Published in final edited form as:

Exp Cell Res. 2007 January 1; 313(1): 22–37.

Focal adhesion kinase signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells

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Abstract

The intracellular signaling events controlling human mesenchymal stem cell (hMSC) differentiation into osteoblasts are not entirely understood. We recently demonstrated that contact with extracellular matrix (ECM) proteins is sufficient to induce osteogenic differentiation of hMSC through an ERK-dependent pathway. We hypothesized that FAK signaling pathways provide a link between activation of ERK 1/2 by ECM, and stimulate subsequent phosphorylation of the Runx2/Cbfa-1 transcription factor that controls osteogenic gene expression. We plated hMSC on purified collagen I (COLL-I) and vitronectin (VN) in the presence or absence of FAK-specific siRNA, and assayed for phosphorylation of Runx2/Cbfa-1 as well as expression of established osteogenic differentiation markers (bone sialoprotein-2, osteocalcin, alkaline phosphatase, calcium deposition, and spectroscopically determined mineral:matrix ratio). We found that siRNA treatment reduced FAK mRNA levels by >40% and decreased ECM-mediated phosphorylation of FAK Y397 and ERK 1/2. Serine phosphorylation of Runx2/Cbfa-1 was significantly reduced after 8 days in treated cells. Finally, FAK inhibition blocked osterix transcriptional activity and the 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 suggest that FAK signaling plays an important role in regulating ECM-induced osteogenic differentiation of hMSC.

Keywords

Human mesenchymal stem cells; focal adhesion kinase; osteogenic differentiation; extracellular matrix

Introduction

Bone-marrow derived human mesenchymal stem cells (hMSC) are a population of self-renewing, multipotent cells that have significant clinical potential in cellular therapies and tissue regeneration. These cells are able to differentiate along several committed phenotypes including osteogenic [1–3], chondrogenic [4], adipogenic [5], cardiogenic [6], and neurogenic [7,8] lineages in response to stimulation by multiple environmental factors.

Cell contact with extracellular matrix (ECM) proteins plays a critical role in regulating hMSC osteogenesis [3,9–11]. Specifically, this interaction triggers osteoblast-specific expression of alkaline phosphatase and osteocalcin mRNAs, and ultimately, mineralization of bone tissue in

a stage-specific sequence [12,13]. Matrix responsiveness during osteogenesis is caused by the aggregation of the $\alpha 2\beta 1$ [9,14–16] and $\alpha v\beta 3$ [17] integrins that subsequently activate intracellular signaling cascades, resulting in the phosphorylation of the osteoblast-specific transcription factor Runx2/Cbfa-1 [15,18,19]. Understanding the molecular signaling mechanisms associated with these events and the osteogenic differentiation of hMSC as a whole, will accelerate the practical application of stem cell engineering.

In virtually all cell types, integrin binding leads to activation of focal adhesion kinase (FAK) [20,21]. FAK disseminates integrin signals by forming complexes with signaling proteins rich in SH2 domains [22]. Association of FAK with src leads to activation of extracellular signal-related kinase (ERK) via the Grb2-Sos-Ras pathway [22,23]. How these pathways are organized in developing bone is not well known, though osteoblasts in osteoporosis or osteoarthritis patients have reduced FAK activity [24]. Inhibition of FAK by herbimycin A, cytochalasin D, or overexpression of antisense FAK mRNA prevents the expression of ERK-dependent alkaline phosphatase activity in osteoblast-like cell lines, consequently preventing further osteogenic differentiation [14]. Despite these observations, the role of FAK signaling in controlling hMSC commitment to the osteogenic phenotype, rather than maturation of committed osteoblasts, has not been examined.

The central regulation of bone differentiation and formation is controlled by the transcriptional activity of Runx2/Cbfa-1. Essential during the osteogenesis of mesenchymal progenitor cells, homozygous deletion of Runx2/Cbfa-1 in mice results in the complete absence of osteoblasts and bone [25,26]. Post-translational modifications and protein-protein interactions play an integral role in regulating this transcription factor [6]. However, the kinases and the specific amino acid residues responsible for the activation of Runx2/Cbfa-1 transcriptional activity are not fully known. Members of the mitogen-activated protein kinase (MAPK) family, including ERK, play a role in controlling Runx2/Cbfa-1 activity in differentiated osteoblasts [18]. Bone-like cells expressing constitutively active MEK, which controls ERK activity, increases expression of the bone protein osteocalcin. In hMSC, pharmacological inhibition of MEK diminishes ECM-induced phosphorylation of Runx2/Cbfa-1 and subsequent osteogenic differentiation [10,11]. Whether FAK controls MEK-activated MAPK signals during osteogenic differentiation in these cells is not known.

