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$\alpha 2\beta 1$ integrin-mediated mechanical signals during osteodifferentiation of stem cells from the Wharton's jelly of the umbilical cord

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Abstract

Introduction. The formation and maintenance of tissues is regulated by various signals triggered by biological, chemical, and physical factors. Data increasingly confirm that matrix or tissue elasticity plays an influential role in regulating numerous cell functions. The aim of the present study was to better understand the regulation of cellular differentiation by mechanical cues. We studied the influence of matrix stiffness on the osteodifferentiation of two cell lineages characterized by different responses: mesenchymal stromal/stem cells isolated from the Wharton's jelly of the umbilical cord (UC-MSCs) with strong stiffness-dependent responses; and bone-derived cells (BDCs), which are insensitive to changes in matrix rigidity. The study also aimed to delineate how matrix stiffness affects intracellular signaling through focal adhesion kinase (FAK) activity — one of the key components in integrin-mediated signaling pathways.

Material and methods. The effect of substrate stiffness on the expression of $\alpha 2$, $\alpha 5$, and $\beta 1$ integrin was studied using real time PCR and Western blot using cells cultured in an osteogenic medium on tunable polyacrylamide gels coated with type I collagen, with elasticities corresponding to Young's moduli of 1.46 kPa and 26.12 kPa. FAK activity was monitored using ELISA assays.

Results. We demonstrate for the first time the changes in the expression of $\alpha 2$, $\alpha 5$, and $\beta 1$ integrin subunits in perinatal stem cells and in adult osteoblast precursor cells during *in vitro* osteogenic differentiation on surfaces characterized by different stiffness. We found that matrix rigidity significantly affects the osteogenic differentiation of UC-MSCs through $\alpha 2$ integrin-mediated mechanotransduction events, though not through the $\alpha 5$ integrin subunit. In BDCs, there were no significant changes in the expression levels of the tested protein associated with varying stiffness.

Conclusions. Our results provide evidence that matrix rigidity affects the osteogenic differentiation of UC-MSCs *via* mechanotransduction events mediated by $\alpha 2$ integrin subunits. *(Folia Histochemica et Cytobiologica 2014, Vol. 52, No. 4, 297–307)*

Key words: umbilical cord; MSC; osteogenic differentiation; mechanotransduction; substrate stiffness; stem cells; integrins

Introduction

Tissue formation and maintenance is regulated by various factors, including biological, physiological, and physical signals transmitted between cells and originating from cell-matrix interactions. In the light of current knowledge, it is relatively easy to comprehend how biochemical factors (such as growth factors, cytokines, or hormones) influence the early stages of formation of cell and tissue. On the contrary, it is much more difficult to imagine and understand how mechanical forces influence biological reactions. Although more and more data is becoming available, the issue of the mechanisms that regulate cell response to mechanical forces, and their insensitivity to such stimuli, remains barely understood.

Many studies have shown a profound dependence of cellular behavior on the stiffness of the cell mi-

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croenvironment [1, 2]. We have previously demonstrated that substrate stiffness exerts varying effects on osteogenic cell differentiation, depending on the level of differentiation. The osteogenic phenotype of human mesenchymal stem cells isolated from the Wharton's jelly of the umbilical cord (UC-MSCs) or bone marrow (BM-MSCs) is regulated by matrix stiffness, whereas osteogenic differentiation of osteoblast precursor cells (BDCs, bone-derived cells) does not vary with substrate stiffness [3, 4].

Although there is strong evidence that physical properties of the matrix can influence cell behavior, it is not clearly known how the mechanical properties of the cell microenvironment are transmitted to the cell through the intracellular biochemical pathways [3, 5, 6].

Osteoblasts interact with the extracellular matrix (ECM) through specific transmembrane receptors dominant among which are integrins. Their binding to proteins adsorbed on the biomaterial surface, and later to the ECM proteins secreted by osteoblasts, is considered the most important process in mechanotransduction [7]. Proteins and ligands binding to the extracellular and cytoplasmic integrin domains forge a link between the extracellular matrix and the actin cytoskeleton. Actin microfilaments are considered one of the key elements in mechanotransduction. Moreover, integrins mediate the attachment of individual cells to basal lamina. Adhesion to the basal lamina regulates the expression of specific genes, as well as the control of proliferation and differentiation [8].

The human integrin family consists of 24 heterodimeric proteins. Each heterodimer consists of one of 18 α or 8 β subunits. We focused on the role of the $\alpha 2$, $\alpha 5$, and $\beta 1$ integrin subunits in addressing the question of whether there are any changes in integrin expression and function during the *in vitro* osteodifferentiation of cells cultured on materials of various stiffnesses. One key component in the integrin-mediated signaling pathways is focal adhesion kinase (FAK). As a signaling molecule, FAK transduces survival signals from integrin receptors, participating in adhesion through an intracellular protein cascade [9]. Knowing that FAK can be autophosphorylated at Y397 upon integrin stimulation [10], we became interested in how FAK reacts to changes in matrix stiffness.

The aim of this study was to examine differences in the cellular levels of the $\alpha 2, \alpha 5, \beta 1$ integrin subunits and of the FAK protein in response to the varying rigidity of the substrate, depending on the type of cells and/or level of differentiation. We therefore decided to isolate two types of cells: (i) UC-MSCs, which are recognized as multipotent and mechanosensitive cells, and (ii) BDCs, which are characterized by an osteoblast phenotype and are not mechanosensitive *in vitro*. Such a comparison will help us to understand the role of the external mechanical determinants in stem cell differentiation, and will also facilitate the use of a translational approach in functional tissue engineering. Controlling or predicting how stem cells differentiate into cells of a specific tissue type is a critical issue in the bioengineering of artificial tissues, in stem cell medicine, and also in pathologies such as tumorigenesis.

