

## CD73 AND CD29 CONCURRENTLY MEDIATE THE MECHANICALLY INDUCED DECREASE OF MIGRATORY CAPACITY OF MESENCHYMAL STROMAL CELLS

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### Abstract

The assumption that mesenchymal stromal cell (MSC)-based-therapies are capable of augmenting physiological regeneration processes has fostered intensive basic and clinical research activities. However, to achieve sustained therapeutic success *in vivo*, not only the biological, but also the mechanical microenvironment of MSCs during these regeneration processes needs to be taken into account. This is especially important for e.g., bone fracture repair, since MSCs present at the fracture site undergo significant biomechanical stimulation. This study has therefore investigated cellular characteristics and the functional behaviour of MSCs in response to mechanical loading.

Our results demonstrated a reduced expression of MSC surface markers CD73 (ecto-5'-nucleotidase) and CD29 (integrin  $\beta$ 1) after loading. On the functional level, loading led to a reduced migration of MSCs. Both effects persisted for a week after the removal of the loading stimulus. Specific inhibition of CD73/CD29 demonstrated their substrate dependent involvement in MSC migration after loading. These results were supported by scanning electron microscopy images and phalloidin staining of actin filaments displaying less cell spreading, lamellipodia formation and actin accumulations. Moreover, focal adhesion kinase and Src-family kinases were identified as candidate downstream targets of CD73/CD29 that might contribute to the mechanically induced decrease in MSC migration.

These results suggest that MSC migration is controlled by CD73/CD29, which in turn are regulated by mechanical stimulation of cells. We therefore speculate that MSCs migrate into the fracture site, become mechanically entrapped, and thereby accumulate to fulfil their regenerative functions.

**Keywords:** CD29, CD73, cytoskeleton, mechanical stimulation, migration, mesenchymal stromal cells.

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### Introduction

It is well accepted that mesenchymal stromal cells (MSCs) are key regulators in homeostasis and in a number of regenerative processes, possibly due to their capacity for self-renewal, multipotency, and, equally, their migratory and trophic activities. This includes their ability to home to tissue sites of repair or to participate in the remodelling response by providing a broad array of paracrine factors.

MSCs also hold great promise as tools for cell-based therapy and tissue engineering approaches. In numerous preclinical and clinical studies, MSCs have shown promising results for the treatment of cardiovascular and immune diseases as well as musculoskeletal injuries. One example of an injury is a bone fracture resulting in the formation of a haematoma, which is known to undergo regeneration while being mechanically strained. The amount of strain is determined by the interfragmentary movement – mainly axial compression (Goodship, 1992; Gardner *et al.*, 1997). These mechanical conditions significantly influence the long-term bone healing outcome. A certain degree of movement is beneficial for fast and uneventful healing, whereas too little or too large movements can lead to so-called non-unions (Klein *et al.*, 2003).

MSCs – key players in bone regeneration – are characterised by their multilineage differentiation capacity and their expression of a typical set of cell surface markers: CD29 (integrin  $\beta$ 1), involved in MSC migration *in vivo* (Ip *et al.*, 2007); the homing receptor CD44 (hyaluronan receptor) (Zhu *et al.*, 2006); the GPI-anchored proteins CD73 (ecto-5'-nucleotidase) and CD90 (Thy-1), signal transduction molecules in the human immune system and mediators of cell-cell and cell-matrix interactions (Hunsucker *et al.*, 2005; Barker and Hagood, 2009); CD105 (endoglin), an integrin- and TGF- $\beta$ -binding molecule (Eliceiri, 2001; Duff *et al.*, 2003) and the adhesion molecules CD106 (VCAM-1) and CD166 (ALCAM) (Chamberlain *et al.*, 2007).

