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Mechanical Integrin Stress and Magnetic Forces Induce Biological Responses in Mesenchymal Stem Cells Which Depend on Environmental Factors

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ABSTRACT

The control of mesenchymal stem cells (MSC) by physical cues is of great interest in regenerative medicine. Because integrin receptors function as mechanotransducers, we applied drag forces to β1 integrins on the apical surface of adherent human MSC. In addition to mechanical forces, the technique we used involved also the exposure of the cells to an inhomogeneous magnetic field. In order to assess the influence of the substrate on cell adhesion, cells were cultured on plain tissue culture polystyrene (TCP) or on coated well plates, which allowed only adhesion to embedded fibronectin or RGD peptides. We found that the expression of collagen I, which is involved in osteogenesis, and VEGF, a factor which stimulates angiogenesis, increased as a result of short-term mechanical integrin stress. Whereas, collagen I expression was stimulated by mechanical forces when the cells were cultured on fibronectin and RGD peptides but not on TCP, VEGF expression was enhanced by physical stimulation on TCP. The study further revealed that magnetic forces enhanced Sox 9 expression, a marker of chondrogenesis, and reduced the expression of ALP. Concerning the intracellular mechanisms involved, we found that the expression of VEGF induced by physical forces depended on Akt activation. Together, the results implicate that biological functions of MSC can be stimulated by integrin-mediated mechanical forces and a magnetic field. However, the responses of cells depend strongly on the substrate to which they adhere and on the cross-talk between integrin-mediated signals and soluble factors. J. Cell. Biochem. 111: 1586–1597, 2010.

KEY WORDS: MESENCHYMAL STEM CELLS; INTEGRIN; MECHANICAL LOADING; BIOLOGICAL RESPONSE

Mesenchymal stem cells (MSC) represent a cell population localized in stem cell niches in a variety of tissues, including bone marrow [Yin and Li, 2006; da Silva Meirelles et al., 2008]. These adult stem cells are required to replace expired cells of the connective tissue or serve to repair damaged tissue [Caplan, 2009]. Therefore, the cells are capable of differentiating into multiple cell lineages of the mesoderm, including osteoblasts, chondrocytes, and adipocytes [Bianco et al., 2008]. In addition, it became evident that MSC are secretory and release a number of bioactive factors which regulate cells in their vicinity [Caplan, 2009]. These trophic effects via soluble factors control regeneration of cells or tissues without generating newly differentiated cells. Functions and phenotype conversions of MSC in a stem cell niche in vivo are controlled by multiple signals of high complexity, which include soluble factors, cell–cell interactions and signals from the extracellular matrix [Scadden, 2006; Discher et al., 2009; Ellis and Tanentzapf, 2009]. The extracellular matrix to which cells adhere provides chemical as well as physical signals [Guilak et al., 2009]. Mechanical interaction with the extracellular matrix is bidirectional and plays a dominant role in cell regulation. Cells are able to sense forces to which they are exposed and generate forces to the substrate, which enable a rearrangement of matrix proteins [Guilak et al., 2009]. Mesenchymal stem cells are also able to sense the elasticity of a substrate, which determines the direction of differentiation or maintains their quiescence [Engler et al., 2006; Winer et al., 2009]. These mechanical interactions are accompanied

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by changes of the cell shape. Cells are flatter on stiff substrates than on soft ones; this involves dramatic alterations in the formation of actin–myosin stress fibers [Zajac and Discher, 2008]. To analyze the complex mechanisms which are involved in mechanical interactions with the extracellular matrix, we applied a technique to mechanically load β1 integrins on the dorsal surface of adherent human MSC. Cells use integrin receptors to specifically adhere to extracellular matrix proteins and to transduce mechanical signals both outside-in and inside-out [Legate et al., 2009; Moser et al., 2009]. In previous studies, we demonstrated that the application of drag forces to integrins on osteoblastic cells induced the accumulation of focal adhesion proteins in the vicinity of the stressed integrins, activation of focal adhesion kinase (FAK) and its localization to the actin cytoskeleton [Rychly et al., 1998; Schmidt et al., 1998; Pommerenke et al., 2002]. These events induced an activation of extracellular signal-regulated kinases (Erk) downstream within the signaling pathway.

To test the combination of mechanical integrin stress on the apical surface of the cells with the effect of the integrin-mediated adhesion to a substrate, cells were plated on tissue culture polystyrene (TCP) and on modified surfaces for more controlled cell adhesion. For this purpose, sP(EO-stat-PO) coatings were applied to well plates and modified with either fibronectin or the fibronectin-derived peptide sequence RGD. Such coatings have already been shown to suppress unspecific adhesion events and only allow adhesion to the immobilized cell adhesion motives [Groll et al. 2005; Gasteier et al. 2007]. The technique we used to mechanically stress integrins is based on paramagnetic beads, which are moved by a magnetic field. Therefore, the magnetic field is another physical factor to which the cells are exposed in our experiments. Previous studies have shown that magnetic fields can induce a physiological response in cells, for example, enhanced proliferation and differentiation [Santini et al., 2009].

The principal findings we present here show that a short-time mechanical loading of integrins as well as the magnetic field affected the expression of differentiation markers and of the vascular endothelial growth factor (VEGF), a factor responsible for vascularization. However, the cell responses depended strongly on both the adhesive substrate and the culture medium. The results emphasize the application of physical factors in strategies to control stem cells for tissue regeneration.

MATERIALS AND METHODS

CELL CULTURE

Human mesenchymal stem cells (hMSC) were isolated from bone marrow, which was obtained during median sternotomy from both male and female patients ranging in age from 63 to 78 years. According to a standard protocol described elsewhere [Muller et al., 2008], interface-enriched cells from a diluted marrow sample (d–1.077 g/ml) were cultured in expansion medium (EM) in 5% CO_2 and at 37°C for 24 h after density gradient centrifugation. Adherent cells were harvested and the purity of mesenchymal stem cells was proved by the absence of the hematopoietic marker CD34 and their ability to differentiate both to osteoblasts and adipocytes. Cells were grown in cell culture flasks using EM for 2–4 weeks and used in the second passage for the experiments. The following cell culture media were used during the experiments: EM: Dulbecco's modified Eagle's medium (DMEM) (high glucose, 4.5 g/L D-glucose; Invitrogen, Carlsbad, CA); osteogenic differentiation medium (ostDIM): DMEM containing 100 nM dexamethasone, 10 mM β-glycerophosphate and 10 μg/ml ascorbic acid (all from Sigma–Aldrich, St. Louis, MO); adipogenic differentiation medium (adiDIM) was obtained from Lonza (Basel, Switzerland) and supplemented with the SingleQuots® provided containing 3-isobutyl-1-methylxanthine, dexamethasone, indomethacin, and insulin (Lonza). All media contained charge-tested 10% fetal calf serum (FCS; PAN-Biotech GmbH, Aidenbach, Germany) and 1% antibiotic–antimycotic solution (Invitrogen).

For the experiments, cells were detached with trypsin/EDTA (0.05% trypsin, 0.02% EDTA) (Sigma–Aldrich), seeded into single wells of 96-well Stripwell plates (Corning, Inc., Corning, NY) or wells, which contained surfaces with immobilized fibronectin or RGD peptides (see below). Cells were grown to subconfluency (80% confluency).

