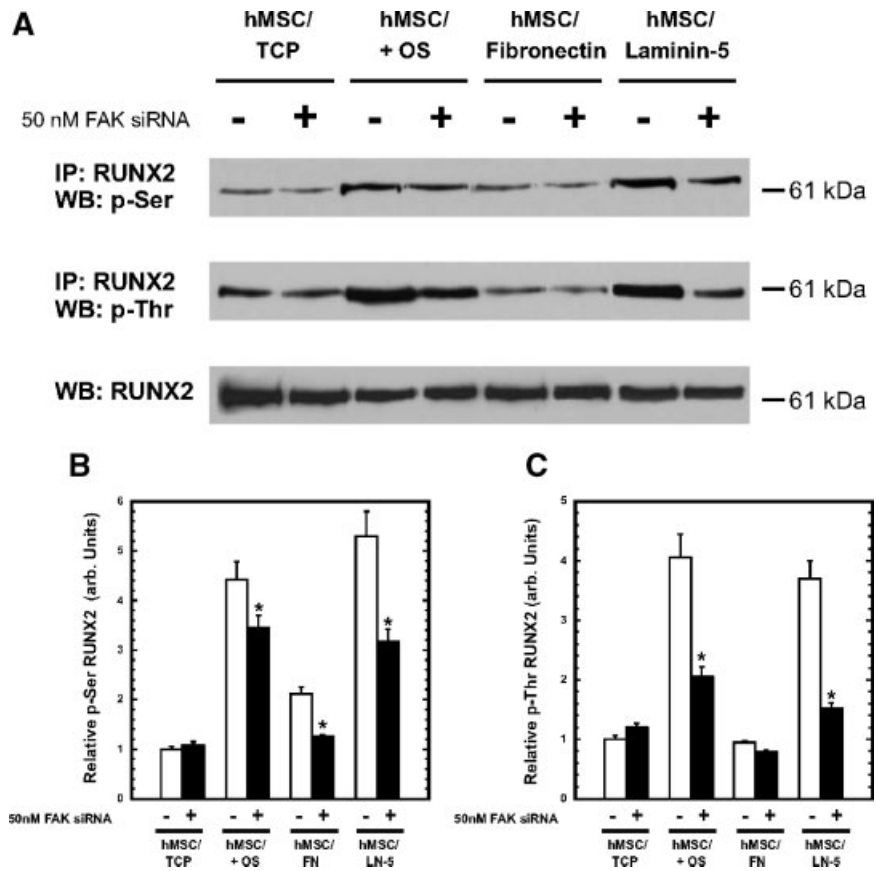

Fig. 4. FAK siRNA knockdown decreases Ln-5-induced phosphorylation of the master bone transcription factor, Runx2/Cbfa-1. **A**: hMSC were transfected with 50 nM Silencer^{TM} validated siRNA against FAK for 72 h then plated on poly-L-lysine, 20 μg/ml FN, or Ln-5 in basal media. The following day (day 0), cells were cultured in fresh control medium or OS medium and samples were incubated for an additional 8 days. Cells were subsequently lysed and Runx2/Cbfa-1 proteins were immunoprecipitated with a Runx2/Cbfa-1 specific antibody. Immunoprecipitated proteins were separated by SDS–PAGE and blotted for phosphorylated serine and threonine, which is indicated by the 61 kDa band. Total Runx2/Cbfa-1 from each lysate was detected by Western blot as a loading control. Densitometric measure of the ratio of band intensities for 61 kDa phospho-serine and threonine to total Runx2/Cbfa-1 are shown in (**B**) and (**C**), respectively. Asterisks indicate significant ($P < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired *t*-test.

hMSC plated for 14 days on various substrates in the presence or absence of FAK siRNA oligonucleotides (Figs. 5 and 6). Cells plated on laminin-5 for 14 days expressed higher levels of bone sialoprotein (Fig. 5B), osteocalcin (Fig. 5C), and osterix (Fig. 5E) mRNA compared to cells plated on tissue culture plastic or purified fibronectin. However, OS and laminin-5-induced osteogenic gene expression was inhibited in FAK knockdown cells. Runx2/Cbfa-1 gene transcription was unchanged in all experimental conditions (Fig. 5D). These results suggest that although FAK expression and signaling is not sensitive to Runx2/Cbfa-1 transcriptional activity, it plays a critical role in its post-translational activation state. Expression levels of novel transcriptional markers for osteogenesis were also assessed by RT-PCR (Fig. 6). Laminin-5 was a potent activator of MEPE, DSPP, and DMP-1 transcriptional expression following 14 day culture. However, FAK knockdown cells plated on laminin-5 expressed decreased mRNA transcriptional levels for these specific osteogenic markers (Fig. 6B–D).