Once MSCs have reached the fracture site – the first ones are evident 24 h after fracture (Phillips, 2005) – they are confronted with a challenging milieu characterised not only by low oxygen and low glucose content, but also by constant mechanical stress (Goodship and Kenwright, 1985; Komatsu and Hadjiargyrou, 2004). Importantly, the latter condition likely affects MSCs, which are known to be mechanosensitive (Wang and Thampatty, 2008). Thus, detailed knowledge about the influence of mechanical loading on MSCs is pivotal for understanding

the physiological processes during bone regeneration in order to develop innovative cell therapy approaches. In recent studies, mechanical loading of MSCs under beneficial healing conditions *in vitro*, stimulated their expression of molecules that are involved in angiogenesis (Kasper *et al.*, 2007a) and matrix remodelling (Kasper *et al.*, 2007b) – key processes in bone regeneration. However, investigations regarding cell autonomous alterations in MSCs, specifically their MSC-characteristics and associated functional changes due to loading are still missing.

Therefore, our first aim was to analyse whether mechanical loading influences the criteria defining MSCs; i.e., their expression of typical cell surface markers and their ability to differentiate along the adipogenic, chondrogenic and osteogenic lineage. Our second aim – based on the first results – was to investigate whether changes in cell surface marker expression by mechanical loading would alter MSC functions, especially proliferation and migration.

While the differentiation potential was unaffected, CD73 and CD29 expression were found to be reduced. Both markers appeared to contribute to morphological changes and to a decrease in the migratory response after loading, possibly *via* FAK/Src family kinase (SFK)- and SFK-signalling. Moreover, the CD73 regulator TNF- $\alpha$  was increased and the CD29 regulator VEGF was decreased in response to loading.

## Materials and Methods

### MSC isolation, culture and characterisation

MSCs were isolated from bone marrow of 10-12 week old male Lewis rats and selected by plastic adherence (Dobson *et al.*, 1999). Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA) supplemented with 10 % foetal calf serum (FCS) (Biochrom AG, Berlin, Germany) and 10 U/mL penicillin plus 100  $\mu$ g/mL streptomycin was used as expansion medium for MSCs. Only cells from passages 2-4 were used for experiments. The MSC-character was validated using flow cytometric analysis for MSC cell surface marker expression (positive: CD29, CD44, CD73, CD90, CD105, CD106 and CD166; negative: CD34, CD45, HLA-DR) and differentiation assays (Dominici *et al.*, 2006).

For flow cytometry the following antibodies were employed: rabbit (anti-rat CD14), mouse (anti-rat CD34):FITC, rabbit (anti-rat CD105), and rabbit (anti-rat CD166) (Santa Cruz Biotechnology, Heidelberg, Germany), Armenian hamster (anti-rat CD29):FITC and mouse (anti-rat CD106) (Biolegend, San Diego, CA, USA), mouse (anti-rat CD45) and mouse (anti-rat CD90) (Acris Antibodies, Herford, Germany), mouse (anti-rat CD44) (AbD Serotec, Düsseldorf, Germany), mouse (anti-rat CD73) (BD Biosciences, Heidelberg, Germany), isotype Armenian hamster IgG:FITC (Biolegend), rat (anti-mouse IgG):PE (BD Biosciences) and donkey (anti-rabbit IgG):Cy5 (Dianova, Hamburg, Germany). Cells were analysed using FACSCalibur. Thirty thousand events were acquired and analysed using the FlowJo software.

To induce osteogenic and adipogenic differentiation,  $1.92 \times 10^4$  MSCs were cultured on 24 well plates and exposed to osteogenic (200  $\mu$ M ascorbic acid, 7 mM  $\beta$ -glycerol phosphate, 0.01  $\mu$ M dexamethasone; 21 d) and adipogenic differentiation media (1  $\mu$ M dexamethasone, 2  $\mu$ M insulin, 200  $\mu$ M indomethacin, 500  $\mu$ M isobutyl-methyl-xanthin; 16 d). Osteogenic differentiation was detected by alizarin red staining. Adipogenic differentiation was determined by Sudan IV/haematoxylin staining. Each experiment was conducted in triplicate. For chondrogenic differentiation, a pellet culture in chondrogenic differentiation media (10 ng/mL TGF- $\beta$ 1,  $10^{-7}$  M dexamethasone, 50  $\mu$ g/mL ascorbic acid, 40  $\mu$ g/mL proline, 100  $\mu$ g/mL pyruvate, 6.25  $\mu$ g/mL ITS, 1.25 mg/mL BSA, 5.35 mg/mL linoleic acid) as described was used for 28 d (Sekiya *et al.*, 2002). Chondrogenic differentiation was detected by Alcian blue staining.