PREPARATION OF SURFACES FOR CELL ADHESION

For comparison with TCP, well plates were coated using NCO-terminated six-arm star-shaped polymers (NCO-sP (EO-stat-PO)) to prevent unspecific protein adsorption. The following biofunctionalization with fibronectin and the peptide sequence GRGDS (RGD peptides) allowed specific cell adhesion [Groll et al., 2005; Gasteier et al., 2007]. The synthesis of NCO-sP(EO-stat-PO) through reaction with isophorone diisocyanante (IPDI) was performed as described elsewhere [Gotz et al., 2002; Groll et al., 2005].

To obtain RGD functionalized surfaces, commercially available CovaLink NH BreakApart Modules (Nunc/Thermo Fisher Scientific, Waltham, MA) were coated with NCO-sP(EO-stat-PO)-solution (10 mg/ml; 9:1 in water:THF) to which GRGDS (1 mg/ml in distilled water; Bachem AG, Bubendorf, Switzerland) was added in the molar ratio of 2:1. The wells were filled with the mixed solution (400 μl per well) and allowed to react for 10 min. Afterwards, the solutions were aspirated using a vacuum system (Vacuboy) and the wells were stored at ambient conditions for at least 12 h to allow complete cross-linking of the coatings. The wells were then washed thrice with sterile water and used for cell culture.

To functionalize the surfaces with human fibronectin (Sigma–Aldrich) the wells were primarily coated with NCO-sP(EO-stat-PO). After 1 h cross-linking time, each well was filled with 50 μl fibronectin solution (50 μg/ml in distilled water) and incubated at room temperature overnight. Again the solution was removed using a vacuum system. Unbound protein was removed by washing thrice with sterile water before the wells were used for cell culture.

DESIGN FOR APPLICATION OF PHYSICAL STRESS

CELL INCUBATION WITH MICROBEADS

To stress integrin receptors mechanically, paramagnetic microbeads were coated with an antibody against the β1 integrin subunit. Preparation of beads and incubation are described elsewhere [Pommerenke et al., 1996]. In brief, paramagnetic microbeads, 2.8 μm in size and coated with sheep anti-mouse antibody (Dynal,

Hamburg, Germany) were used. These beads were incubated with 1 μg of mouse anti-β1 integrin antibody (clone Lia 1/2; Beckman Coulter, Fullerton, CA) for 1 h with shaking. After washing the beads in phosphate-buffered saline (PBS), the beads were resuspended in DMEM. Beads were added to the cell monolayer and incubated for 30 min in 5% CO_2 at 37°C. On average, 5–10 microbeads were attached to the β1 integrin subunit on the apical surface of one cell.

APPLICATION OF PHYSICAL STRESS

A magnetic device, which was described in detail earlier was used [Pommerenke et al., 1996]. Briefly, the system consists of a coil system containing a ferrite core with two differently shaped poles to generate an inhomogeneous magnetic field. The distance between the poles is 1 cm and the average strength of the magnetic field between the magnetic poles was 0.015 T, as measured using a Hall probe. The differences of the magnetic field strength across the length of the cell are insignificant.

A culture well containing the prepared cells was located between the two poles of the device. Drag forces act in the horizontal direction, that is, parallel to the apical cell surface, on the magnetic beads that are attached to the receptors. The forces subjected to one bead were adjusted to 2×10^{-10} N. Because of the varying number and location of beads attached to one cell, differential strains across the cell can occur. A cyclic stress of 1 Hz (0.5 s on, 0.5 s off) was applied for 15 min. For comparison, cells were incubated with anti-β1 integrin antibody coated beads for 30 min for clustering and the magnetic field was applied without beads for 15 min. A scheme of the experimental settings is given in Fig. 1.

FIELD EMISSION SCANNING ELECTRON MICROSCOPY

Cells were seeded on titanium plates for adhesion, incubated with beads and mechanically stressed as described above. During the application of stress, samples were fixed with 4% paraformaldehyde, followed by dehydration using increasing concentrations of ethanol. For visualization, a field emission scanning electron microscope with SCHOTTKY-emitter and GEMINI-optics for ultra-high resolution was used (SUPRA 25 GEMINI FE-SEM with EDX-system QUANTAX/Xflash, Bruker-AXS, Carl Zeiss, Oberkochen, Germany). The application of this technique using a very low beam voltage allows the analysis of adherent cells without conductive coating.

FLOW CYTOMETRY

The expression of integrin subunits of hMSC was determined by flow cytometry using the flow cytometer FACSCalibur (BD Biosciences, Franklin Lakes, NJ). hMSC were prepared for analysis as follows: Cells were detached with trypsin/EDTA (0.05% trypsin, 0.02% EDTA) and washed with PBS. The following monoclonal anti-integrin antibodies against subunits β1 (CD29), α2 (CD49b), α3 (CD49c), α4 (CD49d), α5 (CD49e), α6 (CD49f), αV (CD51) (all from Beckman Coulter), α1 (CD49a) were used for cell staining; as negative control anti-mouse IgG_1 (both from BD Biosciences), and as secondary antibody FITC-conjugated anti-mouse IgG (Fab_2) fragment (Sigma–Aldrich) were used. A total of 10,000 gated events were collected and analyzed using FlowJo software version 5.7.2 (Tree Star, Ashland, OR). Flow cytometric data were obtained from five independent experiments to ensure reproducibility. A representative example is shown for each integrin.

REAL-TIME RT-PCR

Total RNA was isolated using the NucleoSpin RNA II Kit with DNase treatment (Macherey-Nagel, Düren, Germany). After quantifying RNA spectrometrically, 150 ng of total RNA was reverse transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen) for cDNA synthesis. Quantitative real-time PCR assays were performed and monitored in triplicate using an ABI PRISM® 7500 sequence detection system (Applied Biosystems, Darmstadt, Germany). The

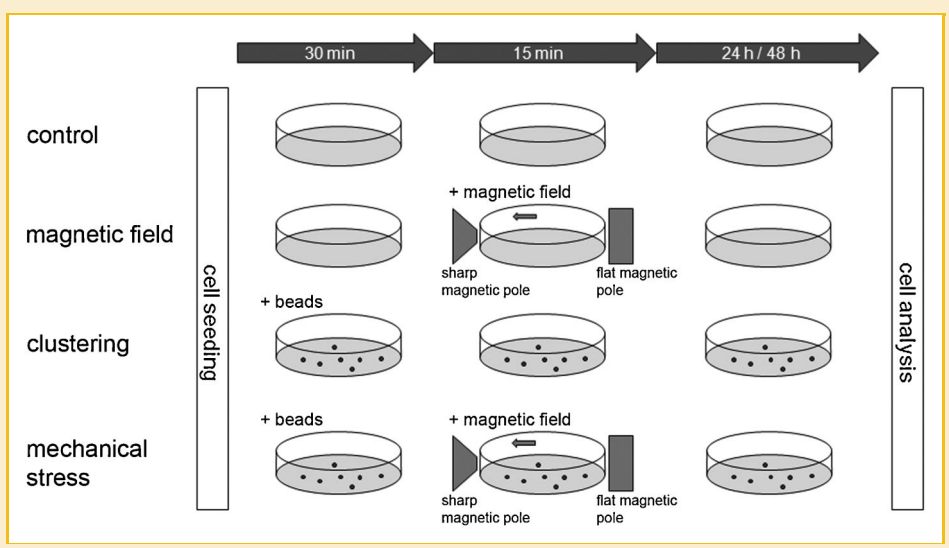

Fig. 1. Scheme of the experimental protocols. We tested four different protocols: Controls without any treatment of the cells, cells were exposed to a magnetic field, integrins were clustered by incubation with antibody-coated beads, and integrins were mechanically stressed by incubation with beads followed by exposure to the magnetic field. Cells were then analyzed for the different parameters after 48 h unless otherwise stated.

