
Student Award Winner in the Undergraduate Degree Category for the Society for Biomaterials, 32nd Annual Meeting, Chicago, Illinois, April 17–20, 2007

Integrin-linked kinase production prevents anoikis in human mesenchymal stem cells

Danielle S.W. Benoit,¹ Margaret C. Tripodi,¹ James O. Blanchette,² Steve J. Langer,³ Leslie A. Leinwand,³ Kristi S. Anseth^{1,2}

¹Department of Chemical and Biological Engineering, University of Colorado, 424 UCB ECCH 111, Boulder, Colorado 80309

²Howard Hughes Medical Institute, University of Colorado, 424 UCB ECCH 111, Boulder, Colorado 80309

³Department of Molecular, Cellular, and Developmental Biology, University of Colorado, 424 UCB ECCH 111, Boulder, Colorado 80309

Received 10 January 2007; accepted 11 January 2007

Published online 2 March 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31292

Abstract: Human mesenchymal stem cells (hMSCs) were infected with an adenovirus expressing integrin-linked kinase (ILK) to understand the role of cell-ECM signal transduction cascades in suppressing anoikis. Survivability of ILK-infected hMSCs encapsulated in poly(ethylene glycol) (PEG) hydrogels, an anoikis-inducing environment, was sustained at 90% over 7 weeks, and survival was attributed to increased protein kinase B (PKB/Akt) activation. hMSCs encapsulated in RGD-modified hydrogels induced an upregulation in ILK production, PKB/Akt activation, and subsequent survival to the same extent of ILK-infected, encapsulated hMSCs. As negative controls, encapsulated hMSCs were infected with cyclization recombinase (a protein not associated with cell survival)-expressing virus, and uninfected hMSCs exhibited very little ILK production, PKB/Akt activation, and survival (~55% after

7 weeks). As a measure of cell–matrix interactions, vinculin was also quantified for the encapsulated hMSCs and found to be 30-fold greater for cells encapsulated in RGD-modified hydrogels and fivefold greater for ILK-infected hMSCs than controls, indicating that cell–material interactions are inducing the cell survivability of hMSCs encapsulated in RGD-modified hydrogels. In sum, ILK infection can support cell survival in the absence of matrix interactions and enable fundamental studies of three-dimensional cell function in response to extrinsic signals, independently of matrix–ligand interactions. © 2007 Wiley Periodicals, Inc. *J Biomed Mater Res* 81A: 259–268, 2007

Key words: three-dimensional cell culture; protein kinase B; poly(ethylene glycol); stem cells

INTRODUCTION

The nature of cell adhesion and subsequent signaling via receptor–ligand interactions provides the cell with vital information about its extracellular environment, regulating a variety of important processes, including proliferation, migration, differentiation,

and even survival.^{1,2} For instance, anchorage-dependent osteoprogenitor cells, such as mesenchymal stem cells (MSCs), require integrin interactions in order to survive.³ Indeed, the interaction of epithelial and endothelial cells with the extracellular matrix (ECM) inhibits default apoptotic pathways, which become activated if cell-ECM interactions are disrupted,⁴ and this process is termed anoikis. Thus, cell-ECM interactions are paramount for maintaining cell viability, and a critical aspect of developing niches for three-dimensional cell culture.

The link between integrins and the ECM takes place at focal adhesions. Focal adhesions consist of a cluster of ECM-bound integrins that serve as a mem-

Correspondence to: K. S. Anseth; e-mail: kristi.anseth@colorado.edu

Contract grant sponsor: National Institutes of Health; contract grant number: DE016523

brane attachment site for actin fibrils inside the cell. Many accessory cytoskeletal proteins concentrate at focal adhesions, as do various signaling proteins. Integrins trigger a number of signaling pathways, some of which are primarily related to cell adhesion. Activated protein kinase B (PKB/Akt) plays a critical role in regulation of adhesion-mediated cell survival signals.^{5,6} Integrin-linked kinase (ILK), first identified as an integrin β 1-cytoplasmic domain-binding protein,⁷ enhances phosphorylation of PKB/Akt, which is essential for PKB/Akt activation⁸ either directly or through a complex involving PINCH-1.⁸ ILK has been implicated in integrin, growth factor, and Wnt signaling pathways.^{5–8} ILK functions as a pivotal effector in the transduction of signals from the ECM, regulating, among others, anchorage-dependent cell progression and the apoptotic pathway.³

While interest in culturing cells in three-dimensions has risen in the past decade,⁹ one of the challenges is identifying suitable matrices to study cell function in a biologically relevant niche. Typical matrices either suffer from a complex array of cell and protein interactions that confound analyses to understand isolated effects of signaling molecules or lead to nonspecific or inadequate cell–matrix interactions to mimic a tissue-like environment. Thus, we were interested in better understanding the intracellular processes that lead to anoikis and then develop strategies to enable one to study cell function in three-dimensions, but in the absence of extracellular interactions. Such an approach could be useful for studying cell function in response to different stimuli, including mechanical and electrical forces, chemical cues, and growth factors, without the presence of adhesive ligands, which could confound or mask trends resulting directly from the stimuli studied. Further, this strategy could prove to be particularly useful when aiming to guide the differentiation of a stem cell down a particular pathway that may be activated or suppressed by specific ligand interactions (e.g., chondrogenic differentiation of MSCs).