Osterix is a transcription factor that is essential for the development of a mature osteogenic phenotype [27]. Genetic knockout of either osterix or Runx2/Cbfa-1 in mice results in the absence of differentiated osteoblasts and no bone formation, but osterix-null mutants express typical chondrocyte marker genes and form cartilaginous tissue [27] while maintaining normal Runx2/Cbfa-1 transcription. Runx2/Cbfa-1 knockout mice lack any osterix transcripts, suggesting that osterix lies downstream of Runx2/Cbfa-1 [27]. Chondrogenic differentiation of mesenchymal progenitor cells requires Runx2/Cbfa-1 activity [28] but not osterix, suggesting that Runx2/Cbfa-1 may be the key regulator of hMSC commitment along the osteogenic or chondrogenic lineages. MAPK signaling pathways are linked to osterix expression in hMSC [29], but the role of FAK signaling in regulation of osterix is entirely unknown.

This study was undertaken to explore the roles of FAK signaling in matrix-induced osteogenic differentiation of hMSC. We present data indicating that FAK activation via the formation of focal adhesions is essential and represents one of the earliest decision making events controlling hMSC commitment to the osteogenic phenotype.

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, poly-L-lysine (cat. # P4832), and purified bovine type I collagen (cat. # C8918) were obtained from Sigma Chemical Co. (St. Louis, MO). Purified human plasma fibronectin (cat. # FC010) and vitronectin (cat. CC080) were from Chemicon International (Temecula, CA). Chick embryo fibroblasts +/- FAK cells (cat. #55-100) were from Biosource International (Camarillo, CA). Rabbit polyclonal IgG anti- G-actin (cat. #AAM01-A) antibody was from Cytoskeleton (Denver, CO). Rabbit polyclonal IgG phospho-specific antibodies against anti- FAK (pY³⁹⁷) (cat. # 44-624G), ERK 1/2 (pTpY^{185/187}) (cat. #44-680G), and Pyk2 (pY⁴⁰²) (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 (Camarillo, CA). Mouse monoclonal IgG antibodies against anti- Pyk2/CAK β (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) and phosphoserine (cat. #AB1603) were purchased from Chemicon International (Temecula, CA). Mouse monoclonal IgG antibodies against anti- Runx2/Cbfa-1 were obtained from MBL International (Watertown, MA). Goat polyclonal IgG antibodies against endoglin (SC-19790) and osteocalcin (SC-18319) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexafluor phalloidin 488 (A-12379) was purchased from Molecular Probes (Eugene, OR). 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 1 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 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/cm² in a T75 flask (75 cm²) for continuous passaging in growth medium (DMEM supplemented with 10% FBS, 1% L-glutamine [29.2 mg/mL], penicillin G [10,000 units/mL] and streptomycin sulfate [10,000 μ g/mL]). Growth 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 induced and plated at densities of 3.1×10^3 cells/cm² in 0.2 mL/cm² of medium on 100 mm Falcon dishes (78.5 cm²). The following day (day 0), we replaced the growth medium with fresh growth medium in the absence (control) or presence of osteogenic supplements (OS: 0.1 μ M 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. For inhibition of MEK 1, PD98059 in DMSO (Calbiochem, San Diego, CA) was added to control and OS mediums at a final concentration of 50 μ M twice in the 8 day period. The final concentration of DMSO never exceeded 0.1 % and the same amount of DMSO vehicle was added to control conditions. Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂.

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 cm²) 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 (no FBS) for 10 min and subsequently mixed with siRNA diluted in serum-deprived DMEM (no FBS) for an additional 10 min to allow transfection complexes to form. Transfection complexes at a final siRNA concentration of 50 µM or 100 µM were dispensed onto the cells and assayed 72 h after transfection. For in vitro osteogenic assays, transfected cells were re-plated 72 h after transfection on tissue culture plastic (± OS), FN, COLL-I, or VN in fresh DMEM medium (10% FBS + 1% GPS) and assayed at 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 Ambion's validated Silencer™ negative control #1 siRNA (cat. #4611) and validated Silencer™ GAPDH siRNA control (cat. #4605) to demonstrate nonspecific effects of gene silencing and to confirmed the transfection procedure, respectively. Non-specific Silencer™ negative control #1 siRNA was incorporated into our osteogenic assays to observe FAK-mediated changes in our cells.

Reverse transcriptase PCR (RT-PCR)

For visual confirmation of siRNA knockdown and 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 after 72 h or tissue culture plastic (± OS), FN, COLL-I, or VN in control media for 14 days, respectively. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). 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 1, which were designed by the Lasergene v5.0 program (DNASTAR, Madison, WI). 0.5 µg of template RNA 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 Chemilmager 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 β-Tubulin or GAPDH values.

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 2X QuantiTect SYBR Green Mix, 0.5 µl of QuantiTect RT Mix, 1 µl of primer found in Table 1, 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 sec denaturation at 94°C, 30 sec of annealing at 58°C, and one 30 sec of extension at 72°C. Samples were assayed in triplicate, and the values were normalized to the relative amounts of β-Tubulin.

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, 1X 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 5000 rpm for 30 sec to discard all the non-labeled proteins. Denatured Runx2 protein was eluted after the addition of 70 µl of 1X denaturing elution buffer containing β-ME and centrifugation. Proteins were diluted in 5X 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 1X PBS + 0.2% Tween-20 (PBST) or 5% BSA in PBST for pY³⁹⁷ 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.