PCR reactions contained cDNA, TaqMan® Universal PCR Master Mix (Applied Biosystems) and assays-on-demand™ gene expression assay mix for detection of alkaline phosphatase (ALP; Hs00758162_m1), Sox 9 (Hs 00165814_m1), peroxisome proliferator-activated receptor γ (PPARγ; Hs 00234592_m1), Collagen Ia (Hs 00164004_m1) and VEGF A (Hs 00900054_m1) (all from Applied Biosystems). Gene expression values were normalized to expression of GAPDH (Hs 99999905_m1) as an endogenous control.

WESTERN BLOT ANALYSIS

Western blot analyses were performed for Akt, phospho-Akt, and phospho-Erk 1/2. Briefly, adherent cells were lyzed using the Bio-Plex™ cell lysis kit (Bio-Rad Laboratories, Hercules, CA). For immunoblotting, 80 μg of total protein were separated by SDS–PAGE and then transferred onto PVDF membranes (Roche, Mannheim, Germany). The membranes were blocked and incubated overnight at 4 °C with the following primary antibodies: rabbit monoclonal anti-Akt (Ser473), rabbit polyclonal anti-phospho-Akt (Ser473), and rabbit monoclonal anti-phospho-p44/42 MAPK (Erk) (Thr202/Tyr204) (all from Cell Signaling Technology, Danvers, MA). As secondary antibody an AP-conjugated monoclonal anti-rabbit IgG (DakoDenmark A/S, Glostrup, Denmark) was used. Protein expression was detected by chemiluminescence using CDP-Star reagent (Roche).

Immunoblots prepared from individual donors were repeated at least three times to ensure reproducibility.

TREATMENT WITH PI-3-K INHIBITOR

To block the activation of Akt we used the PI-3-K inhibitor Ly294002 (Cell Signaling Technology). Cells were preincubated with 10 μM of Ly294002 1 h prior to the application of mechanical integrin stress.

STATISTICAL ANALYSIS

All experiments were repeated at least three times using hMSC from individual donors. Data received from stimulated cells were normalized to data of untreated control cells and results are presented as median values. Error bars represent 25th and 75th percentiles. The Shapiro–Wilk test was used to test normal data distribution. After this, the Mann–Whitney *U*-test was performed

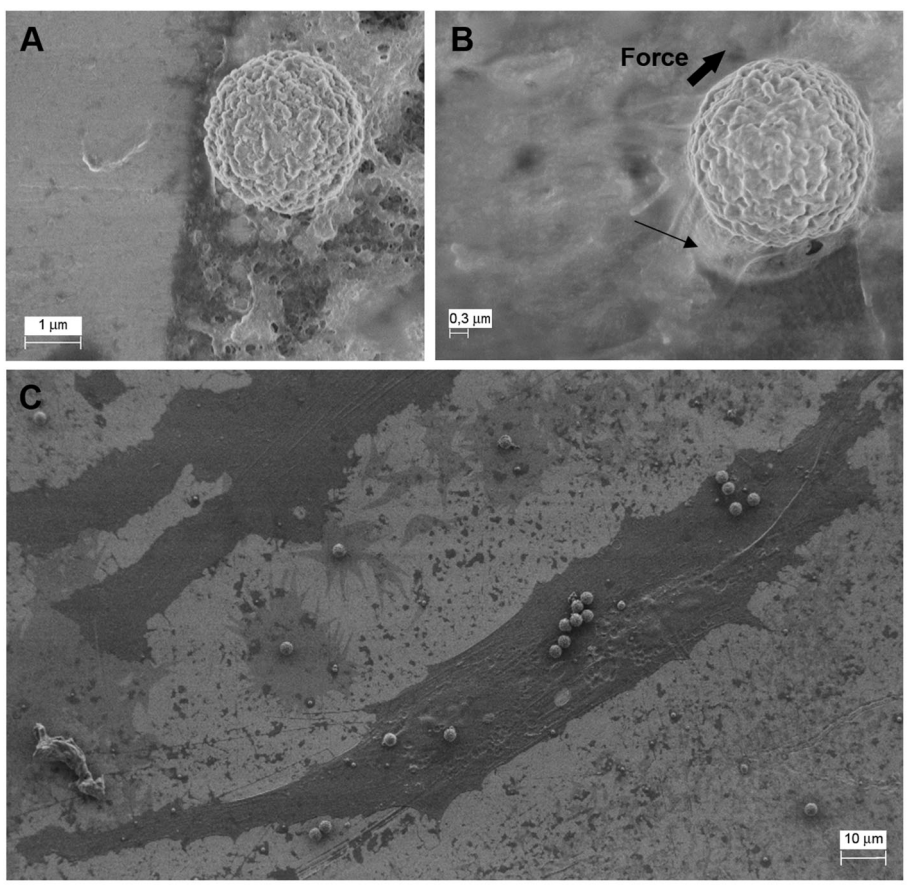

Fig. 2. Field emission scanning electron microscopic images of beads on the apical surface of mesenchymal stem cells. Beads are attached to the surface of the cells by specific binding to integrins. A: Without a magnetic field, distortion of the membrane is not detectable. B: During the application of a magnetic field, beads pull at the receptor in the direction as indicated by the thick arrow. This mechanical stress provokes a small distortion of the cell membrane (thin arrow). C: Image of a whole cell with beads attached to integrins on the apical cell surface. The cell remains spread.

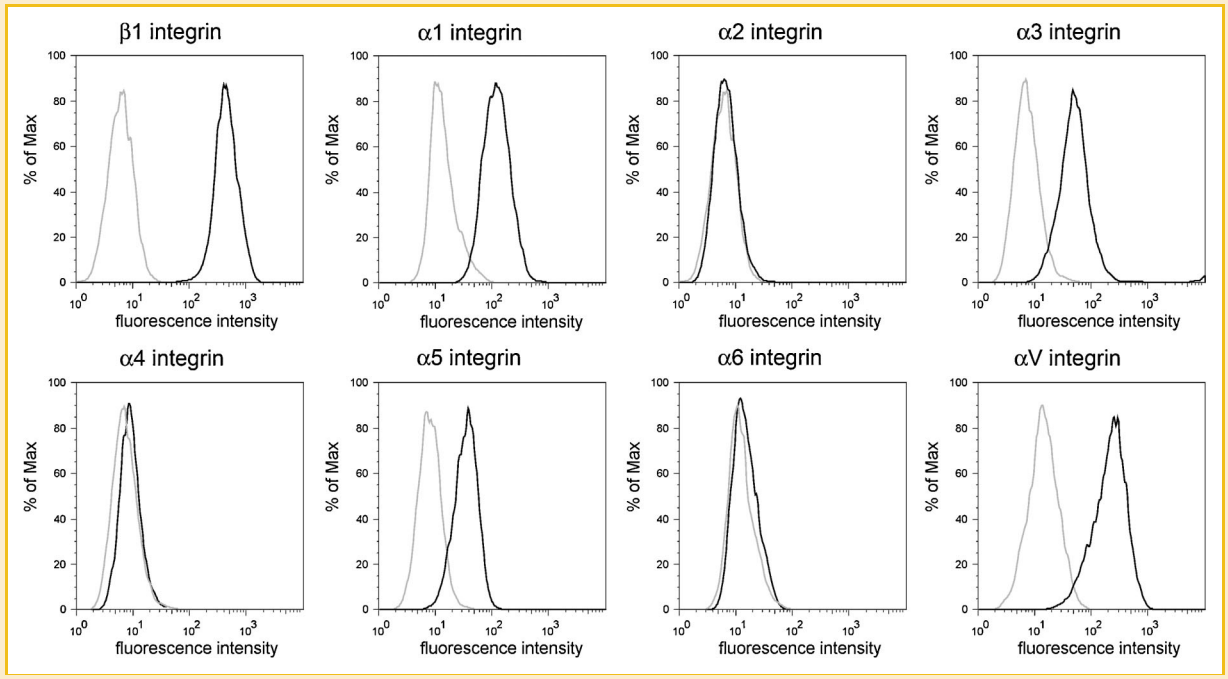

Fig. 3. Flow cytometric analyses of the expression of integrin subunits in mesenchymal stem cells cultured in expansion medium (Weak lines represent the control sample, incubated with a fluorescently labeled secondary antibody; thick lines represent the expression of the integrin subunits labeled with monoclonal anti-integrin antibodies).

when significant overall values using the Kruskal–Wallis test were given. Significance level was set at $P \leq 0.05$. Statistical analyses were performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL).