Material and methods

Fabrication and characterization of gel substrates. Polyacrylamide substrates (PAAM) were prepared according to the previously described protocol (3). Round glass coverslips were treated with 3-aminopropyltrimethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and 0.5% glutaraldehyde (Sigma-Aldrich). PAAM gel solution with the desired concentration of acrylamide and bisacrylamide (Bio-Rad, Hercules, CA, USA) was allowed to polymerize, forming a layer of gel on the slides. After two 10 min sessions of exposure to UV light, the polyacrylamide sheet was washed twice and incubated in a solution of type I collagen (0.2 mg/mL) overnight at 4°C. Sulfo-SANPAH (Pierce, Rockford, IL, USA) was used to link collagen-I to the PAAM gel surface. As a control, glass coverslips were coated with the same density of collagen-I. Before cells were plated, the substrates were soaked in phosphate buffered saline (PBS) and then in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Paisley, UK) at room temperature. Altering the concentration of acrylamide or bisacrylamide monomer results in changes of mechanical properties of polyacrylamide substrates. Higher stiffness of the gel correlates with the higher Young's modulus (E). Substrate stiffness was measured using a PicoPlus 5500 AFM atomic force microscope (Agilent Technologies, USA). Two different stiffness values were obtained for the polyacrylamide gel substrates: $E = 1.46$ kPa (3% acrylamide + 0.3% bisacrylamide), further described as a soft gel; and $E = 26.12$ kPa (8% acrylamide + 0.8% bisacrylamide), further described as a rigid gel. Glass coverslips (GS) served as a high rigidity control ($E > 1$ MPa) whose stiffness has no equivalent in the body's tissues.

Cell isolation and culture. All procedures were approved by the Second Local Ethical Committee of the Medical University of Warsaw (Decision No. KB148/2011). The donors provided informed consent.

The human primary culture of BDCs was isolated from postsurgery trabecular bone chips from the femoral head, based on the protocols described by Kudelska-Mazur et al. [11], with modifications. The experiments were repeated with cells obtained independently from three patients (aged

50–68 years) who underwent hip replacement. The cells of the first passage were used in all experiments.

The human UC-MSCs were isolated from the umbilical cords of three patients, according to the methods described by McElreavey et al. [12], at the Department of Cellular Engineering in the Institute of Oncology in Warsaw, Poland. Low passage number cultures were used for the experiments. The cells were cultured under standard conditions (37°C in 5% CO_2, humidified atmosphere), in a basic culture medium of low-glucose DMEM supplemented with 20% and 10% fetal bovine serum (FBS) for UC-MSC and BDC, respectively, 1% L-glutamine and 1% antibiotic — antimycotic mixture containing 10,000 U of penicillin (base), 10 mg of streptomycin (base), and 25 μg of amphotericin B/mL (Fungizone® Antimycotic, Gibco).

Osteogenic differentiation was induced using DMEM supplemented with FBS (Gibco), 100 nM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerophosphate. Cells were plated onto various substrates at a density of 1×10^5 cells/ /cm^2 and cultured for 1, 7, 14, and 21 days. Media changes were performed every 4 days. All cell culture reagents and chemicals were obtained from Invitrogen (Carlsbad, CA, USA) and Sigma-Aldrich, unless stated otherwise.

Evaluation of cell viability. Viability of the cells was determined by XTT assay (Sigma-Aldrich) based on the capacity of the metabolically active cells to convert the reaction substrate, which was yellow tetrazolium salt (2.3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyloamino) carbonyl]-2H-tetrazolium-hydroxide), into a water-soluble formazan product. Cell proliferation can be measured as an increasing cell number. The XTT cell viability assay provides a simple method for measuring the number of live cells and can thus be used to indirectly monitor proliferation and cell number. The final product of the reaction after 4 hours of incubation at 37°C was measured in a spectrophotometric ELISA reader (FluoStar Optima, MG Labtech, Offenburg, Germany) at 450 nm. The results are presented as absorbance levels; the charts present the results of one representative experiment performed in three independent repeats.

Determination of alkaline phosphatase activity. The osteogenic potential of the cells was evaluated using alkaline phosphatase (ALP) activity measurements in cell lysates with a colorimetric p-nitrophenyl phosphate assay, according to the manufacturer's instruction (Sigma-Aldrich). The amount of the final product was determined by absorbance at 405 nm using an ELISA reader (FluoStar Optima). The absorbance readings were then compared with a standard curve, and ALP activity was calculated and expressed in international units. ALP activity was normalized to the cell viability/number, as measured by the XTT assay, and shown as a ratio of normalized ALP on days 7, 14, and 21 of culturing to normalized ALP on day 1 of the UC-MSC culture on 1.46 kPa substrate.

Cell morphology evaluation. Cells were seeded at a density of 2×10^4 cells/1.9 cm^2 with the culture medium; the following day, cells were treated with differentiating medium [DMEM supplemented with FBS (Gibco), 100 nM dexamethasone, 50 μM ascorbic acid and 10 mM β-glycerophosphate]. On day 4 of the culture, the cells were washed three times with PBS and fixed with cold methyl alcohol for 10 min. The cells were then permeabilized in 0.2% Triton X-100/PBS for 1 min at room temperature and washed three times with PBS. To stain the cytoskeletal proteins, the cells were incubated in darkness with TRITC-conjugated phalloidin (Sigma-Aldrich) in PBS (1:200) for 40 min at room temperature. The cultures were washed three time with PBS and examined with a fluorescent microscope (Nikon, Eclipse TE 2000-U, Tokyo, Japan) connected to a Nikon Digital Sight DS-U1 camera.