MTT viability assays

Cellular viability assays were performed using Sarstedt 96-well suspension cell culture plates. Briefly, hMSC were plated at a density of 5×10^3 cells per well in control medium and incubated overnight. The following day, cells were supplied with fresh medium containing 5mg/ml of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) and incubated for 4 h at 37°C. After aspiration, 100 µl of 10% SDS was added to each well and then the plates were placed back into the incubator for an additional 2 h. Subsequently, absorbance was measured using a TECAN SPECTAFluorspectrophotometer at 570 nm and viability was expressed relative to the untreated control (no siRNA or siPORT™ Amine).

Adhesion assays

Cell adhesion assays were performed as previously described using Sarstedt 96-well suspension cell culture plates [30]. Tissue culture plates were coated with purified ECM proteins (FN, COLL-I, or VN) 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-dairy 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.

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 dH₂O 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 dH₂O five times and left in PBS for 15 min. Cellular specimens were scored according to the quantity and size of precipitated granules.

Immunohistochemistry

hMSC were grown on glass coverslips coated with Collagen-I for 5 h, fixed with acetone, and blocked with PBS/1% bovine serum albumin (BSA) for 30 min. Primary goat antibody for endoglin was diluted 1:200 in 1% PBS _ 1% BSA and added to cells for 1 h along with FITC conjugated phalloidin. TRITC secondary anti-goat antibody was added for 1 h and cover slips were mounted using Prolong antifade medium (Molecular Probes). Cells were visualized with a Nikon TE2000-S inverted fluorescence/phase contrast microscope (Garden City, NY) equipped with a digital SPOT camera.

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, USA) as previously described [31]. The mineral content was calculated based on the spectrally derived mineral-to-matrix ratio (the integrated areas of the phosphate absorbance (900–1200 cm^{-1}) and protein amide I band (1585–1720 cm^{-1})).

Retrovirus Construction and hMSC Transduction

Retroviruses for transduction of hMSC were produced using the MSCV retroviral expression system purchased from BD Biosciences Clontech (Palo Alto, CA). The genes coding for c-myc epitope-tagged FAK and FAK Y397F were PCR-amplified from the pRC/CMV-c-myc FAK expression plasmids kindly provided by Dr. Steven K. Hanks [44] and ligated into the pMSCVneo expression vector as restriction fragments at the *HpaI*-to-*BglII* site. The mouse fibroblast retroviral packaging cell line, PT67, was transfected with the pMSCVneo/c-myc-FAK plasmids using the TransIT-3T3 transfection kit purchased from Mirus Corp. (Madison, WI). Clonal, G418-resistant, transfected PT67 cell lines were screened for multiplicity of infection (MOI) in NIH 3T3 cells. Two cell lines were identified, one containing the exogenous c-myc-FAK wild type gene and one containing an exogenous c-myc-FAK F397 gene, each of which had a MOI in NIH 3T3 cells of 2000–10,000 per mL of virus containing supernatant. The same viral supernatants produced a MOI in hMSC of ~10 per mL. Stems cells were infected with retrovirus by duplicate 24 hour-exposures to viral supernatant containing polybrene (8 $\mu\text{g/mL}$) with an intermediate 24 hour exposure to virus-free hMSC medium. Two polyclonal populations of $\sim 4 \times 10^6$ G418-resistant hMSC ($\sim 760 \mu\text{g/mL}$ active G418 in hMSC medium) were isolated after retroviral infection. Subsequently, these cells and cells from the same lineage but not exposed to viral supernatant (i.e., the control group) were plated in 10-cm tissue culture dishes and glass coverslips with or without collagen I coating. Collagen I coating consisted of exposing 10-cm tissue culture dishes and glass coverslips to calf skin type I collagen (Sigma Chemical Co., St. Louis, MO) at 20 $\mu\text{g/mL}$ in phosphate-buffered saline for 1 h at 37°C prior to plating cell.

Plasmids pRC/CMV-c-myc-FAK and pRC/CMV-c-myc-FAK F397 were kindly provided by Dr. Steven Hanks(steve.hanks@Vanderbilt.Edu)

Statistical analysis

All experiments were repeated a minimum of two times and the representative data were presented as mean $\pm$ S.E where indicated. Statistical analyses were performed using Student's unpaired *t* test, and a *p* value less than 0.05 was considered significant.

Results

Inhibition of FAK expression following siRNA transfection

To provide a direct comparison of hMSC properties before and after the loss of FAK expression, siRNAs specific for FAK were transfected into hMSC using siPORT™ Amine at a final concentration of 50 nM and 100 nM. Our titration studies indicated that siRNA-mediated knockdown of FAK mRNA and protein expression was maximal following a 72 hour transfection compared to 24 or 48 hours (data not shown). Transfection with FAK siRNAs resulted in a significant decrease of endogenous FAK mRNA expression (Fig. 1A, top panel) after 72 h as detected by RT-PCR. To demonstrate the nonspecific effects of gene silencing and to confirm the transfection procedure, hMSC were also transfected with validated Silencer™ negative control #1 siRNA and validated Silencer™ GAPDH siRNA control, respectively. As expected, transfection with validated Silencer™ negative control #1 siRNA had no effect on FAK or GAPDH mRNA expression (Fig. 1A, top and middle panels). However, incubating hMSC with validated Silencer™ GAPDH siRNA resulted in a significant decrease of endogenous GAPDH mRNA expression (Fig. 1A, middle panel) after 72 h, confirming the efficacy of the transfection system. As anticipated, none of the transfected siRNAs decreased the expression of endogenous β -tubulin levels (Fig. 1A, bottom panel).