RESULTS

Our approach of mechanically stressing integrins on the surface of cells involves the incubation with β1 integrin antibody coated beads, which induces a clustering of the receptors, followed by the application of a magnetic field to apply drag forces to β1 integrins coupled to beads. In a field emission scanning electron microscope we observed a slight distortion of the cell membrane when the receptors were stressed by the beads. The cells remained flat during stress with no obvious change in the cell shape (Fig. 2). Thus, the mechanical stress was locally restricted to defined integrin receptors.

Because the mechanically stressed β1 integrin subunit forms heterodimers with various α integrin subunits, which determine the binding specificity and its function, we analyzed the expression of the integrin subunits (Fig. 3). The β1 integrin subunit was highly expressed; we detected the α integrin subunits α1, α3, α5, and αV. Together with the β1 integrin subunit, α5 and αV form receptors for fibronectin and osteopontin. The integrin heterodimers β1α1 and β1α3 can function as receptors for laminin and in addition can bind to collagen I and thrombospondin, respectively.

To determine whether the substrate for cell adhesion controls the mRNA expression of differentiation markers in combination with

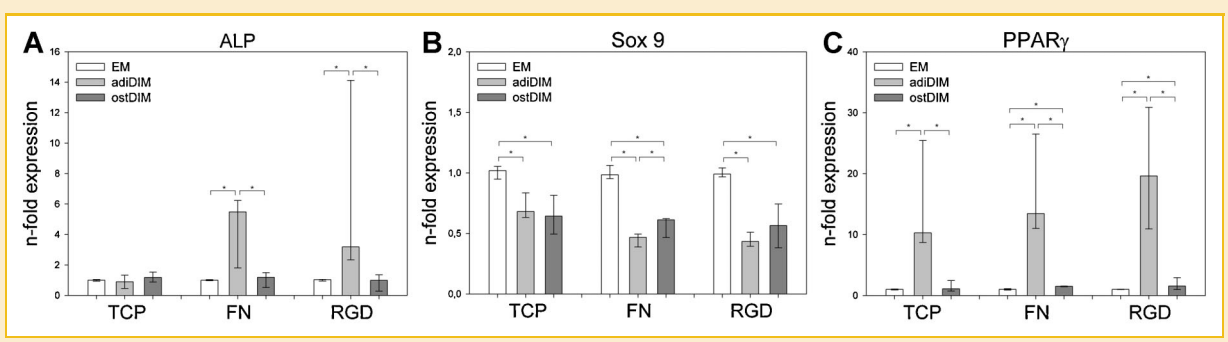

Fig. 4. mRNA expression of markers for differentiation ALP, Sox 9, and PPARγ measured by real-time RT-PCR, when cells were cultured for 48 h in different media and on different substrates. Values were normalized to the cells in the expansion medium. TCP, tissue culture polystyrene; FN, fibronectin; RGD, RGD peptides; EM, expansion medium; adiDIM, adipogenic differentiation medium; ostDIM, osteogenic differentiation medium. Rectangular brackets indicate statistical significance between experimental groups ($^{*}P \leq 0.05$, n = 3).

different media which stimulate cell expansion or differentiation, we cultured the cells on TCP, fibronectin (FN), and RGD peptides (RGD; Fig. 4). Fibronectin and RGD peptides were covalently immobilized on sP(EO-stat-PO) coatings in the well plates, thus preventing unspecific protein adsorption and cell adhesion. After 48 h we found that ALP, a marker for osteogenic differentiation, was differentially expressed on the substrates. On TCP, neither the osteogenic nor the adipogenic medium affected the expression of ALP. However, when the cells were cultured on fibronectin or RGD peptides, we found that ALP expression was increased. Surprisingly, this effect was only detectable in the adipogenic medium. The expression of Sox 9, a marker for chondrogenic differentiation, was reduced when the cells were cultured in media for adipogenic or osteogenic differentiation compared with EM, independent of the substrate for adhesion. PPARγ, a marker of adipogenic differentiation, was strongly upregulated in the adipogenic medium, independent of the matrix for cell adhesion. Together, the culture media determined the expression of different markers for cell differentiation. For ALP, we found a specific effect of the substrate for cell adhesion.

Next, we analyzed the effect of short-time mechanical integrin stress in combination with the adhesion matrix and cell culture media on the expression of differentiation markers. The experimental setup also allowed the examination of the effects of integrin clustering and the magnetic field alone. The results revealed a reduced expression of ALP in the presence of the magnetic field; this was seen on TCP when the cells were cultured in EM, and on FN and RGD peptides when the cells were cultured in adipogenic medium (Fig. 5). Clustering of integrins by incubation of the beads revealed a minor reduction of ALP on TCP in EM as well as in the adipogenic medium on FN and RGD peptides, but additional mechanical integrin stress did not significantly reduce the ALP expression.

For the transcription factor Sox 9, a marker for chondrogenic differentiation, the principal observation was an increase of its expression due to the magnetic field. This was observed only on TCP independent of the culture medium (Fig. 6). The expression of PPARγ was reduced due to the magnetic field on FN and RGD peptides in adipogenic medium. In osteogenic medium, PPARγ increased independent of the substrate (Fig. 7).

Because ALP as a marker for osteogenic differentiation appeared observably sensitive to the substrate and the culture medium, we examined collagen I expression as another marker for osteogenic differentiation. Unlike ALP, collagen I expression was reduced when cultured in an adipogenic medium compared with EM, independent of the substrate (Fig. 8A). After application of a mechanical integrin stress, we found an increase in the expression of collagen I in the adipogenic medium when the cells were cultured on fibronectin and RGD peptides, but not on TCP (Fig. 8B–D).