Real time PCR. Total RNA was extracted from the cell pellet using an RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA concentration was assessed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was subsequently prepared from 250 ng RNA templates using a reverse transcription kit (Applied Biosystems, Foster City, CA, USA). On days 7, 14, and 21, the expression of Runx2, ITGA2 (α2-integrin), ITGA5 (α5-integrin), and ITGBL1 (β1-integrin) was determined by real-time PCR on three different substrates (Assay IDs: Hs00231692_m1, HS00158127_m1, Hs 00233743_m1, Hs00191224_m1; Applied Biosystems, Foster City, CA, USA) using a 7500Fast Real Time PCR System (Applied Biosystems). Detailed information for each TaqMan assay is freely available at http://www.appliedbiosystems.com. Each sample was analyzed in triplicate. The relative expression of the target genes was normalized to the reference gene GAPDH (Hs99999905_m1), and the data was analyzed using the $\Delta\Delta$Ct method [13].

SDS-polyacrylamide gel electrophoresis and western blotting. Protein isolation was carried out using a cell lysis buffer supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). The separation of protein was performed using polyacrylamide gel electrophoresis in denaturing conditions with transfer to Hybond C-extra membrane (Amersham, Amersham, UK). The membrane was blocked with Tris-buffered saline buffer with 0.1% Tween-20 (TBST) containing 5% fat-free dry milk for 30 min at room temperature. Antibodies against α2, α5, and β1 integrins (Applied Biosystem) were used according to the manufacturer's protocol. Incubation was performed overnight at 4°C under mild agitation. Subsequently, the membrane was washed with TBST and incubated with

a secondary antibody coupled to horseradish peroxidase (DAKO, Glostrup, Denmark), diluted in TBST as per the manufacturer's instructions. The signal of the secondary antibody was detected by chemiluminescence (using the ECL detection kit, Amersham), followed by autoradiography [14]. Films were quantified using IMAGE-Quant version 5.2 (Molecular Dynamic, Sunnyvale, CA, USA). The protein levels were determined by normalizing to β-actin.

FAK ELISA assays. ELISA assays were used to determine the total and phosphorylated FAK levels in protein lysates [FAK (Total) ELISA kit (catalogue #KHO0431; Invitrogen) and FAK[pY397] ELISA kit (cat. #KHO0441; Invitrogen), respectively]. The tests were performed according to the manufacturer's protocols.

To deduce the number of cells per sample, 25-μL aliquots of the total protein extracts were taken, and the amount of protein was determined by means of a bicinchoninic acid (BCA) assay (Pierce), according to the manufacturer's protocol. Total FAK and pY397-FAK levels were normalized to cell viability/number, as measured in the XTT test. This assay was repeated three times to allow statistical analysis.

Statistical analysis. Statistical analysis of the results was carried out using nonparametric ANOVA (Kruskal-Wallis test with Dunn's *post hoc* test). Differences at the level of $p < 0.05$ were accepted as significant. The analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

Cell viability

Two types of cells undergoing osteogenesis, UC-MSCs and BDCs, were cultured on collagen-coated polyacrylamide substrates of differing rigidity for up to 3 weeks. The XTT assay was performed to monitor cell survival and growth. Both cell types cultured on all substrates showed increasing cell proliferation activity along culture duration.

Figure 1 shows that the metabolic activity (which represents ongoing proliferation) of UC-MSCs on the softer gel was lower than on the stiffer substrates. Statistically significant differences were observed during the first seven days of culture. On the day 7, the resulting optical density (OD) on the 1.46 kPa, 26.12 kPa, and GS substrates were 0.26, 0.54, and 0.68, respectively.

There were no statistically significant differences between survival and growth of the BDCs in all analyzed conditions and between the proliferation activity of UC-MSCs and BDCs on all elastic materials on days 14 and 21 of the experiment.

Verification of osteogenic differentiation

The induction of osteogenic differentiation in UC-MSCs and BDCs was evaluated by the measurement of

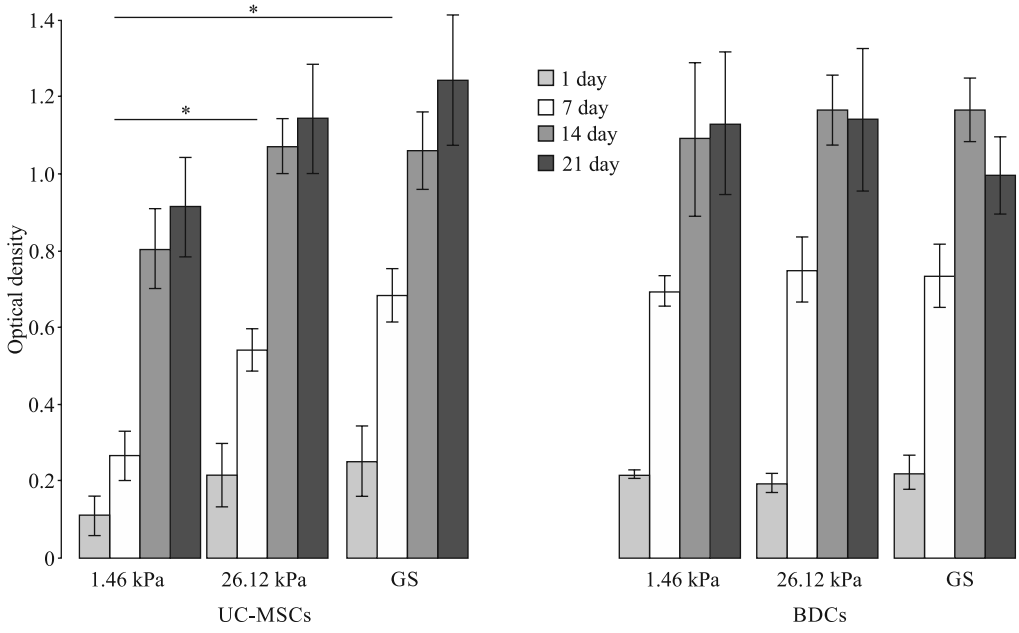

Figure 1. Cell proliferation activity on substrates of differing rigidities determined by XTT assay. Young's modulus: E = 1.46 kPa and E = 26.12 kPa; GS: glass coverslips. During the first seven days of culture, UC-MSCs on the soft gel had lower proliferation activity than on the stiffer substrates. Data are presented as mean ± SD (bars) from three independent experiments. Statistically significant results are indicated (*$p < 0.05$)