The loss of endogenous FAK mRNA expression following 72 h incubation with validated Silencer™ FAK siRNA was also quantitatively analyzed by real-time RT-PCR. Cells treated with FAK siRNA exhibited a 40% reduction in endogenous FAK mRNA as compared to cells transfected with negative control siRNA, siPORT™ Amine alone, or untreated cells (Fig. 1B). Furthermore, there was no significant decrease in FAK expression found between cells treated with a final concentration of 50 nM or 100 nM FAK siRNA. To confirm our transfection procedure, hMSC transfected with GAPDH siRNAs exhibited an approximately 50% decrease in endogenous GAPDH mRNA expression compared to cells transfected with the negative control siRNA, siPORT™ Amine alone, or untreated cells (Fig. 1C). Western blot analysis of whole cell lysates treated for 72 h with FAK and negative control siRNAs further verified the results from real-time RT-PCR (Fig. 1D).

To examine the additional effects of FAK inhibition by siRNA transfection, we analyzed the survival and dynamics of FAK knockdown cells as they adhered to extracellular matrix (ECM) proteins and spread. Cells transfected with FAK siRNA were viable (Fig. 2A) and sustained no appreciable loss of adhesion to ECM proteins compared to hMSC treated with siPORT™ Amine vehicle alone for 30 min (Fig. 2B). Furthermore, cells were assayed for cell spreading and cytoskeleton by plating FAK siRNA transfected cells or cells treated with siPORT™ Amine alone on COLL-I for 5 h (Fig. 2C,D, respectively). Immunofluorescent microscopy exhibited that FAK siRNA-treated cells exhibited delayed spreading after a half an hour adhesion to COLL-I (data not shown), but spread to the same extent as the control cells after 5 h.

Adhesion to COLL-I and VN induces FAK Y397 phosphorylation and is reduced following FAK siRNA transfection

FAK phosphorylation of Y397 occurred in hMSC plated on purified COLL-I or VN for 30 min, as evidenced by reactivity with an anti- FAK (pY³⁹⁷) antibody in an immunoblot (Fig. 3A). Specifically, cells plated on COLL-I and VN had a 2.5-fold and 3-fold greater phosphorylation of FAK, respectively, than cells plated on the non-specific substrate poly-L-lysine (Fig. 3C). Moreover, FAK phosphorylation levels on Y397 increased an additional 1.3-fold and 2.5-fold, respectively, in cells plated on COLL-I and VN for an additional 30 min, but did not change in cells plated on poly-L-lysine.

To assess the activation of Y397 signaling following FAK knockdown by siRNA, hMSC were transfected with 50 nM FAK siRNA for 72 h and plated on the indicated substrates in serum-deprived basal or OS medium for 30 min. FAK knockdown cells exhibited significantly reduced FAK expression (~55%) and FAK phosphorylation on Y397 in all conditions as shown in immunoblot analysis (Fig. 3B). Specifically, FAK activity decreased approximately 2.1-fold and 2.5-fold on COLL-I and VN, respectively, compared to non-transfected cells plated in these conditions (Fig. 3D). Furthermore, FAK Y397 phosphorylation in hMSC plated on FN or cultured on poly-L-lysine in OS medium also exhibited a drop in activation compared to their non-transfected counterparts.

These reductions exhibited in FAK expression and activity did not trigger a compensatory response by PYK2 signaling as evidenced by a lack of increased phosphorylation on PYK2 Y402 when plated in the same conditions described above (Fig. 4A,B).

Knockdown of FAK by siRNA inhibits COLL-I and VN-induced activation of ERK1/2 and Runx2/Cbfa-1 in hMSC