Because mesenchymal stem cells are also a source for bioactive factors, we studied the effect of mechanical forces on the expression of VEGF (Fig. 9). When the cells were cultured on the different substrates, the expression of VEGF decreased in the adipogenic medium and to a lesser extent in the osteogenic medium compared with EM, which was independent of the substrate for adhesion. After application of a mechanical integrin stress, the expression of VEGF increased on TCP in osteogenic medium. In contrast, mechanical integrin stress did not affect

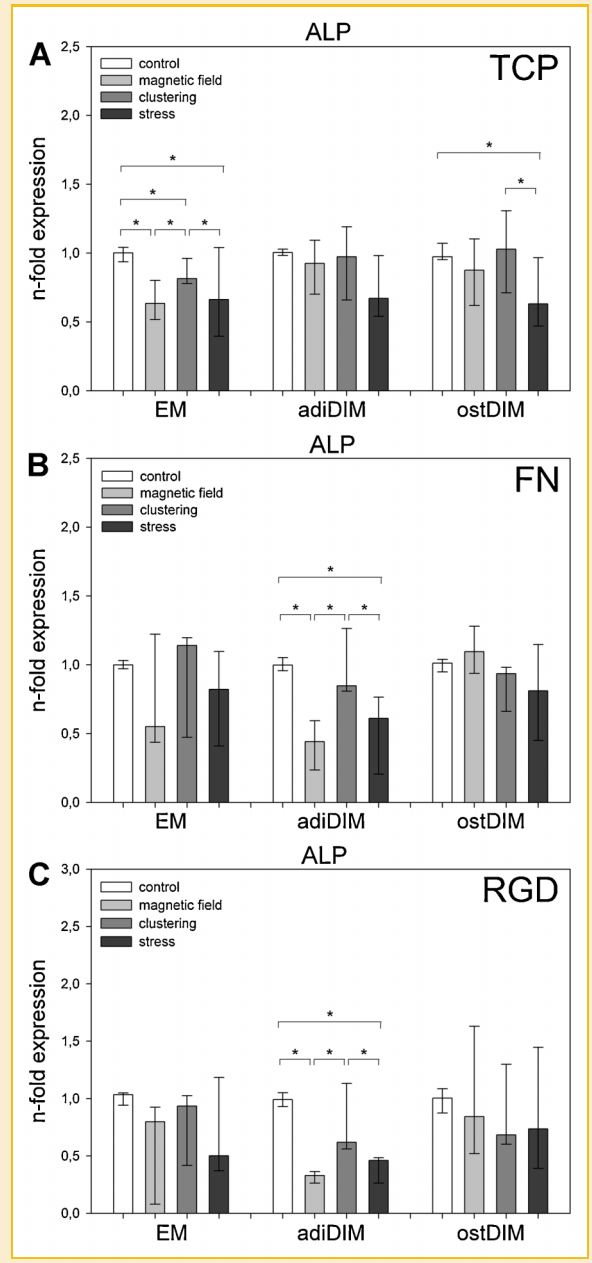

Fig. 5. mRNA expression of ALP after 48 h due to a short-time application of mechanical integrin stress (stress), the magnetic field, and clustering of integrins. Cells were cultured in expansion medium (EM), adiDIM (adipogenic differentiation medium), ostDIM (osteogenic differentiation medium and on different substrates: TCP (A), FN (B), RGD (C). Values are normalized to control cells (control) without any treatment. Rectangular brackets indicate statistical significance between experimental groups ($^*P \leq 0.05$, n = 3).

the expression of VEGF on fibronectin and RGD peptides, which indicates the specific effect of the adhesion substrate.

To address possible intracellular mechanisms for the observed changes in the expression of biologically relevant proteins by the physical factors, we examined the activation of Erk1/2 and Akt. Erk acts as a central signaling protein, which can be activated by multiple integrin-induced pathways, for example by FAK activation

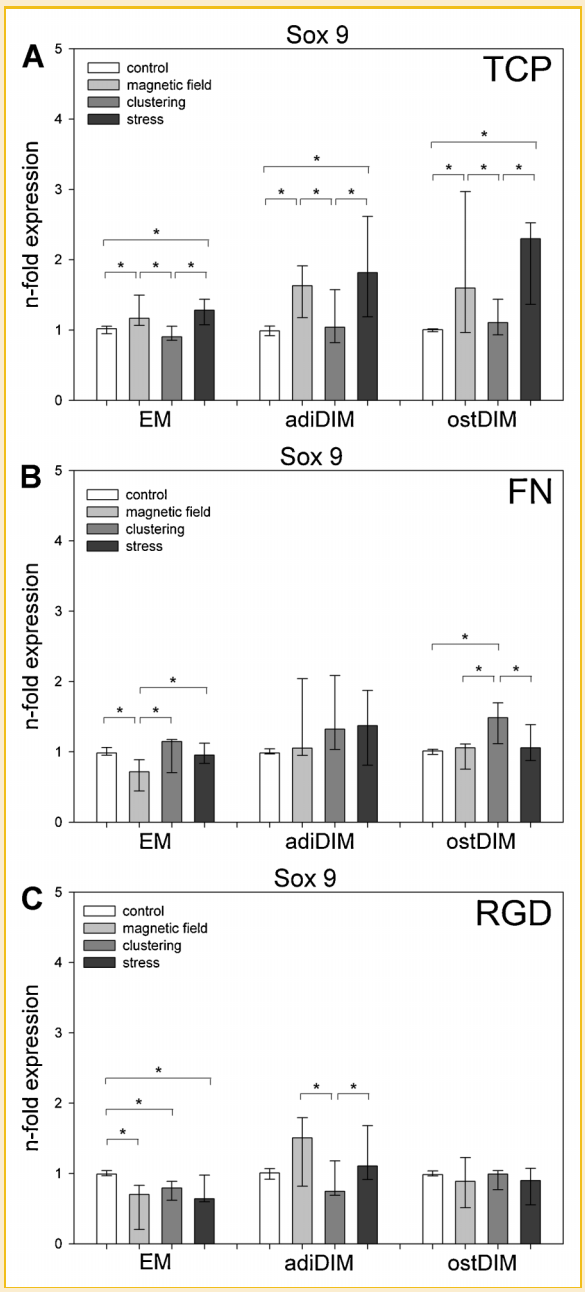

Fig. 6. mRNA expression of Sox 9 after 48 h due to a short-time application of mechanical integrin stress (stress), the magnetic field, and clustering of integrins. Cells were cultured in expansion medium (EM), adiDIM (adipogenic differentiation medium), ostDIM (osteogenic differentiation medium), and on different substrates: TCP (A), FN (B), RGD (C). Values are normalized to control cells (control) without any treatment. Rectangular brackets indicate statistical significance between experimental groups ($^{*}P \leq 0.05$, n = 3).