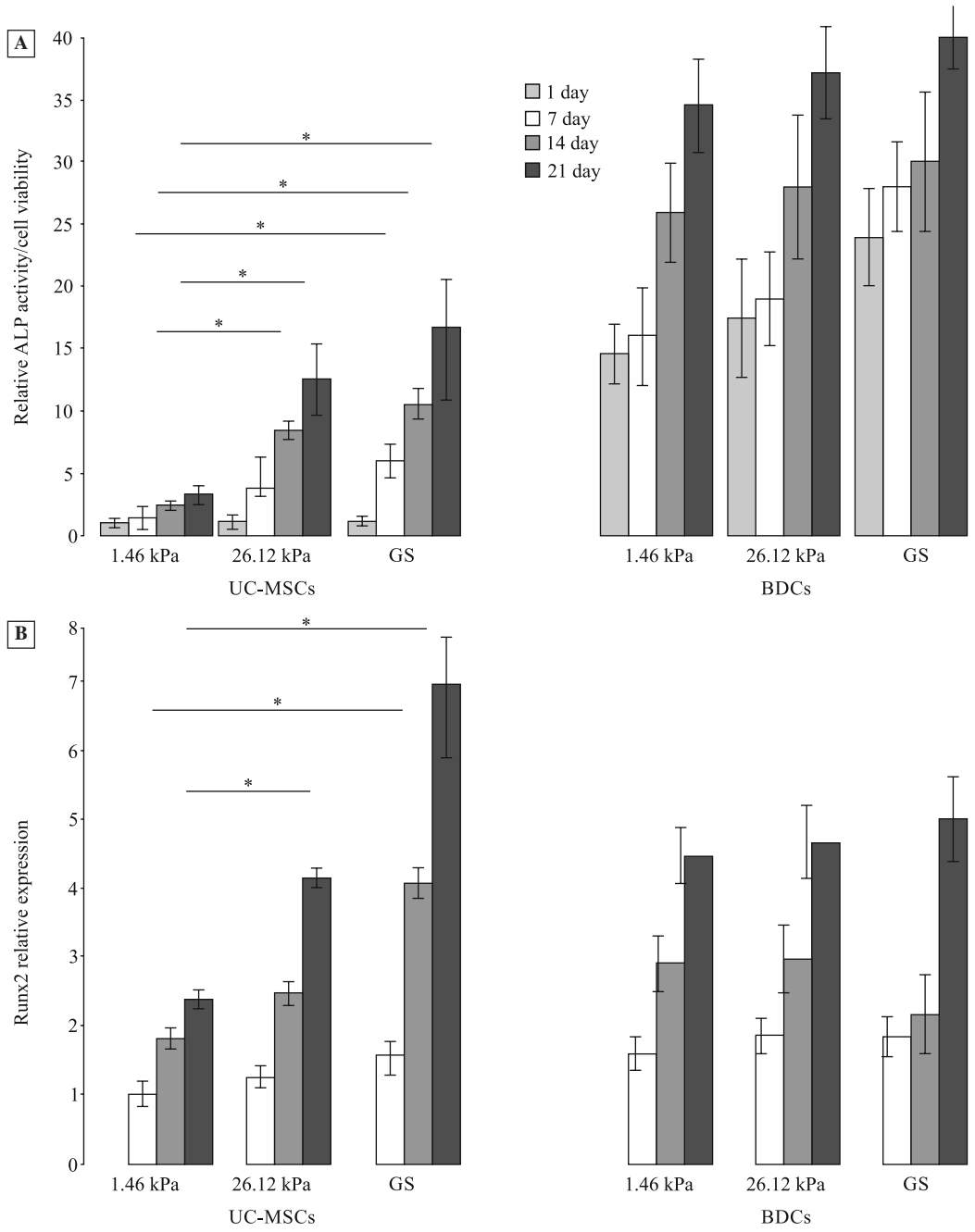

Figure 2. Verification of osteogenic differentiation by the measurements of ALP activity and Runx2 gene expression. All values are expressed as mean ± standard deviation (SD, bars) of three independent experiments. Statistically significant differences between the results are indicated (*$p < 0.05$). **A.** ALP activity assay. ALP activity was measured as described in the Methods. Description of histograms and bars are as for Figure 1; **B.** Relative expression of Runx2 gene as a target -to-reference ratio of mRNA levels determined by real-time PCR. The relative expression of Runx2 was higher in UC-MSCs grown on the more rigid substrates (gel and glass coverslip) than on the softer one. Gene expression was normalized to the reference gene GAPDH

alkaline phosphatase (APL) activity (Figure 2A) and expression of the Runx2 gene (Figure 2B). We found an 11-fold increase in ALP activity between days 1 and 21 of the UC-MSC cultured on the rigid gel, but only a 3.4-fold increase at the same time points for the UC-MSC cultured on soft gel. Moreover, the Runx2 gene expression of the UC-MSCs was higher in UC-MSCs grown on the more rigid substrate than on the softer substrate. Thus, the more rigid substrate (26.12 kPa) accelerated the osteogenic differentiation of the UC-MSCs, while having no effects on the osteo-differentiation of the BDCs.