### Bioreactor experiments

The bioreactor system used has been described previously (Matziolis *et al.*, 2006). Briefly, MSCs were trypsinised, and  $2 \times 10^6$  cells in 350  $\mu$ L of bioreactor medium (culture medium containing 2.4 % Trasylol [Bayer, Leverkusen, Germany]) were mixed with 300  $\mu$ L of fibrinogen/bioreactor medium (1:2) mixture and 50  $\mu$ L of thrombin S/bioreactor medium (1:2) mixture (Tissucol; Baxter, Munich, Germany). This MSC/fibrinogen/thrombin mixture was placed between two spongiosa bone chips and allowed to solidify for 30 min at 37 °C. The sandwich construct was placed into the bioreactor, and 25 mL of bioreactor medium was added. A strain of approximately 20 % at a frequency of 1 Hz was applied in accordance with *in vivo* measurements of interfragmentary movement (Claes *et al.*, 1998). Mechanical loading was carried out for 72 h. Afterwards, cells within the fibrin construct were isolated by 225U trypsin/1 mL PBS. Equal levels of cell viability, activity and apoptosis between loaded and non-loaded MSCs were determined by the electronic cell counter system CASY® DT (Schaerfe System, Germany), a CellTiter96 Aqueous test (MTS test; Promega, Mannheim, Germany) and an Annexin V:FITC assay kit (AbD Serotec, Düsseldorf, Germany), respectively. To determine whether the load-induced effects were persistent effects, loaded and non-loaded MSCs were re-embedded in fibrin and kept in non-loaded bioreactors for another six days. Each MTS test was conducted in triplicates.

### RNA isolation, cDNA synthesis, and quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted using Trizol® Reagent (Invitrogen, Karlsruhe, Germany) and reversely transcribed to cDNA using iScript™ cDNA Synthesis kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. RNA quality was evaluated by visualising the 18S/28S rRNA on a 1.5 % agarose gel. Quantification of the cell surface marker CD29 and CD73 were assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the iQTM SYBR® Green Supermix and the iQTM 5 Multicolor Realtime PCR Detection System and software (Bio-Rad) using the delta-delta-Ct-method. The transcript expression was normalised

**Table 1:** Primer sequences

Protein	Gene	Primer Sequence (forward / reverse)
integrin beta 1 (CD29)	Itgb	5' AATGGAGTGAATGGGACAGG 3' 5' TCTGTGAAGCCCAGAGGTTT 3'
5' ecto-nucleotidase (CD73)	Nt5e	5' GCAAGGAAGAACCCAACGTA 3' 5' TCAGTCCTTCCACACCGTTA 3'
actin cytoplasmic 1 ( $\beta$ -actin)	Actb	5' TGTCACCAACTGGGACGATA 3' 5' GGGGTGTTGAAGGTCTCAA 3'
glyceraldehyde-3-phosphate dehydrogenase	Gapdh	5' ATGGGAAGCTGGTCATCAAC 3' 5' GTGGTTCACACCCATCACAA 3'
elongation factor 1-alpha 1	Eef1a	5' CCCTGTGGAAGTTTGAGACC 3' 5' CTGCCCGTTCTTGGAGATAC 3'

versus the housekeeping gene  $\beta$ -actin (*Actb*), elongation factor 1-alpha 1 (*Eef1a*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primers used in the real-time PCR assay were commercially purchased (Invitrogen; Table 1). Amplification efficiency (E) was assessed to be between 1.8 and 2. Transcripts from five MSC donors were analysed. Each experiment was conducted in triplicate.