Hydroxyapatite crystals formed by the accumulation of phosphate ions produced by alkaline phosphatase activity and calcium into the extracellular space are the quintessential markers for osteogenesis. In order to investigate whether the inhibitory effect of FAK knockdown mediates laminin-5-induced alkaline phosphatase activity and calcium deposition, we transfected hMSC with FAK-specific siRNA and assayed as per mentioned above. FAK knockdown cells inhibited laminin-5-induced alkaline

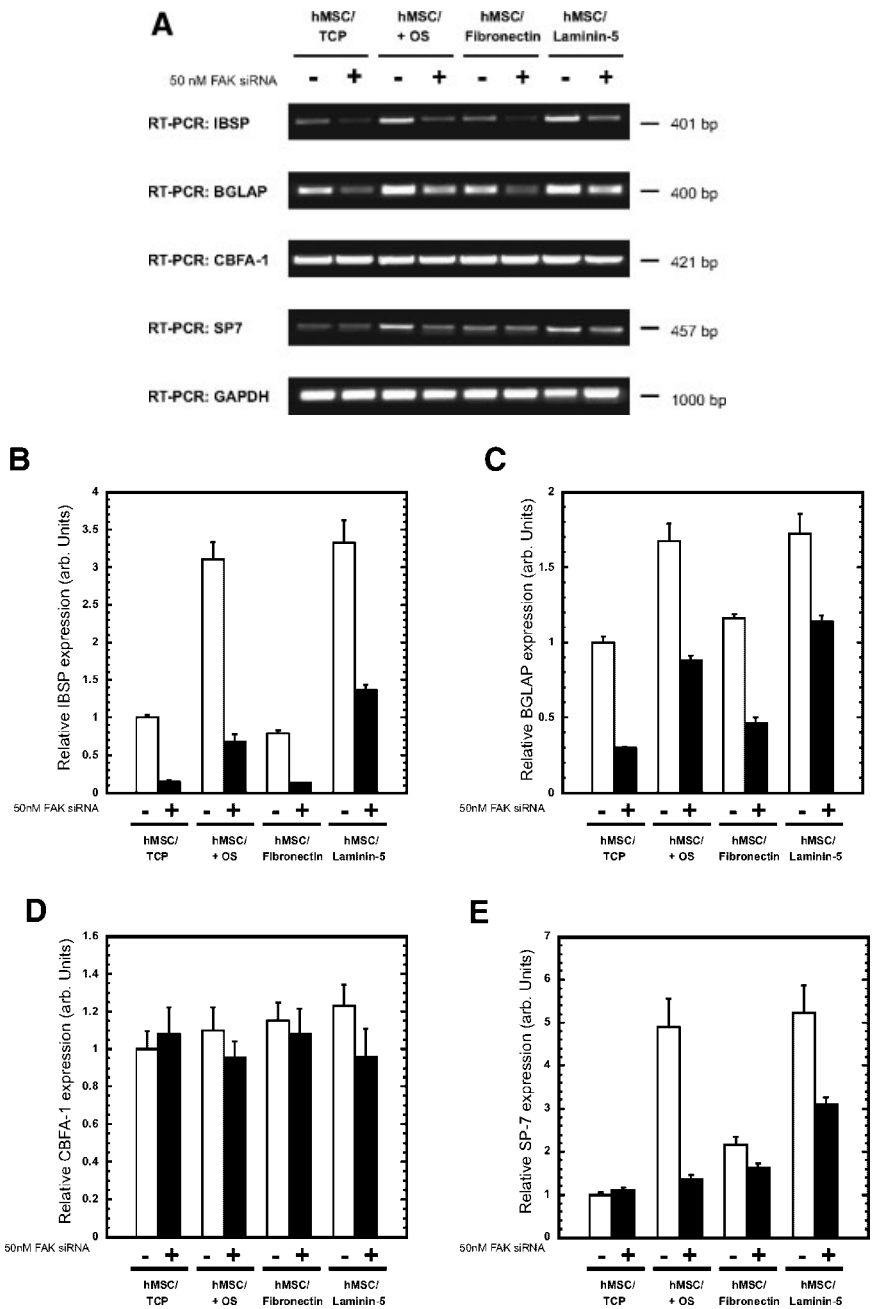

Fig. 5. Osteogenic differentiation of hMSC is sensitive to FAK siRNA knockdown on Ln-5. **A**: hMSC were plated under the same conditions as in Figure 4 and total RNA was isolated following 14 days in culture. RT-PCR was performed on each sample using primers specific for the amplification of the indicated genes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as a loading control. Densitometry of band intensities for bone sialoprotein, osteocalcin, Cbfa-1, and osterix are given in (**B**–**E**), respectively.

phosphatase activity after 14 days as shown by histological staining for the enzyme (Fig. 7A,B). Furthermore, calcium deposition measured by Alizarin Red histological staining and by a colorimetric assay (Fig. 7C,D) demonstrated that untransfected cells plated on laminin-5-induced significant increases in calcium deposition compared to cells plated on tissue culture plastic or fibronectin. However, this increase was eliminated in FAK knockdown cells. Moreover, although OS medium induced the greatest increase of calcium accumulation into the extracellular space after 21 days, transfection with FAK siRNA attenuated these results.