With this in mind, hydrogels synthesized from poly(ethylene glycol) (PEG) are widely used for cell encapsulation and three-dimensional cell culture, and protein adsorption to this synthetic polymer system is minimal, making PEG an ideal system to study cell function in the absence of matrix interactions or in the presence of targeted ligands.¹⁰ By taking a closer look at the cell–ECM signal transduction cascades in PEG gels, we aim to understand important signaling processes that influence cell function, namely survival, and examine complementary strategies to prevent or limit anoikis. Specifically, the viability of anchorage-dependent hMSCs encapsulated in unmodified PEG gels drops from 100 to ~40% over the course of 3 weeks, largely the result of minimal cell–matrix interactions. Because of the lack of

cell–material interactions, the targeted upregulation of ILK can be achieved through PEG materials designed with integrin-specific ligands, such as the cell-adhesive Arg-Gly-Asp (RGD) peptide sequence. For example, when RGD is tethered covalently to PEG-based macromers, human MSC (hMSC) viability is rescued.¹¹ Pinsky et al. studied the survival of isolated hepatocytes and found that RGD conferred survival through the ILK/Akt pathway.¹²

In the work presented here, we specifically study ILK production in response to the adhesive ligand RGD, and to determine whether ILK production is sufficient for survival, hMSCs were infected with an adenovirus expressing ILK. ILK infection and production were first optimized and characterized with respect to stability over the course of the study (i.e., 7 weeks). Then, ILK activity was monitored by PKB/Akt phosphorylation for MSCs encapsulated in RGD-functionalized PEG gels and infected MSCs in PEG gels. Infected hMSCs were encapsulated in a PEG-based, “blank slate” hydrogel, and the resulting viability was monitored and compared with the viability in RGD-presenting hydrogels to see if ILK production, alone, prevents anoikis. Finally, a potential and major difference between these culture systems is the presence of focal adhesions, so vinculin levels of hMSCs and ILK-infected hMSCs cultured in the presence and absence of RGD was monitored in these gel environments.

MATERIALS AND METHODS

Materials were obtained from Sigma-Aldrich unless otherwise specified.

Infection of MSCs with recombinant adenoviruses

Genetic modification of hMSCs was achieved by infection with a recombinant adenovirus. The adenoviruses were generated using the pAdEasy-1 system (MP Biomedicals). Recombinant pShuttle adenoviral vectors with enhanced green fluorescent protein (GFP), cyclization recombinase (CRE), or ILK inserted into the multiple cloning site under the control of a cytomegalovirus promoter were used in these studies. Homologous recombination in DH5 α bacteria with the pAdEasy-1 Ad5 Δ E1/ Δ E3 plasmid created a new plasmid with GFP, CRE, or ILK expression cassette inserted into the E1 region of the adenoviral genome.

hMSC culture

hMSCs were purchased from Cambrex and cultured in growth medium: low-glucose Dulbecco’s modified eagle medium (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Gibco), 0.25% gentamicin (Gibco), and 0.25% fungizone (Gibco). hMSCs after passage 3 were used in this study.

Verification and optimization of ILK infection

Immunoblots were utilized to verify and optimize the production of ILK and subsequent downstream product, phosphorylated protein kinase B (PKB/AKT) (pAKT). hMSCs in 12-well tissue culture treated plates were incubated for 5 days in growth media until 80% confluent and infected with ILK-expressing virus. Viral multiplicities of infection (MOI) of 1, 10, 100, and 10,000 were used for infection. hMSCs were incubated with virus for 48 h, washed, and lysed [mPER (Pierce), with Protease Inhibitor Cocktail Set III (2 μ L/mL of mPER; Calbiochem) and Phosphatase Inhibitor Cocktail Set I (10 μ L/mL mPER; Calbiochem)]. Total protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in PBS with 0.5 wt % Tween 20, 1 wt % bovine serum albumin, and 5 wt % non-fat dry milk at room temperature for 1 h. Membranes were incubated separately with rabbit anti-ILK and rabbit anti-pAKT (Chemicon) overnight at 4°C, then incubated with peroxidase-coupled goat anti-rabbit IgG (Bio-Rad) for 1 h, and bands were detected with the Opti 4CN kit (Bio-Rad). Membranes were scanned and analyzed using ImageJ.

Longevity of infection technique

hMSCs were incubated for 5 days in growth media until 80% confluent and infected with GFP-expressing virus (MOI = 10, which was found to be most efficient for protein expression). The cells were incubated with virus for 48 h, washed, and maintained in growth media. GFP expression was monitored before infection, directly after infection (after washing), and at days 2, 5, 14, 28, and 49, to determine the longevity of the infection procedure.

Synthesis of PEG diacrylate and acrylated RGD

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized as described previously.¹³ First, PEG ($M_w \sim 4600$ g/mol) was dissolved in methylene chloride. Triethylamine (TEA) at 20% molar excess was added dropwise, and the solution was mixed under argon for 5 min. Acryloyl chloride at 20% molar excess was mixed with 10 mL methylene chloride and added dropwise to the PEG/TEA solution, and the final mixture was stirred overnight. The product was precipitated in ice-cold diethyl ether, filtered, and dried in a desiccator. After drying, the PEGDA was redissolved in diH₂O and dialyzed (Spectrum, 1000 M_w cutoff) over 24 h with two distilled water exchanges. ¹H NMR analysis (in chloroform-d, Cambridge Isotopes) on the PEGDA peaks revealed an average of 92% acrylation.

RGDS were synthesized using solid phase methods on an ABI 433A Peptide Synthesizer (Applied Biosystems, Foster City, CA) and following procedures for 2-(1*H*-benzotriazol)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) activation coupling. The peptides, after UV-monitored synthesis, were cleaved from the solid support with a cocktail consisting of 5% phenol, 5% water, and 2.5% triisopropylsilane in trifluoroacetic acid (TFA). The peptide was then washed with copious amounts of ice-cold diethyl ether, redissolved in distilled water, and dia-

lyzed (Spectrum, 500 M_w cutoff) over 24 h with two exchanges of distilled water.

RGDS was coupled to acrylated-PEG following a previously reported method.¹⁴ Briefly, RGDS was dissolved in sodium bicarbonate buffer (50 mM, pH 8.4). Acryloyl-PEG-*N*-hydroxysuccinimide ($M_w \sim 3400$ g/mol, Nektar Therapeutics) was reacted with the peptide while stirring at room temperature for 2 h. The mixture was dialyzed (Spectrum, 1000 M_w cutoff) in distilled water over 24 h with two distilled water exchanges. The dialyzed acrylated-PEG-RGD was lyophilized and stored at 4°C until used. Structures of PEGDA and acrylated-PEG-RGDS are shown in Figure 1.