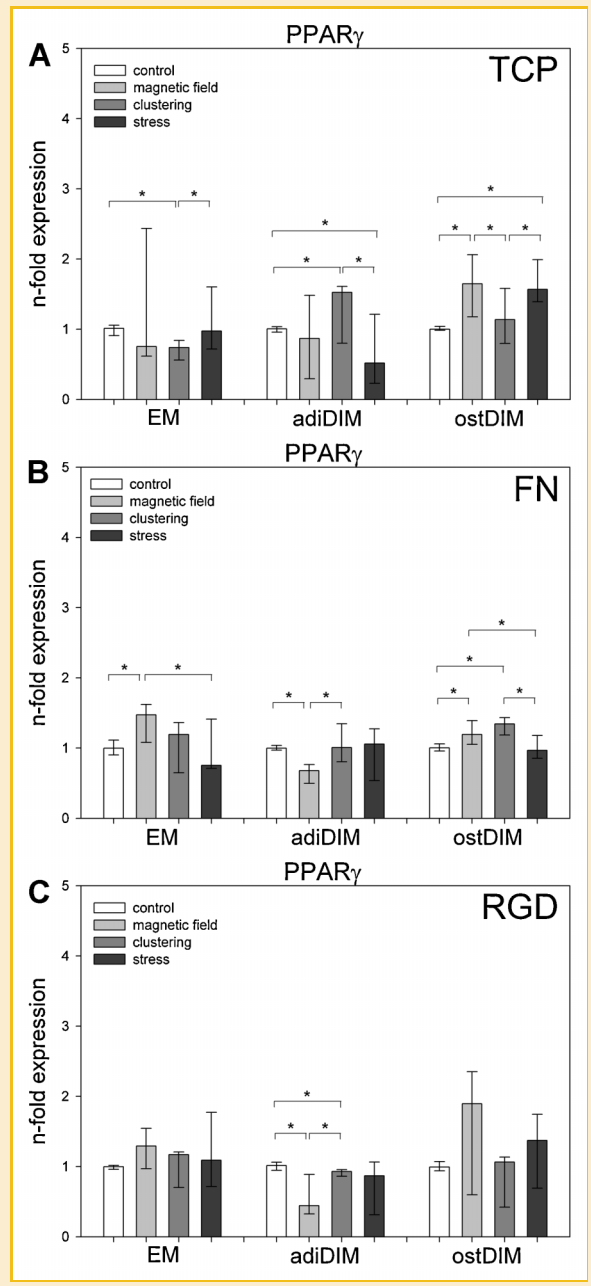

Fig. 7. mRNA expression of PPARγ after 48 h due to a short-time application of mechanical integrin stress (stress), the magnetic field, and clustering of integrins. Cells were cultured in expansion medium (EM), adiDIM (adipogenic differentiation medium), ostDIM (osteogenic differentiation medium), and on different substrates: TCP (A), FN (B), RGD (C). Values are normalized to control cells (control) without any treatment. Rectangular brackets indicate statistical significance between experimental groups ($^{*}P \leq 0.05$, n = 3).

or recruitment of Fyn. Similarly, integrin signaling can activate Akt proximal to Erk activation by multiple mechanisms. In Western blot analyses we found that the physical stresses, induced by integrin clustering pulling on integrins, and the magnetic field induced a transient activation of Erk with its maximum immediately after stress application and a decline after 6 h (Fig. 10A). Mechanical loading, integrin clustering and, to a lesser extent, the magnetic field also induced an activation of Akt, which we detected after 48 h (Fig. 10B). To determine whether increased VEGF due to mechanical integrin loading and a magnetic field depends on activation of Akt, we inhibited PI-3-K, an enzyme which is responsible for phosphorylation of Akt (Fig. 10C). With the exception of cells with

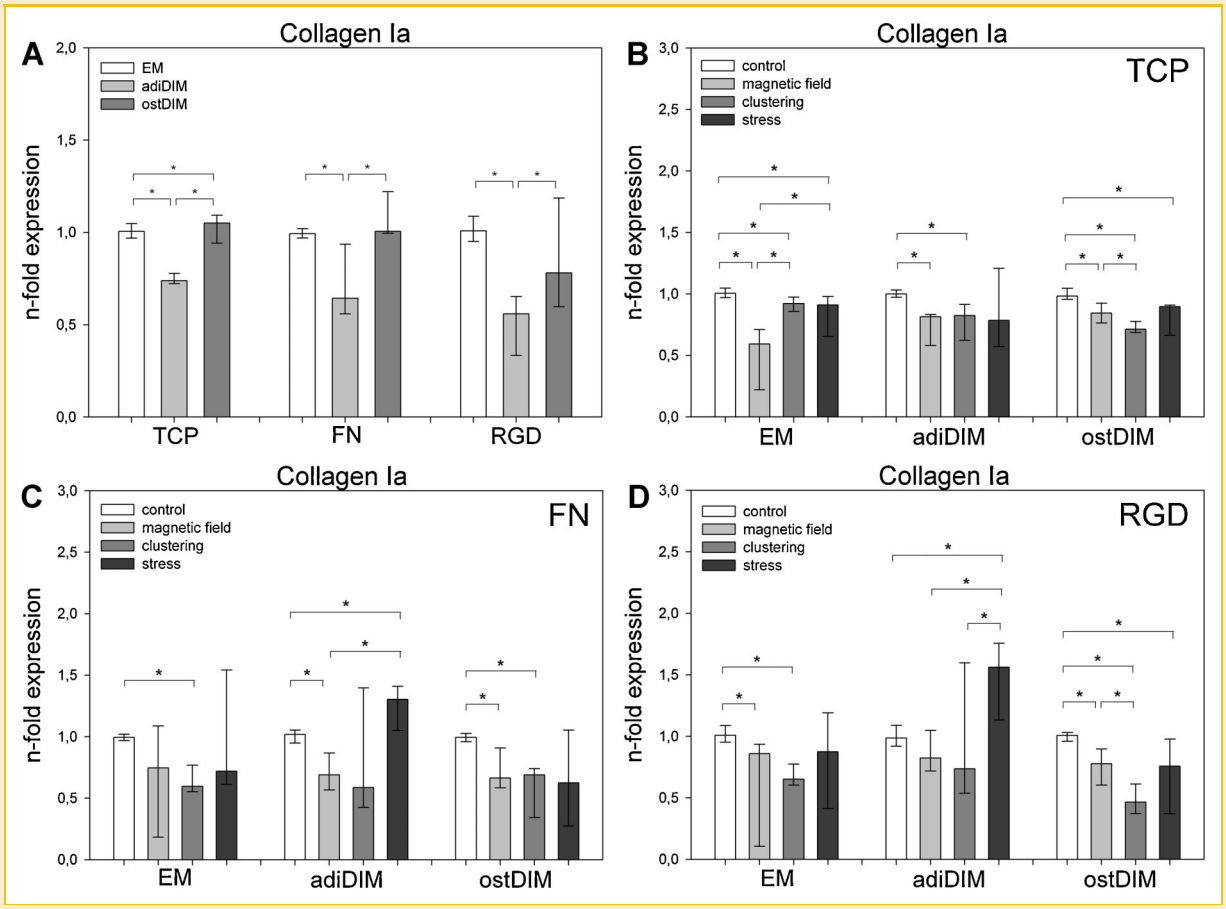

Fig. 8. A: mRNA expression of collagen Ia cultured on different substrates (TCP, FN, and RGD) and in different media (expansion medium, EM; adipogenic medium, adiDIM; osteogenic medium, ostDIM). Values are normalized to cells in the expansion medium. B–D: mRNA expression of collagen Ia after 24 h due to a short-time application of mechanical integrin stress (stress), the magnetic field, and clustering of integrins. Cells were cultured in expansion medium (EM), adiDIM (adipogenic differentiation medium), ostDIM (osteogenic differentiation medium), and on different substrates: TCP (B), FN (C), RGD (D). Values are normalized to control cells (control) without any treatment. Rectangular brackets indicate statistical significance between experimental groups (* $P \leq 0.05$, $n = 3$).

clustered integrins, we observed reduced mRNA expression of VEGF in cells, which were mechanically loaded or exposed to the magnetic field when Akt was blocked. This suggests that Akt activation is involved in the stimulation of VEGF by a physical factor.

DISCUSSION

The aim of this study was to evaluate the role of mechanical forces in the biological response of mesenchymal stem cells. We used an approach for the mechanical loading of cells, which differs from techniques which are widely used to stress a complete cell. We applied a short-time, cyclic, mechanical stress for 15 min to the β1 integrin subunit, which forms a functional heterodimeric adhesion receptor in combination with different α subunits [Hynes, 2002; Schwartz and DeSimone, 2008]. As demonstrated, the short-time mechanical load applied by beads induced a small local distortion of the cell membrane. This load was sufficient to induce activation of Erk and Akt and, as previously shown in an osteoblastic cell line, stimulated extracellular calcium influx with subsequent activation of events in molecular complexes, which are characteristic for focal adhesions [Pommerenke et al., 1996; Schmidt et al., 1998]. Besides other mechanisms, a physical linkage of signaling molecules to the cytoskeleton has been observed [Pommerenke et al., 2002], which gives rise to biochemical reactions and, in consequence, could provoke a biological response of the cells [Vogel and Sheetz, 2009].