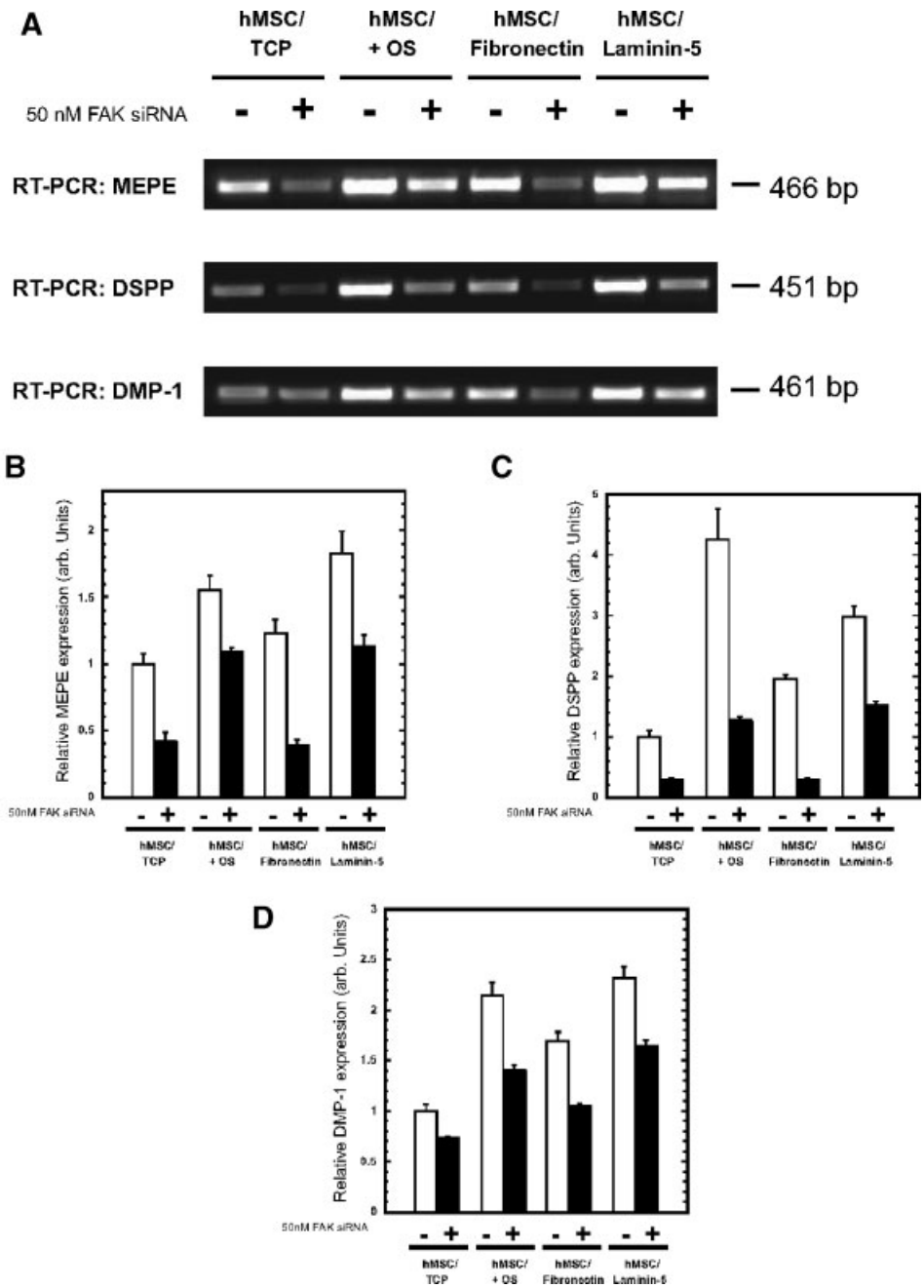

Fig. 6. Osteogenic-specific gene expression of hMSC is FAK dependent on Ln-5. **A**: Following 14 days in culture, total RNA was isolated and RT-PCR was performed for the expression of MEPE, DSPP, and DMP-1. GAPDH was amplified as a loading control and the densitometry of band intensities are given in (**B**–**D**), respectively.

Lastly, we assessed differences in ossification of our untreated controls to siRNA transfected cells by determining the mineral:matrix content of the secreted ECM. We found that FAK knockdown cells exhibited a statistically significant decrease in their mineral:matrix ratio compared to non-transfected control cells (Fig. 8). Representative FTIR spectra of FAK knockdown cultures revealed decreased mineral content compared to their parallel controls (Fig. 8A–D). These results clearly indicate that laminin-5-induced FAK activation stimulates ERK 1/2 and Runx2/Cbfa-1 signaling cascades, and plays a critical role in promoting osteogenic gene expression and subsequent matrix mineralization.