Encapsulation of hMSCs

All hydrogels were formulated by dissolving PEGDA in phosphate-buffered saline (PBS) to achieve a final monomer concentration of 10 wt %. The photoinitiator 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Ciby-Geigy) was added to a final concentration of 0.05 wt %. hMSCs were combined with sterile macromer/initiator solutions (with 5 mM of acrylated RGD for the RGD-containing gels) at a concentration of 25×10^6 cells/mL and photoencapsulated using a longwave ultraviolet lamp (UVP, model XX-20) at an intensity of ~ 4 mW/cm² for 10 min.¹⁵ hMSCs infected with ILK and CRE (MOI = 10), as well as uninfected hMSCs were encapsulated in 10 wt % PEGDA hydrogels. The CRE infected hMSCs and uninfected hMSCs were encapsulated as negative controls. Without any adhesive ligands, hMSCs are known to undergo apoptosis after <2 weeks of culture.^{11,16–17} CRE has no effect on the apoptotic pathways and was used to verify that the infection procedure had no effect on viability. RGD was incorporated as a positive control, as it has formerly been shown to rescue hMSC viability under the same culture conditions.¹⁶ The resulting cell-hydrogel constructs were incubated in 12-well plates. The constructs were cultured for 7 weeks in growth medium. Constructs were removed from culture at days 2, 5, 14, 28, and 49 for LIVE/DEAD and ILK/pAKT production analysis.

Infected hMSC viability in PEG hydrogels

Cell/polymer constructs were cultured for up to 7 weeks. At each time point, a sample of constructs was

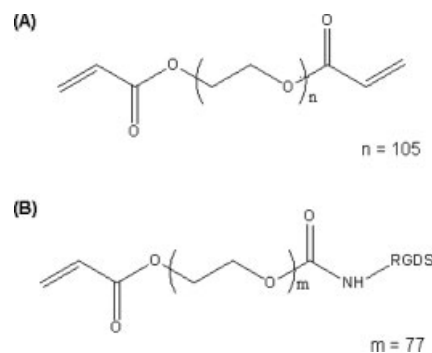


Figure 1. Chemical structures of the multifunctional macromer PEGDA (A) and monovinyl macromers acryloyl-PEG-RGDS (B) used for hydrogel fabrication.

removed from culture and stained using the LIVE/DEAD assay. Cells were placed in 1.5-mL tubes with 1 mL of growth media, treated with 0.5 μ L Calcein substrate (Calbiochem) and 2 μ L of Ethidium Homodimer substrate (Calbiochem), and incubated at 37°C for 30 min. Cell viability and distribution were visualized using confocal microscopy at days 2, 5, 14, 28, and 49. The images were analyzed using ImageJ to determine the percent of live cells present at each time point.

Encapsulated hMSC ILK and pAKT production

Immunoblots were utilized to verify the production of ILK and subsequent downstream product, pAKT, throughout the 7-week encapsulation. After LIVE/DEAD imaging, cell/gel constructs were homogenized in the presence of mPER (Pierce) with Protease Inhibitor Cocktail Set III (2 μ L/mL of mPER; Calbiochem) and Phosphatase Inhibitor Cocktail Set I (10 μ L/mL of mPER; Calbiochem). Total protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in PBS with 0.1 wt % Tween 20, 1 wt % bovine serum albumin, and 5 wt % nonfat dry milk at room temperature for 1 h. Membranes were incubated separately with rabbit anti-ILK and rabbit anti-pAKT (Chemicon) overnight at 4°C, then incubated with peroxidase-coupled goat anti-rabbit IgG (Bio-Rad) for 1 h and bands were detected with the Opti 4CN kit (Bio-Rad). Membranes were scanned and analyzed using ImageJ, normalizing the ILK and pAKT bands to the number of living cells, as analyzed via LIVE/DEAD imaging.

Encapsulated hMSC vinculin quantification

Immunoblots were utilized to verify the production of vinculin throughout the 7-week encapsulation. Vinculin is a protein associated with focal adhesions that form in response to cell-ECM interactions, therefore, should be more prevalent in cells encapsulated in RGDS-functionalized hydrogels than all other formulations.¹⁸ Membranes from above were incubated separately with mouse anti-vinculin (Chemicon) overnight at 4°C, then incubated with peroxidase-coupled goat anti-mouse IgG (Bio-Rad) for 1 h and bands were detected with the Opti 4CN kit (Bio-Rad). Membranes were scanned and analyzed using ImageJ, normalizing to the number of living cells, as analyzed via LIVE/DEAD imaging.

Statistical analysis

Statistical analysis was performed using a one-way ANOVA. Data are presented as mean \pm standard deviation.

RESULTS

Verification and optimization of ILK infection

To determine the necessary viral concentration for infection of hMSCs, different concentrations of ILK-

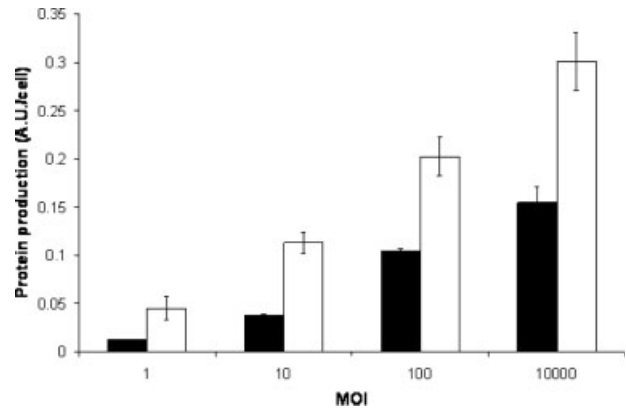


Figure 2. Verification and optimization of ILK/pAKT protein production through analysis of immunoblots prepared from infected hMSCs (ILK – black; pAKT – white). Band densities were determined from several samples and expressed as density (AU)/cell versus MOI. Values are reported as the average of three samples per composition (error bars represent one standard deviation).

expressing virus were utilized, and subsequent ILK production and AKT phosphorylation were measured with immunoblots. As quantified in Figure 2, increasing concentrations of virus caused increased ILK and pAKT production in a dose-dependent manner. MOIs of 10 or greater were determined to be sufficient for infection. Therefore, subsequent infections were carried out with MOI = 10.

Longevity of infection technique

To measure the longevity of the infection technique, cells were infected with GFP-producing viruses under the same conditions as the ILK-expressing virus. As seen in Figure 3, at days 2, 5, 14, 28, and 49, GFP was expressed by the hMSCs. Therefore, at least qualitatively, the infection technique imparts a semipermanent expression of the target protein. A slight decrease in GFP expression was observed over the time course of the study. Adenoviruses are transient; the genes are not necessarily transferred to daughter cells during proliferation. These cells proliferated to a small extent, as characterized visually, but become contact inhibited as the cells occupy more of the tissue culture surface. Proliferation caused decreased production of the product, GFP, in comparison to the number of cells present. However, the cells initially infected with GFP maintained expression of the protein throughout the study.