To assess the role of ECM-induced FAK signaling in the activation of ERK 1/2, hMSC transfected with 50 nM FAK siRNA were assayed as above and immunoblotted with anti-ERK 1/2 (pTpY^{185/187}) antibody to assess activation (Fig. 5A). Specifically, cells plated on COLL-I and VN for an h had approximately 4.1-fold and 5.6-fold greater phosphorylation of ERK 1, respectively, than cells plated on the non-specific substrate poly-L-lysine (Fig. 5B). Similarly, ERK 2 phosphorylation levels were increased 2.4-fold and 3.1-fold, respectively, on these substrates (Fig. 5C). However, addition of FAK siRNA reduced ERK 1 and ERK 2 phosphorylation in all conditions (Fig. 5), demonstrating that ECM-induced ERK phosphorylation occurred through a FAK signaling pathway. In particular, hMSC transfected with FAK siRNA exhibited a significant 3.3-fold and 3.8-fold reduction of ERK 1 phosphorylation on COLL-I and VN, respectively, as compared to untreated cells cultured in the same conditions. Furthermore, ERK 2 phosphorylation in FAK siRNA cells plated on COLL-I and VN exhibited a 1.7-fold and 2.3 fold decrease, respectively. As expected, cells plated on poly-L-lysine and stimulated with OS media also exhibited activation of ERK 1 and ERK 2 [5], but their activity decreased in FAK siRNA cells by 2-fold and 1.6-fold, respectively. Although the plating of hMSC on FN induced phosphorylation of FAK Y397 following 30 min adhesion (Fig. 3A), subsequent ERK 1/2 activation is delayed by an additional hour compared to cells on COLL-I and VN (Fig. 5D). However, FAK knockdown cells exhibited decreased levels of ERK 1/2 phosphorylation compared to mock transfected controls following 60, 90, and 120 min adhesion to FN.

To determine the effect of ECM adhesion on Runx2/Cbfa-1 activation, we assayed for Runx2/Cbfa-1 serine phosphorylation by immunoprecipitation and immunoblot analysis. hMSC plated on COLL-I or VN exhibited increased serine phosphorylation of Runx2/Cbfa-1 compared to cells plated on tissue culture plastic, and that this increased phosphorylation occurred within 8 days of plating (Fig. 6A,C). Strikingly, addition of FAK siRNA reduced

serine phosphorylation of Runx2/Cbfa-1 48% and 62% on COLL-I and VN, respectively, compared to untreated hMSC cultured in the same conditions. To assess the role of FAK signaling events on ECM-induced osteogenic differentiation of hMSC via ERK 1/2 pathways, we included the MEK inhibitor PD98059 in our assays. Addition of PD98059 significantly reduced serine phosphorylation of Runx2/Cbfa-1 in non-transfected cells after 8 days of plating on COLL-I and VN (Fig. 6B,D). Further-more, FAK knockdown cells treated with PD98059 exhibited a further decrease in phosphorylated Runx2/Cbfa-1. These results strongly suggest that FAK signaling pathways control the activation of the master bone transcription factor, Runx2/Cbfa-1, in ECM-induced osteogenic differentiation of hMSC through a ERK 1/2 dependent mechanism. However, its unlikely that any single kinase, including ERK, is wholly responsible for controlling the activity of such a key commitment step of osteogenesis (and chondrogenesis).

Because we observed maximal Runx2 phosphorylation at day 8, we examined the expression levels and activation states of FAK and ERK 1/2 at days 1, 4 and 8 (Fig. 7). We found that, in FAK siRNA treated cells plated on COLL-I, total FAK levels were clearly decreased at day 1, and fully recovered by day 8, with a slight recovery at day 4 (Fig. 7A). In parallel, we found that FAK activation dropped in these cells to the same level in cells plated on tissue culture plastic at day 1 and that this effect persisted through day 8. Total levels of ERK were unaffected by FAK siRNA treatment (Fig. 7B). ERK activation was only observed at day 1 and only in untreated cells plated on collagen I or stimulated with OS media. Finally, Runx2 was not activated at day 1, with a modest increase at day 4 and a pronounced increase at day 8 (Figure 7C). Thus activation of FAK and ERK precedes Runx2 phosphorylation.

Inhibition of FAK signaling by siRNA knockdown disrupts ECM-stimulated osteogenic differentiation of hMSC

To evaluate the role of FAK activity on ECM-induced osteogenic differentiation of hMSC, cells transfected with FAK siRNA were examined for several hallmark markers defining this progression. Firstly, in order to assess the effect of FAK signaling on osteogenic gene expression, we performed RT-PCR analysis on cells plated on various substrates in the presence and absence of FAK siRNA oligonucleotides. Figure 8 reveals that cells plated on COLL-I and VN for 14 days expressed increased levels of bone sialoprotein 2 (Fig. 8B), and osteocalcin (Fig. 8C) mRNAs compared to cells plated on tissue culture plastic and FN, but that FAK siRNA transfection suppressed this expression. Although Runx2/Cbfa-1 gene expression (Fig. 8D) was unchanged in all conditions tested, FAK siRNA knockdown cells exhibited decreased osterix (Fig. 8E) mRNA expression levels on COLL-I and VN compared to untreated cultures. Histological staining of alkaline phosphatase expression (Fig. 9A,C) further confirmed our RT-PCR results.