The experimental setup we used included two integrin-mediated signals, one by pulling on β1 integrin on the apical surface of the cell and one by cell adhesion at the basal surface when the cell attached to varying adhesive substrates. On TCP, cells can adhere to a mix of extracellular matrix proteins both produced by the cells and contained in the culture medium. In comparison, cells were cultured on fibronectin and RGD peptides, which is the classical ligand for the integrin receptor β1α5. It is worth mentioning that in our experiments fibronectin and RGD peptides were immobilized via sP(EO-stat-PO) coatings, which suggests a different protein conformation compared with purely adsorbed proteins [Groll et al., 2004]. In addition, sP(EO-stat-PO) prevents unspecific interactions. The detection of the integrin subunits expressed by the cells revealed

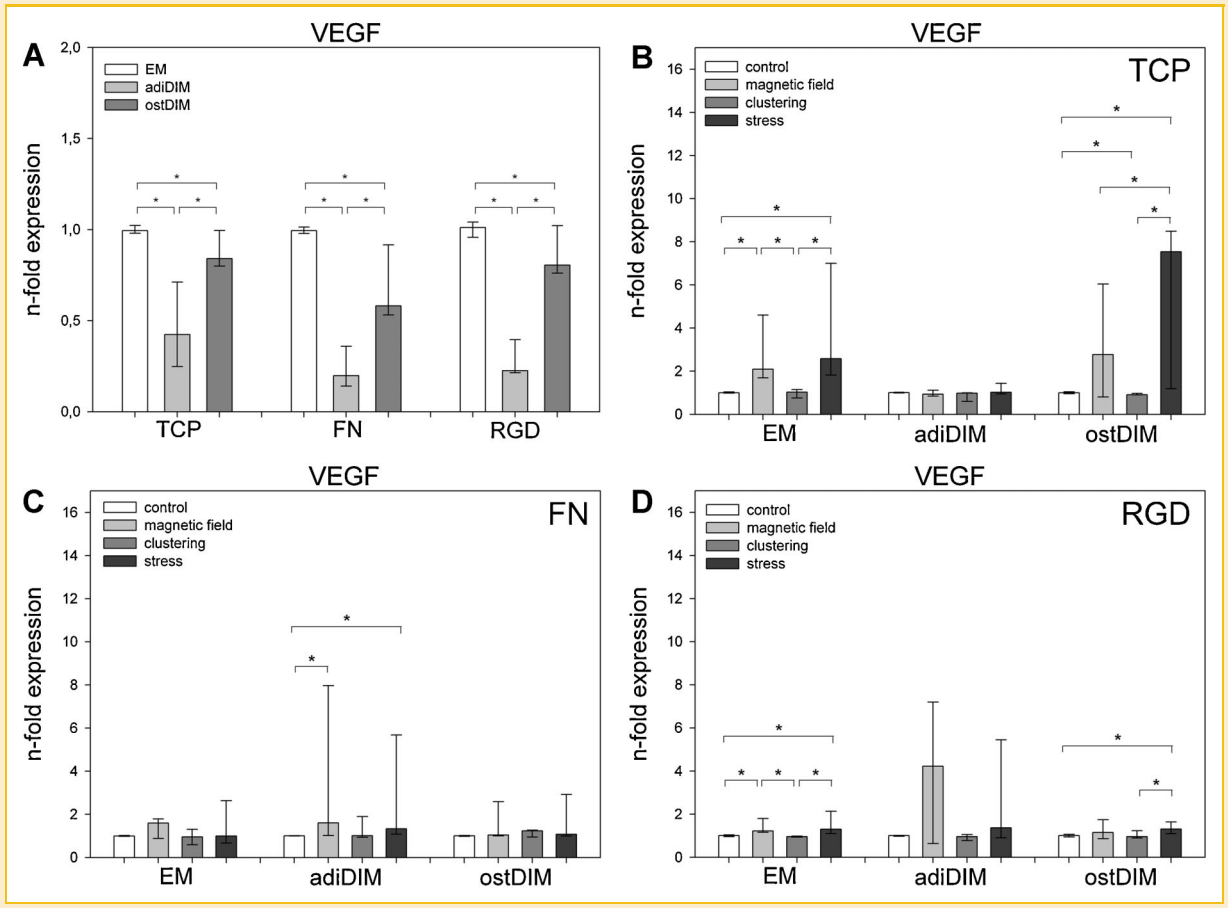

Fig. 9. A: mRNA expression of vascular endothelial growth factor (VEGF) cultured on different substrates (TCP, FN, and RGD) and in different media (expansion medium, EM; adipogenic medium, adiDIM; osteogenic medium, ostDIM). Values are normalized to cells in the expansion medium. B–D: mRNA expression of VEGF after 48 h due to a short-time application of mechanical integrin stress (stress), the magnetic field, and clustering of integrins. Cells were cultured in expansion medium (EM), adiDIM (adipogenic differentiation medium), ostDIM (osteogenic differentiation medium) and on different substrates: TCP (B), FN (C), RGD (D). Values are normalized to control cells (control) without any treatment. Rectangular brackets indicate statistical significance between experimental groups ($^*P \leq 0.05$, n = 3).

that, by the combination of β1 with an α subunit, mechanical stress to β1 may stimulate at least two receptors for fibronectin and laminin, but only one for collagen. The collagen receptor β1α2, which is abundantly expressed in many cell lines, was absent on MSC.

We first studied the effect of the adhesion substrate alone in combination with the medium and found that the adipogenic medium appeared primarily sensitive for the expression of differentiation markers in the cells. As expected, the adipogenic medium strongly stimulated the expression of PPARγ on all substrates but was also able to stimulate the expression of ALP, an osteogenic marker, when the cells were cultured on fibronectin or RGD peptides. This suggests a specific combinatorial effect of the substrate for adhesion with factors in the medium. Previous studies have shown that the coating of TCP with extracellular matrix proteins is required to stimulate ALP expression without additional osteogenic stimulants [Salasznyk et al., 2004; Huang et al., 2009]. The selective effect of the adipogenic medium to increase ALP was unexpected. However, the adipogenic medium, like the osteogenic medium, contains dexamethasone, which has been shown to up-regulate α5 integrin [Hamidouche et al., 2009]. This study also revealed that the activation of α5 integrin promoted osteogenic differentiation via Erk signaling. While in our experiments ALP was stimulated, the expression of collagen I was reduced in the adipogenic medium without the selective effect of the substrate. Although both proteins are involved in osteogenic differentiation, they may be regulated differentially and are at least temporally coordinated during osteogenic differentiation. The expression of ALP occurs earlier and is followed by the expression of collagen I.