Viability of virus-infected hMSCs encapsulated in PEG gels

hMSCs were infected with both ILK-expressing and CRE-expressing (as a negative control) viruses.

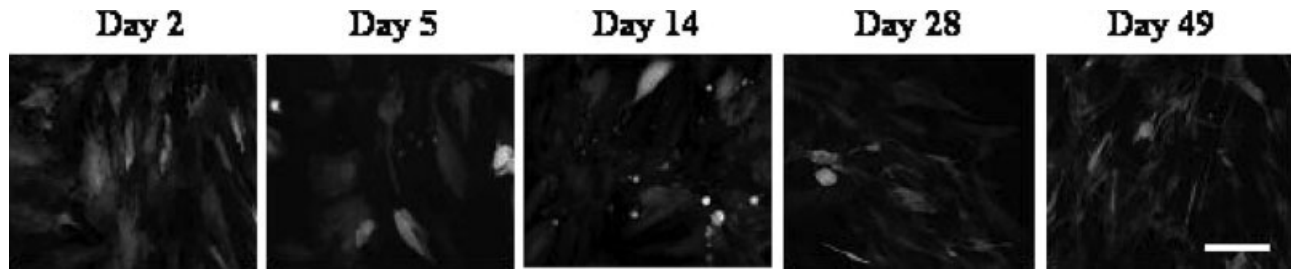


Figure 3. Fluorescent micrographs of hMSCs infected with GFP-expressing virus; bar = 50 μ m. Cells were infected with MOI = 10 and monitored for the longevity of GFP production. GFP fluorescence was converted to greyscale.

These cells and uninfected cells were encapsulated in PEGDA hydrogels. For comparison, uninfected cells were encapsulated in RGD-modified PEGDA hydrogels, a system that provides an avenue for integrin–material interaction. Encapsulated cells were cultured for up to 7 weeks and assessed for viability at days 2, 5, 14, 28, and 49. As Figure 4 shows visually and Figure 5 demonstrates quantitatively, the number of live cells is greater in cells infected with ILK-expressing virus than for CRE-infected cells or for uninfected cells encapsulated PEGDA hydrogels, especially as the study progresses past 5 days. Further, ILK-expressing cells maintained a similar viability to hMSCs encapsulated in RGD-functionalized gels. The total number of cells that fluoresce either green or red reduces significantly between days 28 and 49, indicating that cells undergo apoptosis, and cell debris, including nucleic acids, diffuse out of the gel. As shown in Figure 5, the quantified LIVE/DEAD images revealed viability was sustained at about 90% until day 14 for both uninfected cells encapsulated in RGD-modified hydrogels, as well as ILK-infected cells encapsulated in PEGDA hydrogels. Conversely, uninfected cells or CRE-infected cells encapsulated in PEGDA suffered significant cell death, where only about 40% of the cells remained viable at 7 weeks. Similar to what is shown in Figure 4, the ILK-infected/PEGDA encapsulated and the uninfected/RGD-modified PEGDA encapsulated exhibited a substantial drop in viability between days 28 and 49 from \sim 90 to 65%, perhaps due to increased ECM production and subsequent decreases in diffusional properties. Since these are not degradable gels, the ECM collects in the pericellular space, increasing the time for diffusion of growth factors, nutrients, and wastes. The greatest drop in viability for the negative control gels (CRE-infected and uninfected hMSCs encapsulated in PEGDA) was between days 5 and 14 from \sim 82 to \sim 55%, as expected from previous studies.^{11,16,17}

The trend of viability for cells infected with ILK-producing virus is statistically the same to that of uninfected cells encapsulated in RGD-modified

PEGDA, which confirms the hypothesis that infecting hMSCs with ILK-expressing virus rescues the cells over time in a manner similar to RGD. Additionally, the viability of hMSCs infected with CRE-expressing virus decreases at statistically the same rate as uninfected hMSCs, indicating that infection of CRE does not interfere with the anoikis pathway.

Production of ILK and pAkt by gel-encapsulated hMSCs

To confirm that the hMSCs were producing ILK and affecting the downstream product, pAKT, the samples from the LIVE/DEAD assay were homogenized. Immunoblots for ILK and pAKT were run with the samples, and the quantification of ILK and pAKT production over time is shown in Figure 6. As seen in Figure 6(A), ILK was produced at a similar level by ILK-infected cells encapsulated in PEGDA and infected cells encapsulated in RGD-modified PEGDA over the entire study. Cells in RGD-modified gels showed slightly lower ILK production at day 2, when compared with the ILK-infected cells; however, by day 5, production was statistically the same as for the ILK-infected cells. In addition, control cells (CRE-infected cells and uninfected cells encapsulated in PEGDA) showed production of ILK at levels about 20-fold less than the production by ILK-infected or RGD-modified PEGDA encapsulated cells.

pAKT is also produced at a similar level by ILK-infected cells encapsulated in PEGDA and uninfected cells encapsulated in RGD-modified PEGDA over the entire study [Fig. 6(B)]. Cells in RGD-modified gels showed slightly lower pAKT production during the study, particularly at day 2, where levels are the same as the controls, uninfected cells and CRE-infected cells in PEGDA. Control cells (CRE-infected cells and uninfected cells encapsulated in PEGDA) showed production of pAKT at levels about 15-fold less than the production by ILK-infected or RGD-modified PEGDA encapsulated cells from days 14 to 49 of the study.