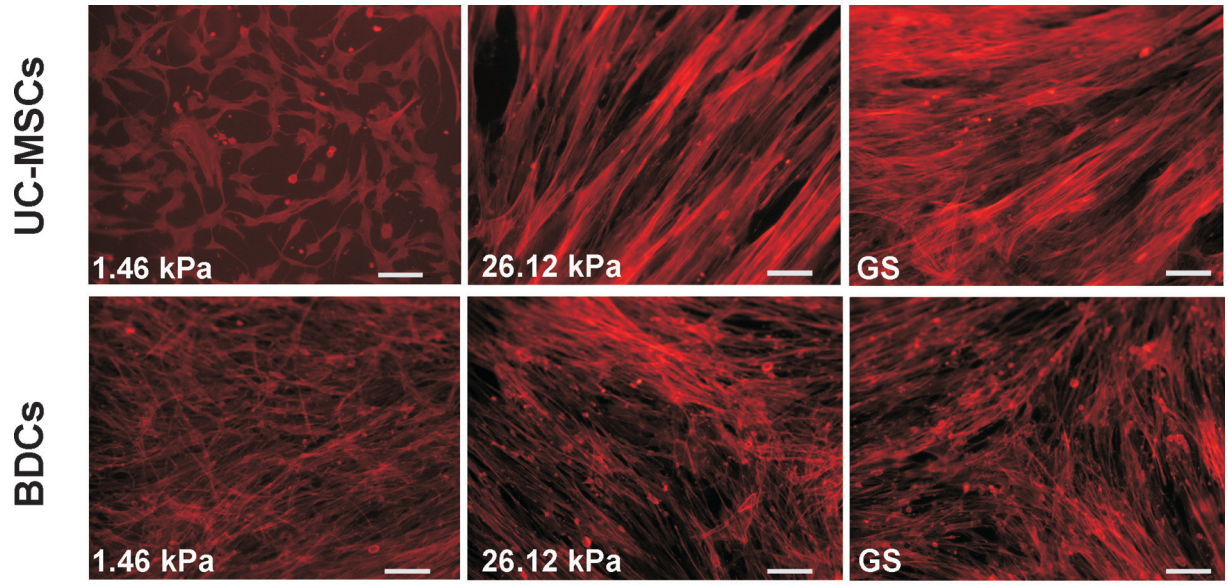

Figure 3. Morphology of cells grown on the tested surfaces on day 7. Actin filaments (red) were visualized by TRITC--phalloidin staining as described in the Methods. UC-MSCs cultured on soft gel (1.46 kPa) are less densely spread and more spindle-shaped than the same cells cultured on the more rigid substrates (26.12 kPa and GS), whereas no such differences were found for BDCs. Bars represent 100 μm

Cell morphology

The morphological observation indicated that, on day 1, both types of cells were well spread over the surface of the GS and elastic substrates (data not shown). At this time, actin microfilaments were not visualized using phalloidin staining. The morphology of the UC-MSCs on the substrates of various degrees of stiffness had evidently changed by day 7. Cells cultured on the soft gel (1.46 kPa) were less densely spread and more spindle-shaped than the same cells cultured on the more rigid substrates (26.12 kPa and glass) (Figure 3). The BDCs were evenly distributed on the surface of all substrates, but their morphology, spreading mode, and actin cytoskeleton organization was similar on soft and rigid gels. Interestingly, after 14 days, we did not observe significant differences in cell morphology or actin cytoskeleton distribution between the UC-MSCs grown on the different substrates (data not shown).

Integrin expression

Cells were harvested at days 7, 14, and 21 to assess the effect of culture duration on the expression of genes for the $\alpha 2$, $\alpha 5$, and $\beta 1$ integrin subunits. Generally, the relative expression levels of the tested genes increased with time. The relative expression of genes encoding $\alpha 2$ and $\beta 1$ integrin subunits were higher in UC-MSCs grown on the more rigid substrate than on the softer one (Figure 4A, C, left column). On the day 7, the relative expression of the $\alpha 2$ subunit gene was 2.6-fold higher in UC-MSCs grown on the more rigid gel, and 3.6-fold higher on the control glass coverslips, than on the softer substrate. Similarly, on day 14 of the culture, the $\alpha 2$ subunit mRNA levels were 1.7-fold and 2-fold ($p < 0.05$) higher, respectively. The results demonstrated that $\beta 1$ subunit gene expression increased on the stiffer substrate (1.5-fold), as compared to the softer substrate, over 3 weeks of culture, whereas the expression of $\alpha 5$ integrin subunit gene remained at similar levels (Figure 4B, left column). No such differences were observed between any of the analyzed genes in the BDCs (right column). The expression of the $\alpha 2$ and $\beta 1$ integrin subunit genes was relatively constant as a function of increasing stiffness in the BDCs. The relative levels of integrin $\beta 1$ subunit mRNA in the UC-MSCs were higher, but the $\alpha 5$ subunit mRNA level was reduced on all surfaces, as compared to the BDCs, in cells cultured on all substrates. For the BDCs, there were differences in $\alpha 5$ subunit gene expression on the tested substrates (increasing from 1.2-fold to 1.7-fold).

Because the differences in the investigated processes (gene expression and actin cytoskeleton organization) between the UC-MSCs and BDCs were most significant on day 7 of the culture, we decided to verify the protein level specifically on this day. Western blot analysis of the human UC-MSCs grown on the rigid surfaces showed increased levels of $\alpha 2$ integrin