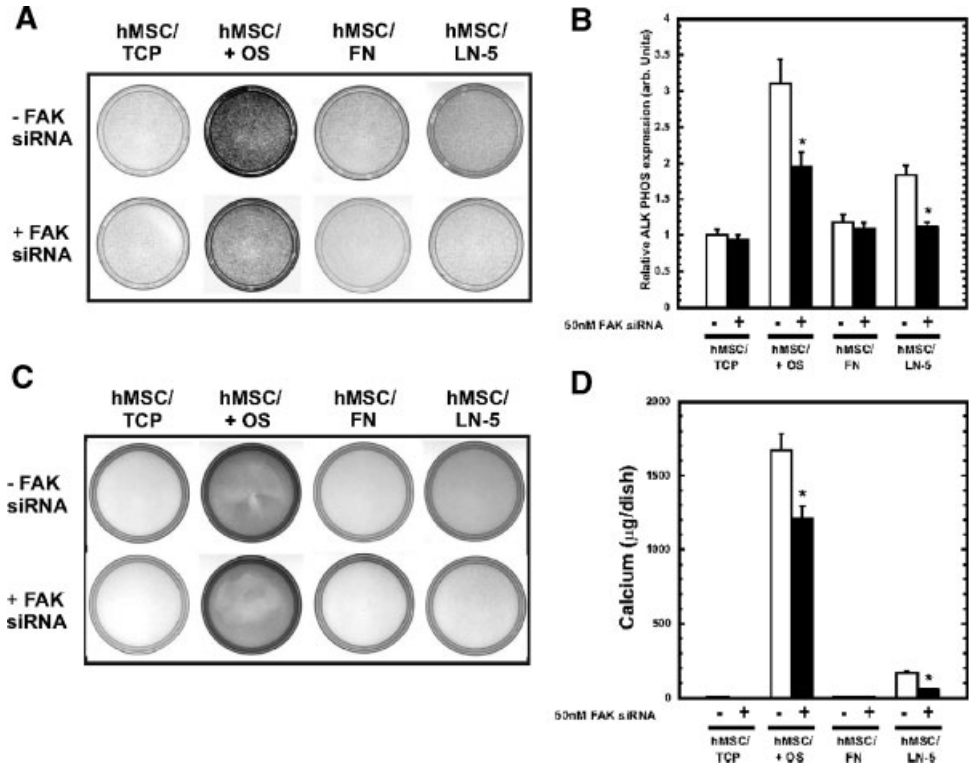

Fig. 7. FAK siRNA knockdown decreases the osteogenic-specific marker expression of alkaline phosphatase and a calcified matrix in hMSC plated on Ln-5. hMSC were plated for 14 or 21 days under the same conditions as in Figure 4, then stained for alkaline phosphatase activity (b) or calcium with Alizarin Red (**C**), respectively. The top and bottom rows indicate cultures transfected in the absence and presence of 50 nM validated Silencer ™ FAK siRNA, respectively. Cells expressing alkaline phosphatase and calcified matrix appear dark in cell culture plates. **B**: Densitometry of plates shown in **panel A**. Asterisks indicate significant ($P < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired *t*-test. **D**: Colorimetric assessment of total calcium in hMSC. Cells were plated for 21 days as for panel C, then lysed and total calcium assayed as described in Materials and Methods. Asterisks indicate significant ($P < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired *t*-test.

DISCUSSION

Collagens are the most abundant proteins in the human body [Wieczorek et al., 2000; Gullberg and Lundgren-Akerlund, 2002; Kotch and Raines, 2006], accounting for nearly a quarter of protein mass. Of these, Collagen-I (Col-I) is most common, being found in nearly every tissue [Westermarck et al., 1995; Gutierrez and Perr, 1999; Kjaer, 2004]. Specificity within the ECM of tissues therefore results not only from differences in collagen structure and assembly, but also from the non-collagenous constituents in each tissue. For example, in healthy bone, Col-I represents ~95% of the protein mass [Datta et al., 2006] such that the remaining 5%–10% of ECM proteins, including Laminin-5, play a critical role in determining the distinct function and organization of bony tissues, and between individual bones in a single organism.

How these non-collagenous ECM proteins contribute to bone formation is poorly understood. Our results suggest that Ln-5 upregulates the expression level of numerous proteins found in the bone microenvironment and which play necessary roles for bone formation and function. Some of these are members of the Small Integrin-Binding LIgand, N-linked Glycoprotein (SIBLING) family of proteins, including osteopontin, bone sialoprotein, osteocalcin, DMP-1, DPP, and MEPE.

Though DMP-1 knockout mice lack a distinct skeletal phenotype [Feng et al., 2003], DMP-1 is essential for osteocyte maturation [Rios et al., 2005]. DMP-1 expression is associated with mineralizing tissues; being expressed by hypertrophic chondrocytes, osteoblasts, and odontoblasts [D'Souza et al., 1997]. DMP-1 has high affinity for calcium and hydroxyapetite (HA) and may function as both a regulator of bone