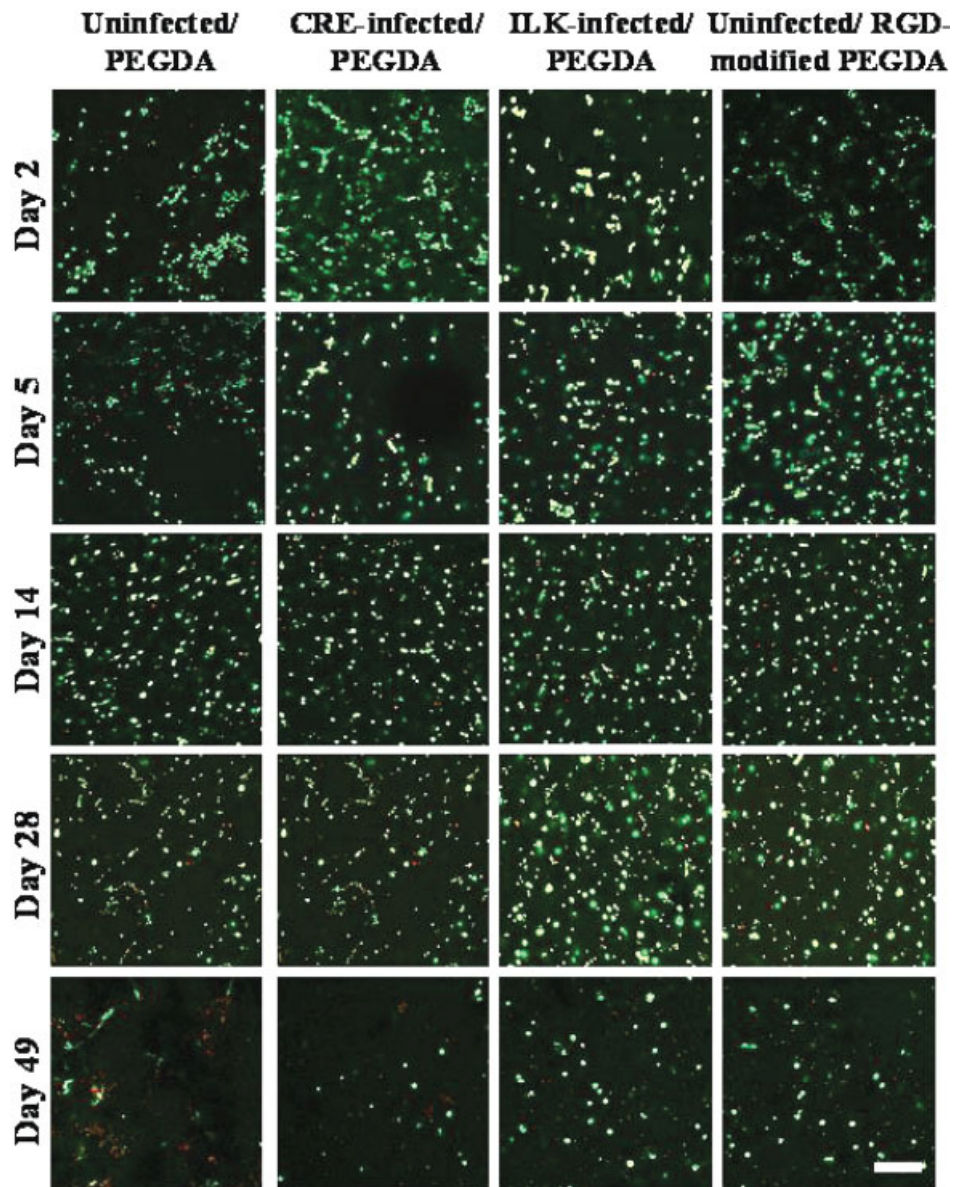


Figure 4. Effect of RGD incorporation or infection by ILK- or CRE-expressing virus particles on hMSC viability inside PEGDA hydrogels. Uninfected hMSCs were encapsulated in unmodified PEGDA or PEGDA containing 5 mM Ac-PEG-RGD. Infected hMSCs (with ILK or CRE-expressing virus with MOI = 10) were encapsulated in unmodified PEGDA. Encapsulated cells were cultured for 7 weeks and viability was assessed at days 2, 5, 14, 28, and 49 using the LIVE/DEAD assay (bar = 200 μ m). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Vinculin quantification of gel-encapsulated hMSCs

While cell survival and pAKT/ILK production were similar in the ILK-infected cells in PEG and the uninfected cells in PEG-RGD gels, a potential and major difference between these systems is the presence of focal adhesions. Vinculin is a prominent protein associated with focal adhesions, and quantification of vinculin for the encapsulated hMSCs was performed using an immunoblot prepared from the LIVE/DEAD homogenized samples. Data is shown over time in Figure 7. Vinculin is found at a similar

level by ILK-infected cells encapsulated in PEGDA and uninfected cells encapsulated in RGD-modified PEGDA at day 2. However, cells in RGD-modified gels showed about twofold greater vinculin levels throughout the rest of the study compared with ILK-infected cells encapsulated in PEGDA. ILK-infected hMSCs produced a moderate amount of vinculin, perhaps due to feedback regulation of ILK. In addition, control cells (CRE-infected cells and uninfected cells encapsulated in PEGDA) exhibited very low levels of vinculin (\sim 30-fold less than cells in RGD-modified gels). Focal adhesions involving vinculin

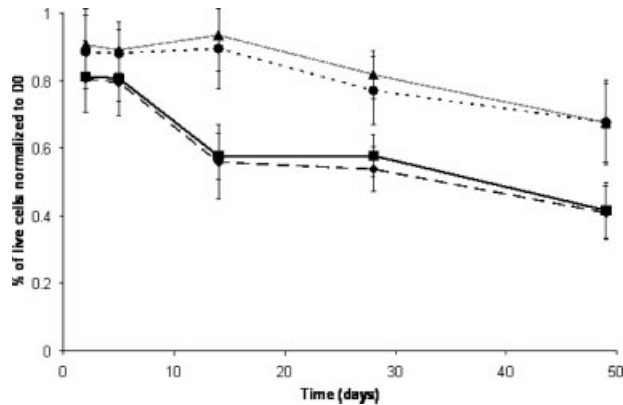


Figure 5. Effect of RGD incorporation or infection by ILK- or CRE-expressing virus particles on hMSC viability inside PEGDA hydrogels: 10% PEGDA + uninfected hMSCs (—◆—), 10% PEGDA + CRE-infected hMSCs (—■—), 10% PEGDA + 5 mM RGD + uninfected hMSCs (—▲—), and 10% PEGDA + ILK-infected hMSCs (—●—). Uninfected hMSCs were encapsulated in unmodified PEGDA or PEGDA containing 5 mM Ac-PEG-RGD. Infected hMSCs (with ILK or CRE-expressing virus with MOI = 10) were encapsulated in unmodified PEGDA. Encapsulated cells were cultured for 7 weeks and viability was assessed at days 2, 5, 14, 28, and 49 using the LIVE/DEAD assay; series of LIVE/DEAD images for each time point and treatment were quantified using ImageJ and the results are shown over time. Error bars represent one standard deviation.

are specialized structures involving close apposition of the plasma membrane to the ECM, especially fibronectin.¹⁹ therefore the increased presence of vinculin in RGD-modified gels is consistent with the high level of cell–matrix interactions.