An important marker of osteogenic differentiation is the accumulation of calcium in the extracellular space and its organization into hydroxyapatite crystals. We measured calcium deposition by Alizarin Red histological staining and by a colorimetric assay (Fig. 9B,D) and observed that untreated cells plated on COLL-I or VN induced significant increases in calcium deposition compared to cells plated on tissue culture plastic or FN, and this increase was eliminated in hMSC treated with FAK siRNA. Moreover, although OS medium induced the greatest increase of calcium accumulation, cells transfected with FAK siRNAs and cultured in OS medium for 21 days exhibited a significant reduction in calcium deposition. Lastly, we assessed the osteogenic differentiation between our untreated cells and our FAK knockdown cells by determining the mineral:matrix ratio of the secreted ECM, the quintessential hallmark of ossification (Fig. 10). We found that FAK siRNA-treated cells exhibited a significant decrease in this ratio compared to control cells (Fig. 10E). Representative FTIR spectra of FAK knockdown cultures showed decreased mineral content compared to their parallel controls (Fig.

10A-D). Together these results demonstrate that ECM-induced FAK activation stimulates ERK 1/2 and Runx2/Cbfa-1 signaling cascades and plays a critical role in promoting the osteogenic differentiation of hMSC.

To confirm the siRNA FAK results we repeated selected assays using hMSC expressing either wildtype (WT) c-myc-FAK or a mutant form, F397 c-myc-FAK. Cells were plated on COLL-I for 21 days and assayed for both ERK activation (Fig. 11A) and osteocalcin levels (Fig. 11B). Cells expressing WT c-myc-FAK on either tissue culture plastic or COLL-I showed higher levels of both osteocalcin and phosphorylated ERK as compared to the inactive F397 mutant expressing cells. These results both confirm previous siRNA findings that FAK activation stimulates ERK 1/2 phosphorylation and also stimulates expression of osteogenic markers.

Discussion

The signaling mechanisms linking integrin binding with the commitment of hMSC to the osteogenic lineage are essentially unknown. Our results suggest that FAK, ERK, Runx2/Cbfa1, and osterix constitute important elements of the signal transduction machinery controlling this process. Considerable work has focused on the role of integrin-mediated signaling during progression of osteoblast differentiation [15,17,18,32,33], but our work focuses on a much earlier step, when hMSC are still capable of differentiating into multiple cell types. Clearly, a different set of decisions are being made by hMSC at this earlier step of osteogenesis, in that down-regulation of adipogenic, chondrogenic, neural, and muscular genes is occurring at the same time that osteogenic genes are being upregulated [34,35].

Because such a wide variety of signals affect hMSC differentiation, it is very likely that no single signaling pathway is responsible for regulating this ECM-induced commitment step. Rather, a network of signaling pathways is likely at work, and FAK is well suited to integrate these signaling activities. Autophosphorylation of FAK initiates the formation of dynamic molecular complexes that contain numerous signaling proteins (e.g., src, p85 regulatory subunit of PI3-kinase, phospholipase C γ , Grb-7, and Shc) [22]. These pathways are not completely redundant, and may be activated to differing degrees by different integrin/ECM interactions. Partial knockdown of FAK, as our siRNA achieved, might for example disrupt fibronectin-activated signaling pathways that are more sensitive to total FAK levels than collagen-stimulated pathways. In other cells, these FAK complexes are sensitive to a wide variety of extracellular stimuli beyond ECM binding, including glucocorticoids [36], peptide growth factors [37], and mechanical stress [38]. These same stimuli contribute to osteogenic differentiation on hMSC [5,39,40], and our data support the idea that FAK mediates this response.

What these stimuli also share in common is that they converge on MAPK family members during osteogenic differentiation of hMSC. We have already established that signaling by the MAPK family members ERK1 and ERK2 are critical for stimulating osteogenic gene expression in hMSC [10,11], and the data presented here establish a link between FAK and ERK *during hMSC differentiation*. This is of course not the first report that FAK activates ERK family members, but it is the first demonstration that specific ECM proteins induce hMSC differentiation via the FAK-ERK pathway. Significant cross-talk also occurs between MAPK, bone morphogenic proteins (BMP)/Smads (which are also regulators of bone formation) [7], and protein kinase D [29] signaling cascades in osteoblasts. Together these observations suggest that FAK phosphorylation of ERK 1/2 via MEK may be only one of many mechanisms for regulating Runx2/Cbfa-1 in our cells.

Our study predicts that suppression of FAK in animals should disrupt bone formation. We obtained heterozygous knockout mice from Dr. Dusko Ilic at the University of California-San

Francisco, and compared bone development in four of these mice (two adult males, two adult females) with age and strain-matched control mice. Faxitron analysis of bone density revealed significant variation between these mice, and these variations were confirmed by microCT analysis of individual bones in each animal. However, no statistically significant differences between heterozygotes and control animals were found (data not shown). These findings are not as disappointing 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, hundreds of papers report that FAK plays a significant role in cell migration in vitro, and this is now well accepted. However, there are no differences in healing of skin wounds in either keratin 14-Cre [41] or keratin 5-Cre [42] keratinocyte-specific FAK knockout mice. Moreover, FAK-null keratinocytes do not survive in vitro, but are quite viable in these animals. Thus, as-yet-unknown, functionally redundant signaling mechanisms may be able to accommodate FAK knockout in some tissues.