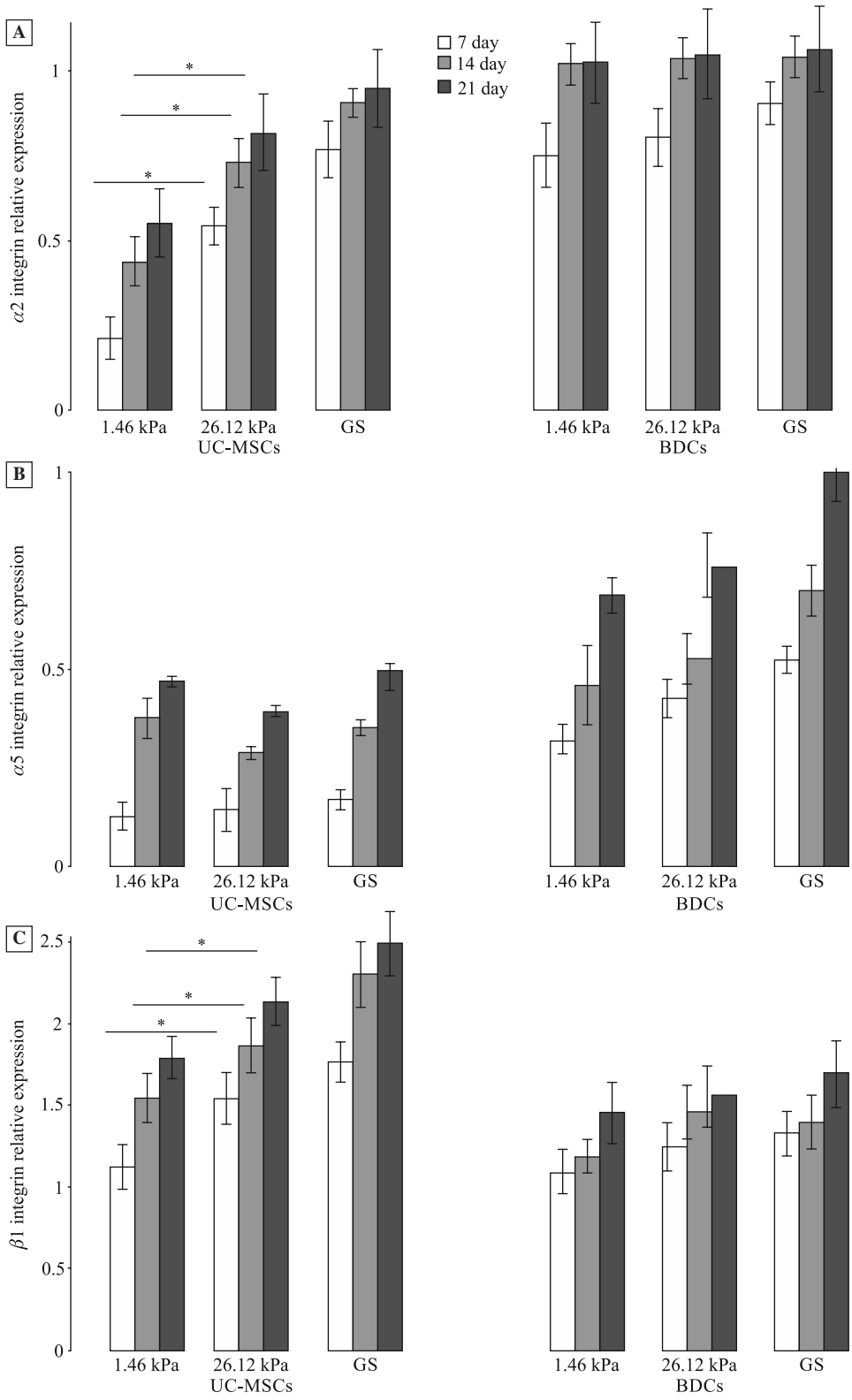

Figure 4. Relative gene expression of integrin subunits: $\alpha 2$ (**A**), $\alpha 5$ (**B**), and $\beta 1$ (**C**) in UC-MSCs and BDCs cultured on substrates of differing stiffness. The relative levels of $\alpha 2$ and $\beta 1$ subunits mRNAs were higher in UC-MSCs grown on the rigid substrate (26.12 kPA) than on the softer one (1.46 kPa). The expression of the $\alpha 5$ integrin subunit gene did not differ between UC-MSCs cultured on substrates of differing stiffness. The expression of all the studied genes at the given time did not differ in the BDCs cultured on substrates with different stiffness (right column). Y axis: the relative levels of the mRNAs, as determined by real-time PCR. The gene expression was normalized to the reference gene GAPDH. All values are expressed as mean ± SD (bars) of three independent experiments. Statistically significant differences between the results are indicated (*$p < 0.05$)

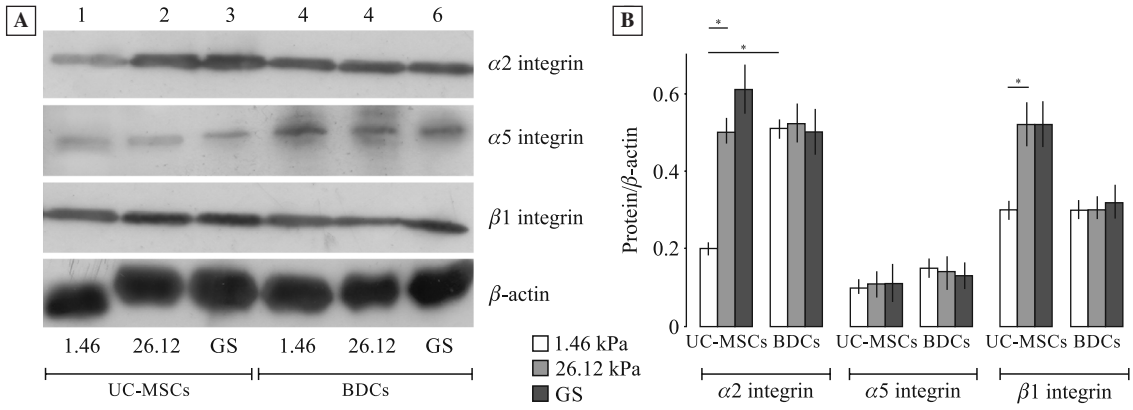

Figure 5. Effects of substrate stiffness on protein levels of the integrins in cells cultured for 7 days. Protein expression was determined by western blot analysis, as described in the Methods. **A.** Representative gels; **B.** The relative quantities of the integrin subunits after normalization, with the level of β-actin used as control. All the values are expressed as means ± SD of three biological replicates. Statistically significant differences between the results are indicated (*$p < 0.05$)