#### Transwell migration assay

Random migration (i.e., equal concentrations of bioactive molecules in the both compartments) was measured by a modified Boyden chamber assay (Falk *et al.*, 1980) using polycarbonate filters (8  $\mu$ m pore size; Nunc, Wiesbaden, Germany) coated with and without Collagen I (100  $\mu$ g/mL; Pure Col®, Inamed Biomaterials, Fremont, CA, USA), which is the most abundant extracellular protein of bones (Rossert and de Crombrughe, 2002). MSCs ( $4 \times 10^4$ ) were seeded onto the filters and incubated for 5 h at 37 °C. Equal cell seeding was validated by an MTS test. Non-migrated cells were removed from the upper side of the filter by scraping, and remaining migrated cells were stained with 10  $\mu$ g/mL Hoechst-33342 (Invitrogen). The average numbers of migrated cells from five microscopic fields (1 mm x 0.8 mm) per filter (0.47 cm<sup>2</sup>) were analysed using the NIH ImageJ software package (<http://rsb.info.nih.gov/nih-image/>). Each experiment was conducted in duplicate.

#### Blocking and induction assays

A CD29 specific antibody, Armenian hamster (anti-rat CD29), (10  $\mu$ g/mL; BD Biosciences), a CD73 inhibitor, adenosine 5'-( $\alpha$ ,  $\beta$ -methylene) diphosphate (APCP; 120  $\mu$ M; Sigma-Aldrich, Munich, Germany) and PP2 (10  $\mu$ M; Calbiochem, Merck KGaA, Darmstadt, Germany) were employed in migration assays. Each experiment was conducted in duplicate.

#### Multiplex analysis and ELISA

Multiplex analysis (Milliplex Rat Cytokine/Chemokine Panel Premix 24 Plex and Milliplex Rat Bone Panel

2; Millipore, Schwalbach, Germany) of conditioned media (CM) were performed by the manufacturer using duplicates. Enzyme-linked immunosorbent assays (ELISAs) (monoclonal antibodies for TNF- $\alpha$  and FAK (pTyr397) from R&D Systems, Wiesbaden, Germany) were performed according to the manufacturer's instructions using triplicates. TNF- $\alpha$  was detected in 30-times concentrated CM. For standardisation, CM corresponding to equal cell numbers was applied. FAK (pTyr397) was detected in cell lysates and normalised to total protein content as determined by the Bio-Rad DC Protein Assay.

#### Immunocytochemistry

Staining of actin fibres of *para*-formaldehyde-fixed and permeabilised cells was achieved by incubation with Alexa 594-conjugated phalloidin (6.6 nM; Invitrogen). Nuclei were counterstained with 10  $\mu$ g/mL Hoechst-33342 (Invitrogen). Each experiment was conducted in duplicate.