In later stages of osteogenic differentiation (in osteoblast-like cells), fluoride (fluoroaluminate) treatment leads to co-activation of FAK and its analog, proline-rich tyrosine kinase-2 (PYK2), and subsequent downstream stimulation of MAPKs including ERK 1/2 [43], implying that the two kinases may act cooperatively. However, siRNA knockdown of FAK in our cells results in no compensatory increase in PYK2 phosphorylation on Y402 and still leads to a reduction in ERK activity, suggesting that at this earlier stage of differentiation, at least, FAK appears to be acting alone.

Several signaling molecules (BMP-2, IGF-I, PKD, p38, JNK, and ERK 1/2) play some role in the transcriptional upregulation of osterix expression in hMSC [7], possibly by regulating Runx2/Cbfa-1 activity. We find that transcriptional levels of Runx2/Cbfa-1 remain unchanged in response to FAK knockdown (Fig. 8A,D), but those stimuli that lead to the highest degree of ERK 1/2 phosphorylation (Fig. 5) also induce the most serine phosphorylation of Runx2/Cbfa-1 (Fig. 6) in our cells, suggesting that FAK-ERK signaling targets osterix expression but not Runx2/Cbfa-1 transcription. Together, these findings suggest that FAK, ERK, Runx2/Cbfa-1, and osterix form a decision-making pathway that directly affects hMSC fate determination.

This pathway also functions to maintain osteogenic differentiation in committed cells: inhibiting with ERK activity with dominant negative mutants of ERK 1/2 or the MEK1 inhibitor U0126 blocks osterix expression and matrix mineralization [29]. Blocking Runx2/Cbfa-1 activity using a dominant negative Runx2/Cbfa-1 construct and an ubiquitination mediator for Runx2 degradation decreases BMP-2 induction of osterix [29]. We may therefore be observing the assembly of this important pathway in our uncommitted cells, and suggest that FAK activation is an essential early step in this process.

Acknowledgements

This work was supported by the National Institutes of Health Grant 1R01-EB002197 (G.E.P.).

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Fig. 1. siRNA-mediated knockdown of FAK in hMSC on the mRNA and protein level. (A) hMSC were transiently transfected in triplicate with 50 nM and 100 nM of Silencer™ validated siRNA against FAK, GAPDH (positive control), Silencer™ negative control #1, or siPORT™ Amine alone. Relative FAK and GAPDH mRNA levels were assessed after 72 h using RT-PCR and real-time RT-PCR (B,C). FAK mRNA knockdown activity in FAK siRNA transfected cells is approximately 40% as compared to cells transfected with negative control siRNA or siPORT™ Amine alone, or untreated cells. (D) hMSC were lysed and probed for FAK expression by western blot by FAK-specific antibodies after 72 h incubation with 50 nM validated Silencer™

siRNA against FAK or SilencerTM negative control #1, or siPORTTM Amine alone. Immunoreactivity to G-actin was used as a loading control.

Fig. 2. hMSC survive, adhere, and spread following siRNA-mediated knockdown of FAK expression. (A) Cells were transfected with 50 nM and 100 nM of Silencer™ validated siRNA against FAK or siPORT™ Amine alone for 72 h and assayed for cellular viability using MTT. (B) 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, 20 µg/ml COLL-I, or 20 µg/ml VN 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. (C) hMSC were assayed for cell spreading as per Fig. 2B by plating FAK siRNA transfected cells or cells treated with siPORT™ Amine alone (D) on COLL-I for 5 h., then fixed and stained for endoglin and Phalloidin (10X magnification).

Fig. 3.

Adhesion to insoluble COLL-I and VN induces FAK Y397 phosphorylation and is attenuated following FAK siRNA transfection. (A) hMSC were plated for 30 and 60 min on poly-L-lysine, 20 μ g/ml FN, 20 μ g/ml COLL-I, or 20 μ g/ml VN in serum-deprived DMEM, then lysed and probed for activated FAK by western blot with phospho-Y397 FAK-specific antibodies. Total FAK was detected by western blot with a non-phospho-specific antibody. Densitometric measures of band intensity for FAK are shown in (C). (B) hMSC were transiently transfected with 50 nM Silencer™ validated siRNA against FAK for 72 h followed by culture in serum-deprived medium overnight. Cells were plated on the indicated ECM proteins for 30 min, then assayed for western blot as above. Densitometric measures of band intensity for FAK are shown

in (D). All gels shown are representative of 3 experiments. Asterisks indicate significant ($p < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired t test. OS-treated cells were used as a positive control for osteogenic differentiation.

A **B** **Fig. 4.**

PYK2 activity does not compensate for FAK expression knockdown by siRNA. Cells were plated for 30 min on the indicated substrates and conditions and assayed for phosphorylated pY402 PYK as per Fig. 3B. Densitometric measures of band intensity for PYK are shown in (B). OS-treated cells were used as a positive control for osteogenic differentiation.

Fig. 5.