Table 1. Effects of substrate stiffness on the activation of total Focal Adhesion Kinase (FAK) and autophosphorylated FAK at tyrosine residue 397 (FAK[pY397])

Substrate stiffness	Total FAK (units/mL ± SD)				FAK[pY397] (units/mL ± SD)			
	1 day	7 day	14 day	21 day	1 day	7 day	14 day	21 day
	UC-MSCs							
1.46	1.07 ± 0.25	4.24 ± 0.46	5.2 ± 0.21	6.9 ± 0.31	0.5 ± 0.11	1.9 ± 0.21*	1.9 ± 0.51*	2.1 ± 0.31*
26.12	1.6 ± 0.38	6.1 ± 0.51	6.3 ± 0.41	6.5 ± 0.14	0.56 ± 0.19	3.4 ± 0.41	4.2 ± 0.61	4.9 ± 0.62
GS	1.8 ± 0.19	5.42 ± 0.48	5.92 ± 0.5	6.97 ± 0.19	0.6 ± 0.12	4.1 ± 0.37	4.5 ± 0.31	5.3 ± 0.57
	BDCs							
1.46	2.07 ± 0.11	4.37 ± 0.39	4.81 ± 0.19	5.38 ± 0.48	1.3 ± 0.25	3.0 ± 0.25#	4.1 ± 0.45#	4.3 ± 0.55#
26.12	2.08 ± 0.2	4.74 ± 0.48	5.13 ± 0.4	5.44 ± 0.38	1.4 ± 0.18	3.1 ± 0.45	3.9 ± 0.5	4.5 ± 0.47
GS	2.11 ± 0.19	4.85 ± 0.38	4.95 ± 0.28	5.63 ± 0.39	1.4 ± 0.17	3.2 ± 0.43	4.1 ± 0.32	4.5 ± 0.36

Data are shown as mean ± SD from three independent experiments; *statistically significant differences between levels of FAK[pY397] in UC--MSCs cultured on soft versus stiff substrates ($p < 0.05$); #statistically significant differences between BDCs and UC-MSCs cultured on the soft substrates ($p < 0.05$)

subunit and unaltered levels of the integrin α5 subunit. UC-MSCs cultured on the stiffer surface exerted an increased level of β1 integrin subunit (Figure 5). In the BDCs, there were no significant changes in the expression of protein levels in a stiffness-dependent manner.

Focal adhesion kinase activation during osteogenic differentiation on matrices of differing stiffness

According to hypothesis that extracellular matrix stiffness regulates the activity of FAK, important mediators of integrin-mediated signaling — total and phosphorylated FAK (FAK[pY397]) — were monitored using ELISA assays. We decided to use an ELISA assay because of its greater sensitivity than Western blot analysis. The data are presented in Table 1.

The relative levels of FAK[pY397] to total FAK decreased from 46% to 30% between days 1 and 2 of the UC-MSC culture on soft gel.

When the UC-MSCs were cultured on the studied substrates, the relative levels of FAK[pY397] to total FAK ratio increased with increasing stiffness of the underlying substrate. Specifically, on day 7 of the culture, the ratio for the soft gel ($E = 1.46$) was determined to be 44%. The ratio increased significantly to 55% and 75% on the stiff gel ($E = 26.12$) and GS, respectively.

On day 14 of the UC-MSC culture, the differences between the soft and rigid gel were even more pronounced. At this time, the FAK[pY397] to total FAK ratios were 36% and 66%, respectively. By day 21 of the culture, these ratios had changed to 30% on the soft gel and 75% on the rigid gel. This trend clearly shows that, in the absence of any other cues,

FAK phosphorylation at tyrosine residue 397 in UC--MSCs depends on the mechanical properties of the substrates.

When the BDCs were cultured on the substrates tested here, there were no statistically significant differences between the FAK[pY397]/FAK ratio at any time point (Table 1).

Discussion

The role of mechanical signals in the regulation of the behavior and fate of cells has been much less studied and is less understood than the role of biochemical stimuli. Increasing understanding of how epigenetic factors, such as the mechanical environment, regulate stem cell fate is being obtained through the use of tightly controlled *in vitro* models. PAAM, in allowing changes to its elasticity without modifications of the chemical space, is a convenient tool for verifying the hypothesis that substrate stiffness affects cell differentiation, and thus the formation of tissues. In our study, different stiffness values were used: a soft gel (E = 1.46 kPa) corresponds to the stiffness of brain tissue, while the rigid gel (E = 26.12 kPa) corresponds to bone tissue. The glass coverslips (GS) provided high rigidity. Our previous studies show that substrate stiffness may regulate the osteogenic differentiation of UC-MSCs and BM-MSCs, but does not affect the *in vitro* response of BDCs to osteogenic stimuli [3, 4, 15]. We suspect that the stronger osteogenic response of BM-MSCs and UC-MSCs to the more rigid substrate can be attributed to the fact that they are more sensitive to mechanical stimuli during differentiation and tissue formation — in contrast to more committed cells, such as BDCs. This may be related to the different origins and biology of the stem cell types, but the real question concerns how cells feel and receive information about environmental stiffness, and how they transduce this into intracellular signals.