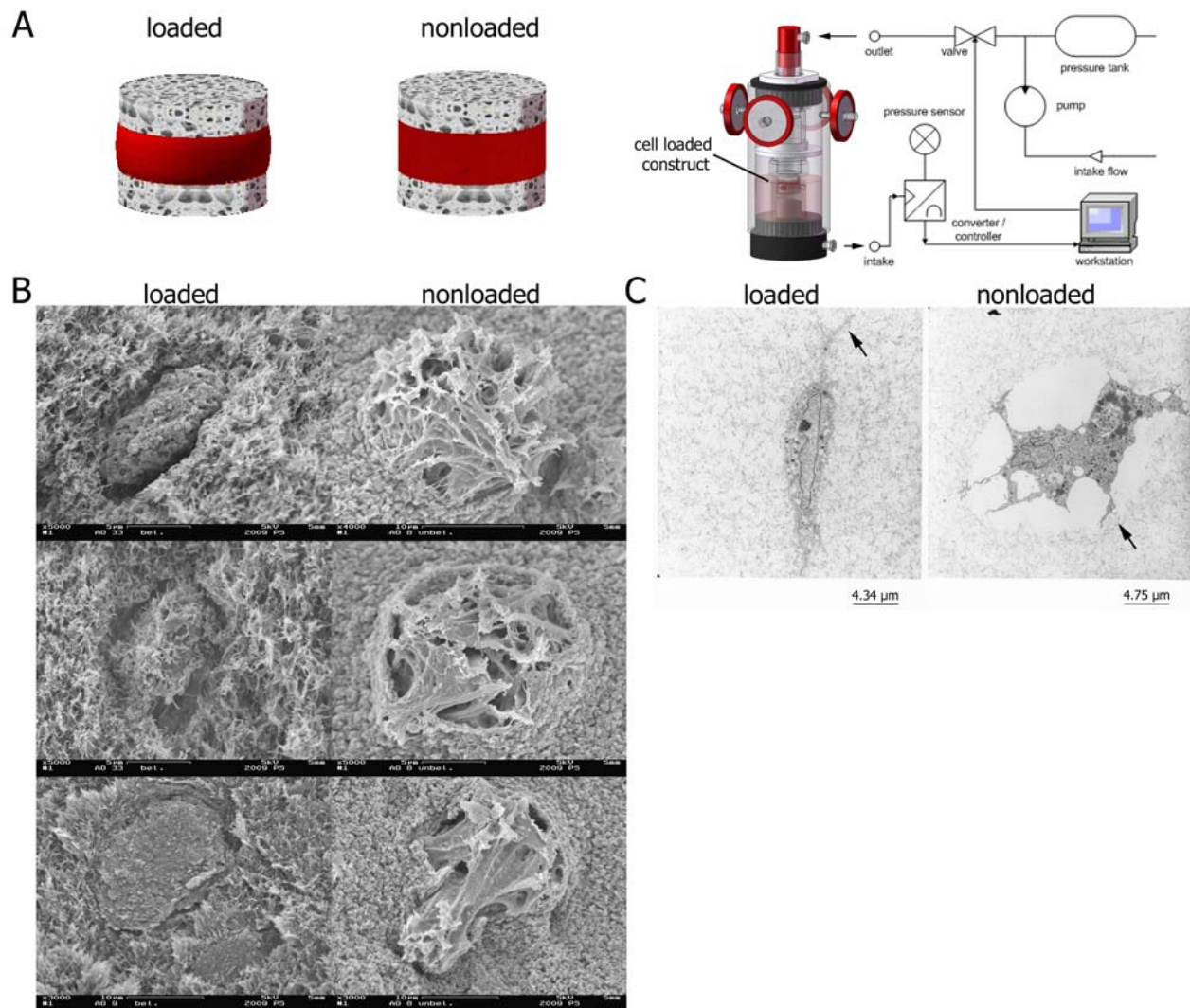
#### Transmission electron microscopy

MSCs in fibrin constructs were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Specimens were post-fixed for 2 h in 2 % osmium tetroxide at room temperature, followed by dehydration in a graded series of ethanol. Samples were immersed with propylene oxide as an intermedium, with a mixture of propylene oxide and epoxy resin, with pure epoxy resin and finally polymerised at 60 °C. Ultra-thin sections (70 nm) were prepared with an ultra-microtome (Ultracut S, Leica Microsystems, Wetzlar, Germany) and mounted on electron microscopy copper grids, 300 mesh. Sections were stained with uranyl acetate and lead citrate and investigated in the transmission electron microscope EM 906 (Zeiss, Oberkochen, Germany).

#### Scanning electron microscopy

MSCs in fibrin or on migration filters were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Samples underwent a graded ethanol series and





**Fig. 1. Mechanical stimulation of MSCs embedded in a 3D fibrin construct.** (A) Left: MSCs were embedded in fibrin, placed between two cancellous bone chips and mechanically stimulated. Shown are schematic pictures of loaded and non-loaded MSC/fibrin constructs. Right: Experimental set-up and control chart to investigate the effect of mechanical loading of MSC/fibrin constructs. (B) Shown are three representative pictures of loaded and non-loaded MSCs after three days detected by SEM. Note the differences in the 3D morphology and cell-fibrin interaction after loading. (C) Shown are loaded and non-loaded MSCs after three days detected by TEM.

were immersed in 100% hexamethyldisilazane (HMDS) to replace the ethanol. Finally, samples were air dried under the fume hood overnight. Fibrin samples were mechanically fractured to investigate cells within the gel. Finished samples were mounted and fixed on aluminium stubs, and coated with gold/palladium using the MED 020 sputter coater (Baltec, Lichtenstein). SEM examination of the samples was performed using a Zeiss Digital Scanning Electron Microscope (DSM 982 Gemini, Zeiss). Each experiment of MSCs on migration filters was conducted in duplicate.