DISCUSSION

Development of biomaterial niches for three dimensional cell culture is an important area for basic research, serving as relatively simple and chemically well-defined models of ECM, allowing studies of ECM signals controlling cell adhesion, spreading, proliferation, differentiation, matrix degradation, as well as viability. Previous reports demonstrated that hMSCs encapsulated in PEG hydrogels undergo substantial apoptosis after <2 weeks in culture.^{11,16,17} Survival of anchorage-dependent cells, such as hMSCs, requires integrin-mediated adhesion to ECM components, including collagens, fibronectin, and laminins.³ Integrins regulate cell survival, among other cell functions, and are believed to mediate signaling effects by binding and activating different intracellular proteins. One of these is ILK.⁶ Here, we utilized ILK in a chemically defined three-dimensional scaffold to gain a better understanding of the anoikis pathway of hMSCs. After determining the

best infection titer, longevity of the infection, and ensuring that ILK and the downstream product, pAKT, were produced, hMSCs were infected with ILK-expressing and CRE-expressing adenovirus (negative control) and then encapsulated in PEGDA hydrogels. As controls, uninfected hMSCs were encapsulated in PEGDA and RGD-modified PEGDA, which has been shown to maintain hMSC viability. Viability and ILK and pAKT production were assessed over a 7-week period. ILK production, by

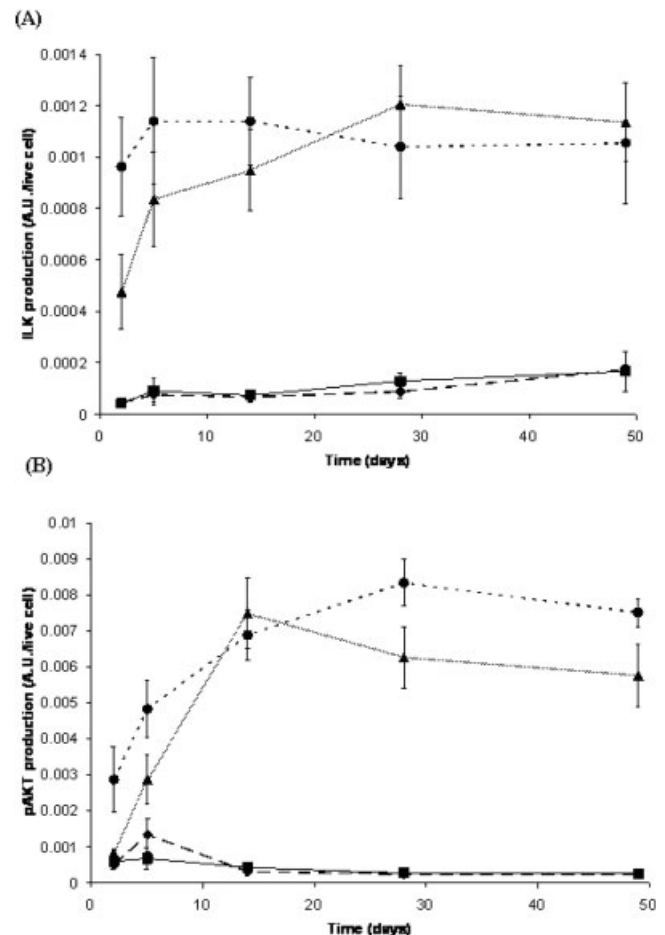


Figure 6. Verification of ILK/pAKT protein production through immunoblots prepared from encapsulated hMSCs: 10% PEGDA + uninfected hMSCs (—◆—), 10% PEGDA + CRE-infected hMSCs (—■—), 10% PEGDA + 5 mM RGD + uninfected hMSCs (—▲—), and 10% PEGDA + ILK-infected hMSCs (—●—). A: The graphic analysis of ILK levels over the 7-week experiment. B: The graphic analysis of phosphorylated PKB/Akt levels over the 7-week experiment. Uninfected hMSCs were encapsulated in unmodified PEGDA or PEGDA containing 5 mM Ac-PEG-RGD. Infected hMSCs (with ILK or CRE-expressing virus with MOI = 10) were encapsulated in unmodified PEGDA. Encapsulated cells were cultured for 7 weeks and protein production was assessed at days 2, 5, 14, 28, and 49 using immunoblots. Band densities were determined from several samples and expressed as density (AU)/cell over time. Values are reported as the average of three samples per composition (error bars represent one standard deviation).

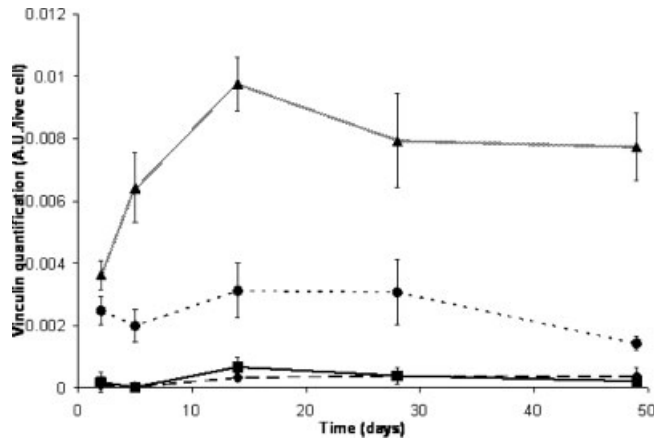


Figure 7. Quantification of vinculin through immunoblots prepared from encapsulated hMSCs: 10% PEGDA + uninfected hMSCs (—◆—), 10% PEGDA + CRE-infected hMSCs (—■—), 10% PEGDA + 5 mM RGD + uninfected hMSCs (—▲—), and 10% PEGDA + ILK-infected hMSCs (···●···). Uninfected hMSCs were encapsulated in unmodified PEGDA or PEGDA containing 5 mM Ac-PEG-RGD. Infected hMSCs (with ILK or CRE-expressing virus with MOI = 10) were encapsulated in unmodified PEGDA. Encapsulated cells were cultured for 7 weeks and protein production was assessed at days 2, 5, 14, 28, and 49 using immunoblots. Band densities were determined from several samples and expressed as density (AU)/cell over time. Values are reported as the average of three samples per composition (error bars represent one standard deviation).