FAK siRNA knockdown inhibits ERK 1/2 activation on COLL-I and VN. (A) Cells were plated for 1 h on the indicated substrates and conditions and assayed for phosphorylated pTpY185/187 ERK 1/2 as per Fig. 3B. Densitometric measure of band intensity for ERK 1 and ERK 2 in panel (A) 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 significant ($p < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired t test. FAK knockdown and mock transfected control cells were plated for an additional 30 and 60 min on FN and assayed as above (D). OS-treated cells were used as a positive control for osteogenic differentiation.

Fig. 6. FAK siRNA knockdown reduces COLL-I and VN-induced phosphorylation of Runx2/Cbfa-1. (A) hMSC were transfected with 50 nM Silencer™ validated siRNA against FAK for 72 h then plated on tissue culture plastic, 20 μ g/ml FN, 20 μ g/ml COLL-I, or 20 μ g/ml VN in basal media. The following day (day 0), media was replaced for OS-treated conditions and samples were cultured for an additional 8 days. FAK knockdown and mock transfected control cells were also plated on TCP ($\pm$ OS) or COLL-I in the presence or absence of the MEK inhibitor, PD98059 (50 μ M), on day 0 (B). At the end of the 8 day incubation period, cells were 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 phospho-serine,

which is indicated by the 61 kDa band. Total Runx2/Cbfa-1 from each lysate was detected by western blot as a loading control. Quantitation of the 61 kDa band in each lane is shown for 8 day samples (C,D). Single and double asterisks indicate significant ($p < 0.05$) reductions in parallel untreated samples (white bars), using Student's unpaired t test. OS-treated cells were used as a positive control for osteogenic differentiation.

Fig. 7.

Activation of FAK and ERK precedes Runx2 phosphorylation. Cells were cultured for 1, 4 or 8 days on the indicated substrates and conditions and assayed for (A) FAK activation (upper panel) and total FAK (lower panel) as per Fig 3B; (B) ERK activation (upper panel) and total ERK (lower panel) as per Figure 5; and (C) Runx2 phosphorylation (upper panel) and total Runx2 as per Figure 6.

A

Fig. 8.

Osteogenic differentiation of hMSC is FAK-dependent on COLL-I and VN. (A) Cells were plated under the same conditions as in Fig. 6, and total RNA was isolated after 14 days in culture. RT-PCR was performed on each sample, using primers specific for amplification of the indicated genes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as a loading control. Densitometry of band intensities for bone sialoprotein 2, osteocalcin, cbfa-1, and osterix are given in panels B-E, respectively. Cells cultured in OS medium served as the positive control in all osteogenic differentiation assays. OS-treated cells were used as a positive control for osteogenic differentiation.

Fig. 9.

FAK siRNA knockdown decreases alkaline phosphatase activity and the expression of a calcified matrix on COLL-I and VN. hMSC were plated for 14 days or 21 days under the same conditions as in Fig. 6, then stained for alkaline phosphatase activity (A) or calcium with Alizarin Red (B), 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. (C) 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 B, 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. OS-treated cells were used as a positive control for osteogenic differentiation.

Fig. 10.

FAK siRNA knockdown decreases matrix mineralization. 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 COLL-I (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.

Fig. 11.

Dominant negative FAK decreases ERK 1/2 activation and expression of osteocalcin in hMSC on COL-I. Cells expressing either WT c-myc-FAK or a dominant negative form F397 c-myc-FAK were grown on COL-I for 21 days and assayed for ERK activation (A) or osteocalcin protein levels (B) by western blotting. Equal amounts of protein from cell lysates were loaded in each lane.

TABLE 1

Gene Name	Primer Sequences	Product Size (bp)
PTK2 (Focal-Adhesion Kinase) NH_005607	Forward 5' - GCGCTGGCTGGAAAAAGAGGAA - 3' Reverse 5' - TCGGTGGGTGCTGGCTGGTAGG - 3'	475
TUBB2 (Tubulin Beta-2) NM_001069	Forward 5' - GGATCGACCCAGGCAGTTA - 3' Reverse 5' - GGCGTTGTAGGGCTCCACCAC - 3'	470
IBSP (Bone Slatoprotein 2) NM_004967	Forward 5' - ACAACACTGGGCTATGGAGAGGAC - 3' Reverse 5' - ACTTGTGGTGGGGTTGTAGGTTC - 3'	401
BGLAP (Osteocalcin) NM_199173	Forward 5' - CGCAGCCACCGAGACACCAT - 3' Reverse 5' - AGGGCAAGGGGAAGAAGAGGAAAGAA - 3'	400
CBFA1 (Core binding factor alpha 1) NH_004348	Forward 5' - ATGGCGGGTAACGATGAAAAT - 3' Reverse 5' - ACGGCGGGGAAGACTGTGC - 3'	421
SP7 (Osterix) NM_152860	Forward 5' - AACCCCCAGCTGCCACCTACC - 3' Reverse 5' - GACGCTCAGCTCATCCGAACG - 3'	457
GAPDH (Glyceraldehyde -3-phosphate dehydrogenase) NM_002046	Forward 5' - GCGCTGGCTGGAAAAAGAGGAA - 3' Reverse 5' - GGTGAGCACAGGGTACTTTATT - 3'	1000