Furthermore, we applied mechanical forces to integrins on the apical cell surface to study the expression of differentiation markers. We found that collagen I was increased by mechanical integrin stress in the adipogenic medium, but only in combination with fibronectin or RGD peptides as substrates. This suggests that the intracellular signaling induced by integrins at the apical surface interferes with both signals from soluble factors and from the extracellular matrix. Similarly, in endothelial progenitor cells the combined effect of environmental factors controlled the cell differentiation. Cells cultured on a polymer fiber mesh together with VEGF in the medium induced an angiogenic activation of progenitor cells, whereas in a co-culture with osteoblasts, VEGF

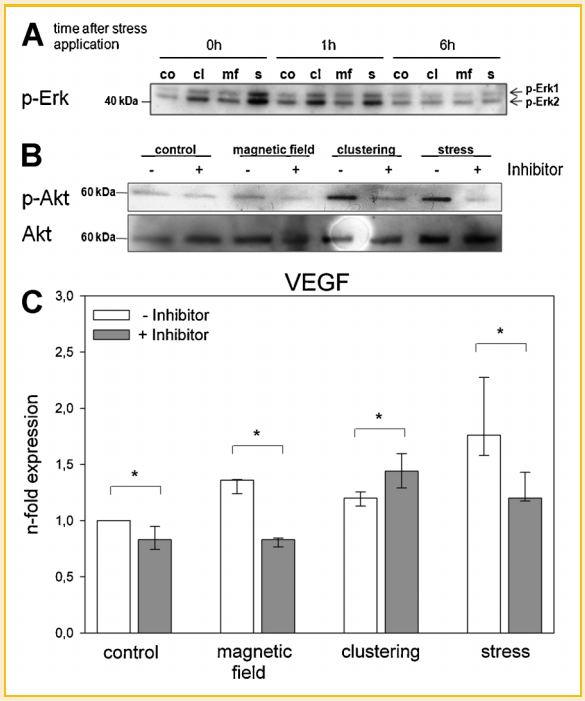

Fig. 10. Intracellular signaling of physically stressed mesenchymal stem cells on TCP. A: Western blot analyses of time dependent activation of Erk 1/2. Note the transient phosphorylation of Erk 1/2 mainly due to mechanical stress immediately after stress application, which declines after 6 h (co—untreated control cells, cl—clustering, mf—magnetic field, s—application of mechanical stress). B: Activation of Akt and the inhibition of PI-3-K. Akt is phosphorylated due to clustering and mechanical integrin stress. Phosphorylation of Akt is inhibited by the PI-3-K inhibitor Ly294002 (control—untreated control cells, clustering, stress—application of mechanical stress; Blots are a representative example of three independent experiments). C: Influence of PI-3-K inhibitor on the mRNA expression of VEGF after 48 h when cells were physically stressed in the expansion medium. With the exception of integrin clustering, VEGF expression decreased due to the inhibition of PI-3-K. Rectangular brackets indicate a significant difference by treatment with PI-3-K inhibitor ($^{*}P \leq 0.05$, $n = 3$).

was not required to stimulate differentiation of endothelial progenitor cells. This effect might be caused by direct cell–cell contacts or by the increased formation of collagen I in this assay [Fuchs et al., 2009a,b]. In regard to the osteogenic differentiation of MSC by mechanical forces, it appeared from other studies that only longer mechanical stress, which involves the whole cell in combination with a strong osteogenic stimulus like BMP-2, can induce osteogenic differentiation. This was measured by an increased expression of Runx2, collagen I or osteopontin [Thomas and el Haj, 1996; Kreke et al., 2008; Sen et al., 2008]. However, it was also reported that a longer cyclic tensile strain alone without additional osteogenic supplements is sufficient to induce osteogenic differentiation [Sumanasinghe et al., 2006].

We then studied the effect of the extracellular matrix, the differentiation medium and mechanical integrin stress on the expression of VEGF. Mesenchymal stem cells contribute to the regeneration and repair of tissue not only by their ability to differentiate into different specific phenotypes but also by the secretion of bioactive factors that may influence organogenesis and regeneration, which is of equal importance [Caplan, 2009]. VEGF is a factor determining activation, proliferation, and migration of vascular endothelial cells [Kinnaird et al., 2004]. Therefore, MSC might control angiogenesis in bone marrow, where mesenchymal cells are localized in the vicinity of microvessels, or in other organs by the secretion of VEGF [Sorrell et al., 2009]. When mechanical integrin stress was applied in our experiments, we observed a stimulation of VEGF expression. However, the effect depended both on soluble factors and on the extracellular matrix. Hence, the expression of VEGF in MSC is under mechanical control but depends on factors from the microenvironment.

There are various concepts for a cross-talk between the signaling done by integrins and growth factors. Inside the cell, integrin signaling pathways share the signaling pathways activated by growth factors [Clark and Brugge, 1995]. Besides this cooperation in the downstream signal transduction, receptors may cross-talk via membrane–proximal interactions. In addition, the extracellular matrix can serve as a binding site for growth factors and integrate signals sent by integrins and growth factors [Hynes, 2009]. In our assays, integrin-mediated mechanical stress and factors in the medium provoked opposite effects. While collagen I and also VEGF were reduced in a specific differentiation medium, in combination with mechanical integrin stress the expression of both proteins was enhanced.

Using our device to mechanically stress integrins, we were also able to test the influence of the magnetic field alone. The experiments revealed that the magnetic field stimulated the expression of Sox 9 and reduced the expression of ALP and PPARγ. Similar to the mechanical integrin stress, the effects of a magnetic field depended on the type of adhesive substrate and the culture medium. Like mechanical integrin stress, the magnetic field acted oppositely to the effect of the medium alone. Previous studies with the osteoblast cell line MG-63 and rat osteoblasts demonstrated that exposure to a magnetic field for several days stimulated the osteogenic differentiation, detected by increased ALP activity and osteocalcin production [Lohmann et al., 2000; Selvamurugan et al., 2007]. Although not obvious, the magnetic field induced a small increase of VEGF expression in an EM on two substrates in our experiments, which correlates with findings showing that magnetic fields are able to affect the release of growth factors in osteoblasts and endothelial cells [Lohmann et al., 2000; Tepper et al., 2004; Hopper et al., 2009].

Concerning an intracellular mechanism that is involved in the physical induction of VEGF, we found that the PI-3-K/Akt pathway plays a role. Activation of Akt regulates numerous biological activities, including survival, proliferation, and angiogenesis [Cantley, 2002; Jiang and Liu, 2008]. In our experiments, we were able to demonstrate the connection between VEGF expression and signaling pathways induced by physical factors, which involve Akt activation. Mechanical stimulation of integrins in our assay activated FAK, as we have shown previously [Pommerenke et al., 2002]. FAK activation leads to a recruitment of PI-3-kinase to focal adhesions and its activation [Legate et al., 2009]. A central consequence of PI-3-K activation is activation of Akt. As we have shown here, blocking of PI-3-K leads to a reduction of VEGF

expression as one among other possible biological consequences. The reason why VEGF expression was not reduced by PI-3-K inhibition during integrin clustering is not known. Although little is known about signaling pathways induced by magnetic fields, our experiments and other data provide evidence that cellular signals induced by mechanical and magnetic forces share similar intracellular pathways. The application of a magnetic field was shown to increase the membrane rigidity and interact with calcium channels, which modify the calcium ion flux [Barbier et al., 1996; Baureus Koch et al., 2003; Chiu et al., 2007]. Furthermore, changes in the organization of the cytoskeleton and focal adhesions have been demonstrated [Manni et al., 2002; Delle Monache et al., 2008].

Taken together, our data emphasize the role of physical factors for the expression of both differentiation markers and growth factors in MSC. The data further clearly revealed a strong interrelationship between mechanical forces, the extracellular matrix and soluble factors. The findings have important implications for strategies in regenerative medicine. The release of VEGF by the cells to stimulate angiogenesis could be mechanically tuned, for example by the appropriate stiffness of the material serving as a matrix [Dumas et al., 2009]. The data further support the application of external magnetic fields to stimulate tissue regeneration, as it has been shown in experimental approaches to aid in the faster repair of bone and to enhance cutaneous wound healing [Aaron and Ciombor, 1993; Strauch et al., 2007].

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