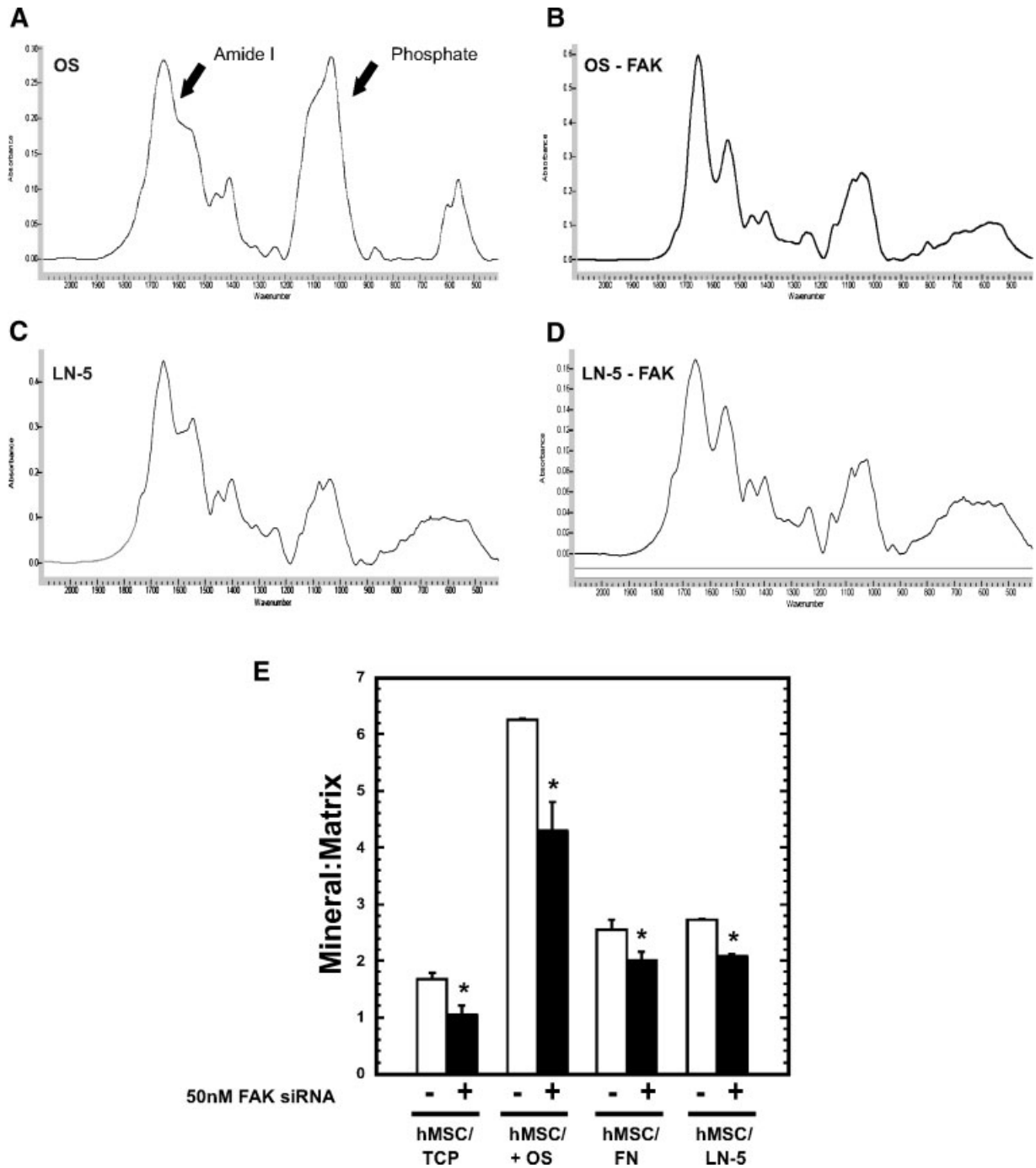

Fig. 8. FAK siRNA knockdown decreases Ln-5-induced matrix mineralization in hMSC. Representative FTIR analysis of hMSC transfected without or with 50 nM validated Silencer™ FAK siRNA plated on tissue culture plastic cultured with OS medium (**A**, **B**), and Ln-5 (**C**, **D**) for 28 days. The mineral:matrix (number) is computed by comparing the area of the phosphate peak (mineral) to the amide peak (protein) shown. **E**: Osteogenic differentiation of hMSC plated on indicated substrates for 28 days, as assessed by indicated measurements of mineral to matrix ratio. By comparison, fully differentiated human osteoblasts cultured on tissue culture plastic for 21 days have a ratio of approximately 5.4. Asterisks indicate significant ($P < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired *t*-test.

mineralization and as a signal transducer [Tartaix et al., 2004]. C3H10T1/2 and MC3T3-E1 cells expressing antisense RNA to DMP-1 exhibit decreased levels of the osteogenic marker proteins alkaline phosphatase and osteocalcin, while transfection of these cells with DMP-1 cDNA results in the appearance of an odontoblastic phenotype [Narayanan et al.,

2001]. Introduction of FAK siRNA decreases Ln-5-induced expression of DMP-1 by ~20% in our cells, demonstrating that FAK links Ln-5 binding to DMP expression.