Since the properties of material surfaces — such as their topography, chemistry, or surface — play an essential role in osteogenic cell activity, adhesion and growth (mainly through integrins which are the primary receptors for cellular interactions with the extracellular matrix macromolecules) [16–19], we decided to check the differences in $\alpha 2$, $\alpha 5$, and $\beta 1$ integrin subunit gene and protein expression between UC-MSC and BDC cell types. In summary, after comparing the $\alpha 2$, $\alpha 5$, and $\beta 1$ expression levels in these two cell types, varying the substrate stiffness, we found different patterns of their expression: in UC-MSCs, the $\alpha 2$ and $\beta 1$ integrin subunits play an important role in determining osteoblast behavior, and $\alpha 2$- and $\beta 1$-dependent signaling is an important target for stimulation by stiffness of the osteogenic response of developmentally young naive stem cells. We found a diminished expression of the $\alpha 5$ subunit with ongoing differentiation on the mRNA and protein levels in UC-MSCs, compared to preosteoblast BDCs. Previous studies have demonstrated that matrix stiffness controls the formation of $\beta 1$-integrin/FAK complexes, and that $\beta 1$ integrin has been shown to be important in mediating the response of human bone-marrow-derived MSCs to mechanical stimulation [20]. In osteoblasts, the $\alpha 2\beta 1$ heterodimer, consisting of the $\alpha 2$ and $\beta 1$ subunits, is essential for the interaction with type I collagen, while $\alpha 5\beta 1$ integrin binds to surface-attached fibronectin [21]. In our experiments, the $\alpha 2$ integrin subunit gene and protein showed higher expression than did $\alpha 5$, which may be related to the coverage of collagen substrates and their extracellular concentration, being much higher than fibronectin assembled into the extracellular matrix. Our findings are in agreement with the results of Olivares-Navarrete and coworkers, who showed that $\alpha 2\beta 1$ integrin mediates osteoblast response to titanium (Ti) surfaces, and that downregulation of $\alpha 5\beta 1$ does not affect the differentiation of human osteosarcoma cell lines MG63 grown on Ti [22]. We hypothesize that the elasticity of the substrate plays a role in the conformational stability of $\alpha 2\beta 1$ integrin's bond to its ligand.

The major link connecting the extracellular matrix to the actin cytoskeleton through integrin receptors is focal adhesion kinase (FAK) [23]. The integrin receptors that cluster in the cell membrane when these receptors bind ligands trigger a conformational change in the associated FAK to allow for the autophosphorylation of residue Y397. The Y397 phosphorylation of FAK has been implicated in the osteogenic differentiation of mesenchymal stem cells [24]. In UC-MSCs, rigid substrates can induce the formation of large focal adhesions more efficiently than can flexible substrates of the same chemical composition. Focal adhesions grow under tension, generate strong adhesion, and induce upstream signaling. Peng and coworkers [25], in the mechano-sensitive model generated in their Monte Carlo study, showed the enhancement and acceleration of integrin clustering with substrate stiffness. The simulations reflect the synergy between substrate rigidity, reaction energies, and integrin density in determining the clustering status [25]. The results of Gershlak et al. also support our observations: they found that gel stiffness positively correlates with the number of $\beta 1$ integrin bonds formed and with FAK phosphorylation at the Y397 residue in mesenchymal stem cells [26]. Since we observed the UC-MSCs' weak osteogenic response to

soft substrates and lower total FAK phosphorylation at Y397, it is possible that shorter focal adhesions to soft substrates would result in lower levels or lower stability of $\beta 1$ subunit complexes (in our experiments $\alpha 2\beta 1$).

Both tested cell types differ in their degree of potential for differentiation: UC-MSCs are multipotent or even pluripotent stem cells, considered to be more primitive stem cell populations than BDCs, which are unipotent stem cells that have traditionally been considered osteoblastic cells. Several studies have reported that cells mimic their *in vivo* behavior better when they are cultured on substrates whose rigidity resembles that of the cell type's native tissue matrix; however, we did not observe such a correlation supporting the osteogenic differentiation of BDCs by stiffer substrates [3, 4, 6, 27]. It has, however, been observed that the osteogenic differentiation of dental follicle cells is supported by soft matrices [28]. It is thus difficult to formulate a universal principle.

The mechanism through which stem cell differentiation is affected by substrate rigidity is not well understood. One hypothesis to explain the substrate stiffness-dependent differences among cells suggests that BDCs, as cells that are adapted to the rigid environment (precalcified bone $E \sim 100$ kPa; calcified bone $E \sim 4$ GPa), may not demonstrate a sensitivity to relatively small changes in substrate stiffness (1.46 kPa––26.12 kPa), while for UC-MSCs, this difference may be noticeable and sufficient for changes in phenotype. Perhaps multipotent or even pluripotent stem cells like UC-MSCs have a unique mechanism for inducing a stem cell lineage specified by ECM elasticity, and which functions only in the early stages of development.

The mechanism by which matrix stiffness affects cell behavior across integrins and FAK-mediated signaling is poorly understood. In this report we have for the first time compared the changes in $\alpha 2$, $\alpha 5$, and $\beta 1$ integrin subunit levels in umbilical cord stem cells and adult osteoblast precursor cells during *in vitro* osteogenic differentiation on surfaces of different stiffness. We found that matrix rigidity significantly affects the osteogenic differentiation of UC-MSCs through $\alpha 2$ integrin-mediated mechanotransduction events, and not through the $\alpha 5$ integrin subunit. In BDCs, there were no significant stiffness-dependent changes in the expression of $\alpha 2$, $\alpha 5$, or $\beta 1$ or in the activation of the FAK protein. What determines the reception of physical stimuli and the sensitivity of cells to these signals? There is a clear need for integrated genomic and proteomic approaches to define how these signaling pathways are regulated, and how they overlap in cell behavior. This is especially interesting, since cancer-transformed cells are defective in their responses to mechanical signals from the matrix, as compared to normal cells [29]. Several *in vitro* and *in vivo* studies have demonstrated the association between the regulation of integrin expression and cancer [8, 30, 31]. Changes in the integrin pattern during malignant transformation are highly dependent on the type of cancer, and the integrin expression pattern allows normal cells to be distinguished from cancer cells. Besides their protumorigenic effects, integrins can also suppress tumorigenic transformations in specific circumstances. Knowledge of the response to such regulatory factors is crucial in understanding integrins' role in the proliferation, migration, differentiation, and regeneration of normal cells and tumor cells.

Understanding how cells respond to stiffness signals from the microenvironment should increase the efficiency of stem cell therapies, but also of cancer therapies, and may thus have important clinical implications.

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