itself in the absence of cell–matrix interactions, was found to maintain viability of hMSCs. This observation was verified by LIVE/DEAD imaging coupled with immunoblots verifying the mechanism of the sustained production of ILK and pAKT from both the ILK-infected cells and the cells encapsulated within the RGD-modified hydrogels. These data provide strong support for the hypothesis that ILK production is sufficient to suppress anoikis.

Previous work has established that integrin-mediated cell anchorage is essential for cell survival. The interaction of epithelial and endothelial cells with the ECM inhibits default apoptotic pathways, which become activated if cell-ECM interactions are disrupted.⁴ Although the engagement of certain integrins results in increased expression of the antiapoptotic protein Bcl-2, as well as in suppression of caspase activity,^{20,21} several other studies implicate the phosphorylation of AKT in the maintenance of cell survival and inhibition of apoptosis through cell-ECM interactions. Constitutively active forms of AKT blocked anoikis in epithelial cells, whereas pharmacological inhibitors of phosphorylation enhanced anoikis, which could be overcome by AKT,²² clearly linking anoikis and AKT. ILK phosphorylates AKT on Ser473,²³ and this modification is essential for the complete activation of AKT.

Others have studied the effects of ILK expression in response to integrin–ligand interactions. The application of antibodies against the fibronectin receptor ($\alpha 5\beta 1$, $\alpha 3\beta 1$ integrins) and the vitronectin receptor ($\alpha v\beta 3$, $\alpha v\beta 5$ integrins) leads to activation of ILK in mammary epithelial cells.²⁴ In addition, Pinkse et al. showed that ILK, specifically, is activated in response to hepatocyte interactions with the RGD peptide, causing an increased and longer activation than when treated with anti- $\beta 1$ integrin antibody.¹² RGD has also been shown to activate ILK in human glomerular mesangial cells, which results in an increased secretion of transforming growth factor $\beta 1$.²⁵

The exploration of peptides to rescue cells from anoikis has been applied in several systems. Activation of integrins that use a peptide derived from laminin, EIKLLIS, protects hippocampal neurons from anoikis.^{26,27} Additionally, the $\alpha 3\beta 1$ integrin activation by EIKLLIS peptides led to activation of ILK and pAKT.²⁷ However, in studies utilizing peptides to confer adhesion to nonadhesive biomaterials, unanticipated changes in cell functions have occurred. For example, ECM production by smooth muscle, endothelial cells, and osteoblasts was greatest on substrates that were the least cell adhesive, while cells on highly adhesive surfaces displayed significantly decreased ECM production.^{28,29} This result suggests a role of adhesion-mediated signaling events in the regulation of ECM synthesis. As matrix production is a critical component for the creation of engineered tissues, these findings emphasize the importance of tailoring materials to contain the optimal types and concentration of ligands that results in the desired cellular response, in addition to improved viability.

Currently, there is a plethora of research focused on identification of an appropriate three dimensional cell culture niches for use in basic biology research. PEG hydrogels, as described here, provide many advantages for cell encapsulation and culture; however, cells do not interact with highly hydrated gels because of limited protein adsorption on this “blank slate” environment. This bioinert microenvironment prevents interactions of integrins and other cell surface receptors with the gel, dramatically reducing survivability of anchorage-dependent cells. However, to initiate fundamental studies of cell–material interactions, PEG-based biomaterials provide an especially useful platform; PEG-based materials are resistant to nonspecific protein interactions, and thus, can be used to test systematically selected cell signaling molecules or other extrinsic signaling such as mechanical and/or electrical stimulation and study how this influences subsequent cellular functions. A common strategy to increase survivability in such gels is by incorporation of cell-adhesive ligands such as RGDs. Incorporation of such ligands, as demon-

strated here, increases vinculin, an important focal adhesion protein, indicating that cell-material interactions and, therefore, signaling via integrins is occurring. Therefore, by introduction of adhesive ligands, one is not only testing extrinsic signals but also the ligand of choice. From a fundamental standpoint, use of ILK-infected cells can alleviate these confounding effects.

While ILK expression is sufficient to elicit viability of cells in otherwise apoptotic conditions, ILK overproduction can cause other effects, specifically in terms of differentiation. For instance, by increasing intracellular levels of ILK, p21 is blocked, decreasing the hypertrophic differentiation of growth plate chondrocytes, which is a process necessary for endochondral ossification.³⁰ Somasiri et al. found that overexpression of ILK induced a mesenchymal transformation in mammary epithelial cells.³¹ In separate studies, ILK activity was found to be necessary for the suppression of differentiation of C2C12 myoblasts³²; however, Miller et al., using L6 myoblasts, found that ILK overexpression actually increased myoblast differentiation.³³ Clearly, ILK has varied effects on differentiation that are highly dependent on cell type. In the work described here, no significant change in phenotype was observed due to ILK overexpression based on the evaluation of histology and immunohistology of samples at day 49 (data not shown).

CONCLUSIONS

In exploration of anoikis of hMSCs, it was found that ILK production was found to be sufficient in suppressing the anoikis pathway in hMSCs. ILK is a pivotal effector in the transduction of signals from the ECM. Through incorporation of the integrin ligand, RGD, as well as by infecting the cells with ILK-expressing adenovirus, viability was rescued in anoikis-conferring conditions. The role of ILK in cell viability is important for the understanding how intracellular cytoskeletal and signaling proteins connect and communicate with the ECM for the rational design three dimensional cell culture niches.

Fellowship assistance is awarded graciously from the U.S. Department of Education's Graduate Assistantships in Areas of National Need program, the American Association of University Women, and the National Science Foundation to DSWB and from the Undergraduate Research Opportunities Program at the University of Colorado to MCT.