A second, bone-specific SIBLING protein induced by Ln-5 is DSPP [Qin et al., 2002]. DSPP is cleaved into two distinct peptides, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP, also known as phosphophoryn), that play separate roles in osteogenesis [MacDougall et al., 1997]. DPP is highly phosphorylated, and thus has a high affinity for calcium, a trait similar to most SIBLING proteins. DPP induces osteogenesis via the well-known MEK-ERK MAPK pathway [Jadlowiec et al., 2004, 2005]. We have shown that Ln-5 activates this pathway directly [Klees et al., 2005], and in this study, we establish that FAK lies upstream of MEK-ERK. It is possible, then, that because they share common components of a signaling pathway, that Ln-5 and DPP act cooperatively in inducing osteogenesis in hMSC.

Ln-5 binding also induces expression of MEPE, another SIBLING protein that binds calcium and HA. MEPE is highly expressed in osteoblasts, and has both pro- and anti-ostoegenic activity. Mice that lack MEPE have increased bone mass as well as an increased osteoblast population [Gowen et al., 2003], yet a short peptide fragment of MEPE (containing both an RGD sequence and a glycosaminoglycan binding region) increases osteogenesis through a FAK-ERK pathway [Hayashibara et al., 2004]. It is possible that MEPE and Ln-5 act synergistically, in that both bind heparin sulfate [Utani et al., 2003] and utilize a FAK-ERK to stimulate osteogenesis.

Ln-5 has significant effects on expression of non-SIBLING proteins as well. For example, our data demonstrate that adhesion to Ln-5 stimulates expression of the bone gene osterix. Mesenchymal stem cells from osterix null mice fail to produce osteoid and cannot differentiate into mature osteoblasts [Nakashima et al., 2002]. Not only does osterix turn on osteogenic genes, but it also negatively regulates genes that induce chondrogenesis [Tai et al., 2005], and recent studies by Miyazaki and colleagues have shown that Ln-5 suppresses chondrogenic differentiation [Hashimoto et al., 2005]. This proposes a possible role of Ln-5 at the point when vascularization of the calcified cartilaginous matrix occurs and hMSC begin to differentiate into osteoblasts. Ln-5 activation of osterix may therefore help trigger the switch from chondrogenesis to osteogenesis in these stem cells. Finally, Ln-5 also stimulates the phosphorylation of Runx2, another osteogenic transcription factor that appears to act upstream of osterix [Kim et al., 2006]. That Ln-5 stimulates both Runx2 activation and osterix expression suggests it is capable of inducing osteoegensis; that these events are inhibited by knockdown of FAK shows that Ln-5 is acting via FAK to promote osteogenic differentiation in vitro.

These results, therefore, suggest that knockdown of FAK should interfere with bone formation in vivo. Complete knockout of FAK results in embryonic lethality in mice [Peng et al., 2006]. Instead, we turned to heterozygous FAK knockout mice, which express 50% less FAK than normal mice (kindly provided by Dr. Dusko Ilic at University of California, San Francisco). Faxitron analysis of bone density revealed variation between these mice and control mice, and microCT analysis of individual bones confirmed these findings. However, no statistically significant differences between heterozygotes and control animals were found (data not shown).

These findings are not as surprising as they may appear at first. Instead, how FAK functions in vivo may be far more complicated than our in vitro studies suggest. For example, it is well established that FAK plays a significant role in cell migration in vitro. However, keratin 14-Cre [McLean et al., 2004] or keratin 5-Cre [Essayem et al., 2006] keratinocyte-specific FAK knockout mice exhibit no differences in wound healing compared to control mice. Moreover, keratinocytes taken from these animals do not survive in vitro, but are quite viable in vivo. This suggests that some unknown factors act exclusively in vivo to maintain cellular viability and function in haploinsufficient mice. Similarly, our in vitro siRNA treatment achieved 43%–45% (±6%) knockdown, and we observed a dramatic loss of osteogenic differentiation, presumably because these unknown factors are not present in our cultures. Thus, it is possible that functionally redundant signaling mechanisms triggered by these unknown factors may be able to accommodate FAK knockout in some tissues, including bone and skin.

Overall, our results suggest an important role for FAK in the early commitment steps of hMSC differentiation along the osteoblastic

lineage, induced by either soluble OS factors or laminin-5.

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