References

1. Koenig A, Grainger D. Cell adhesion-dependent signaling pathways on biomaterials surfaces. In: Dillow A, Lowman A, editors. *Biomimetic Materials and Design*. New York: Marcel Dekker; 2002. p 187.
2. Longhurst C, Jennings L. Integrin-mediated signal transduction. *Cell Mol Life Sci* 1998;54:514–526.
3. Ishaug SL, Thomson RC, Mikos AG, Langer R. Biomaterials for organ regeneration. In: Meyers RA, editor. *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*. New York: Wiley-VCH; 1995. pp 86–93.
4. Howe A, Aplin AE, Alahari SK, Juliano RL. Integrin signaling and cell growth control. *Curr Opin Cell Biol* 1992;10:220–231.
5. Alahari SK, Reddig PJ, Juliano RL. Biological aspects of signal transduction by cell adhesion receptors. *Int Rev Cytol* 2002; 220:145–184.
6. Nicholson KM, Anderson NG. The protein kinase B/Akt signaling pathway in human malignancy. *Cell Signal* 2002;14: 381–395.
7. Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC, Dedhar S. Regulation of cell adhesion and anchorage-dependent growth by a new β 1-integrin-linked protein kinase. *Nature* 1996;379:91–96.
8. Wu C. PINCH, N(i)ck and the ILK: Network wiring at cell-matrix adhesions. *Trends Cell Biol* 2005;15:460–466.
9. Abbott A. Cell culture: Biology's new dimension. *Nature* 2003;424:870–872.
10. Langer R, Tirrell DA. Designing materials for biology and medicine. *Nature* 2004;428:487–491.
11. Nuttelman CR, Tripodi MC, Anseth KS. In vitro osteogenic differentiation of human mesenchymal stem cells photoencapsulated in PEG hydrogels. *J Biomed Mater Res A* 2004;68: 773–782.
12. Pinske GGM, Jiawan-Lalai R, Bruijn JA, de Heer E. RGD peptides confer survival to hepatocytes via the β 1-integrin-ILK-pAkt-pathway. *J Hepatol* 2005;42:87–93.
13. Sawhney AS, Pathak CP, Hubbell JA. Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-*co*-poly(α -hydroxy acid) diacrylate macromers. *Macromolecules* 1993; 26:581–587.
14. Hern DL, Hubbell JA. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. *J Biomed Mater Res* 1998;39:266–276.
15. Bryant SJ, Nuttelman CR, Anseth KS. Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts. *J Biomater Sci Polym Ed* 2000;11:439–457.
16. Nuttelman CR, Tripodi MC, Anseth KS. Synthetic hydrogel niches that promote hMSC viability. *Matrix Biol* 2005;24:208–218.
17. Nuttelman CR, Benoit DSW, Tripodi MC, Anseth KS. The effect of ethylene glycol methacrylate phosphate in PEG hydrogels on mineralization and viability of encapsulated hMSCs. *Biomaterials* 2006;27:1377–1386.
18. Stupack DG, Cheresch DA. Get a ligand, get a life: Integrins, signaling and cell survival. *J Cell Sci* 2002;115:3729–3738.
19. Yamada KM, Miyamoto S, Aota S, Katz BZ. Fibronectin and integrin signaling and cytoskeletal regulation. *FASEB J* 1996; 10:2433.
20. Ginocotti FG, Ruoslahti E. Integrin signaling. *Science* 1999; 285:1028–1032.
21. Cardone MH, Alvesen GS, Widmann C, Johnson G, Frisch SM. The regulation of anoikis: MEK-1 activation requires cleavage by caspases. *Cell* 1997;90:315–323.
22. Dedhar S, Williams B, Hannigan G. Integrin linked kinase: A regulator of integrin and growth factor signaling. *Trends Cell Biol* 1999;9:319–323.
23. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: Roles of integrin aggregation and occupancy of receptors. *J Cell Biol* 1996;135:1633–1642.

24. Khwaja A, Rodriguez-Viciano R, Wennstrom S, Warne PH, Downward J. Matrix adhesion and ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J* 1997;16:2783–2793.
25. Ortega-Velazquez R, Diez-Marques ML, Ruiz-Torres MP, Gonzales-Rubio M, Rodriguez-Puyol M, Rodriguez PD. Arg-Gly-Asp-Ser (RGDS) peptide stimulates transforming growth factor β 1 transcription and secretion through integrin activation. *FASEB J* 2003;17:1529–1531.
26. Gary DS, Milhavel O, Camandola S, Mattson MP. Essential role for integrin linked kinase in Akt-mediated integrin survival signaling in hippocampal neurons. *J Neurochem* 2003;84:878–890.
27. Tashiro K, Moniji A, Woshida I, Hayashi Y, Matsuda K, Tashiro N. An IKLLI-containing peptide derived from the laminin α 1 chain mediating heparin-binding, cell adhesion, neurite outgrowth and proliferation, represents a binding site for integrin α 3 β 1 and heparan sulphate proteoglycan. *Biochem J* 1999;340:119–126.
28. Mann BK, Tsai AT, Scott-Burden T, West JL. Modification of surfaces with cell adhesion peptides alters extracellular matrix deposition. *Biomaterials* 1999;20:2281–2286.
29. Benoit DSW, Anseth KS. The effect on osteoblast function of colocalized RGD and PHSRN epitopes on PEG surfaces. *Biomaterials* 2005;26:5209–5220.
30. Beier F. Cell-cycle control and the cartilage growth plate. *J Cell Physiol* 2005;202:1–8.
31. Somasiri A, Howarth A, Goswami D, Dedhar S, Roskelley CD. Overexpression of the integrin-linked kinase mesenchymally transforms mammary epithelial cells. *J Cell Sci* 2000;114:1125–1136.
32. Huang Y, Li J, Zhang Y, Wu C. The roles of integrin-linked kinase in the regulation of myogenic differentiation. *J Cell Biol* 2000;150:861–871.
33. Miller MG, Naruszewicz I, Kumar AS, Ramlal T, Hannigan GE. Integrin-linked kinase is a positive mediator of L6 myoblast differentiation. *Biochem Biophys Res Commun* 2003;310:796–803.