#### Statistical analysis

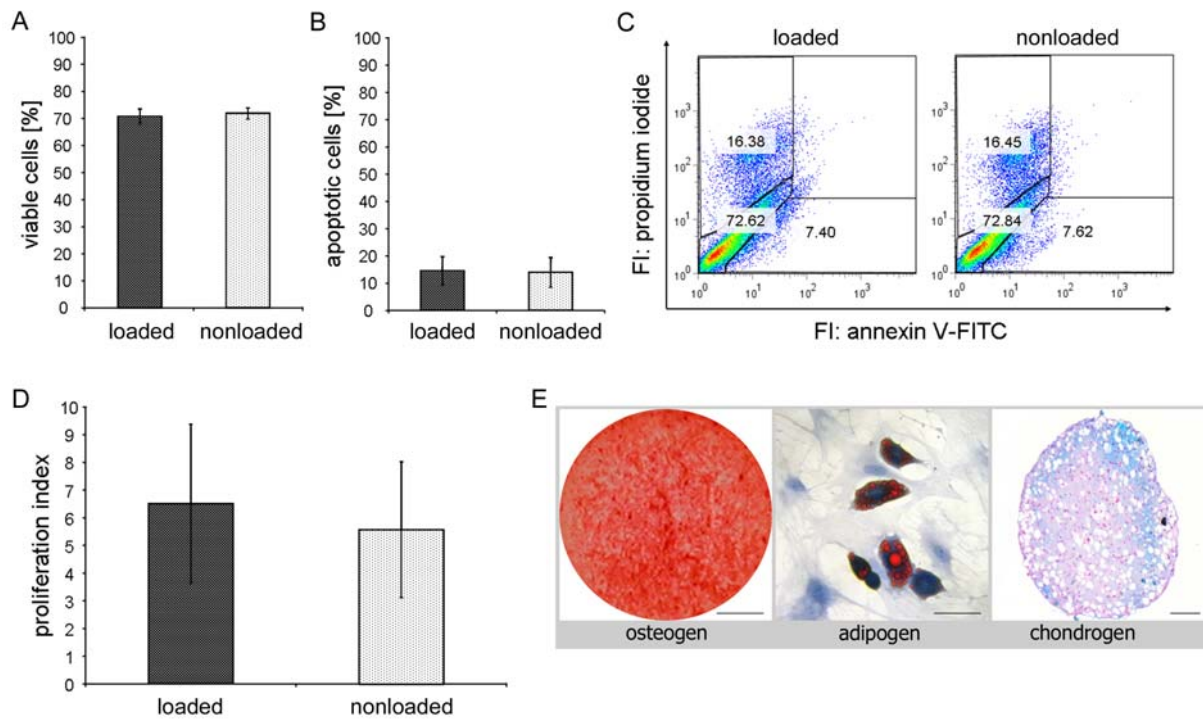
The SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA) was used for statistical evaluation. If not stated otherwise, results from at least five independent experiments were analysed for statistical significance using the paired Student's *t* test. The *t*-test assumption of normality was tested using the Shapiro-Wilk normality

test. When performing multiple pair-wise comparisons, one-way or two-way analysis of variance (ANOVA, repeated measures) were performed, and *p*-values were adjusted using Bonferroni's *p*-value adjustment multiple comparison procedure. Results are presented as mean  $\pm$  standard deviation (SD). Gene expression data was statistically analysed using REST<sup>®</sup> 2008 (Corbett Research/Qiagen, Hilden, Germany) (Pfaffl *et al.*, 2002). REST<sup>®</sup> 2008 was developed by M. Pfaffl and Qiagen for exclusive analysis of gene expression data from quantitative real-time PCR experiments. Results are presented as median  $\pm$  min/max. A significance level was set at  $p < 0.05$ .

#### Results

##### Characterisation of MSCs after mechanical loading

In order to mechanically stimulate MSCs, a compression bioreactor system was employed to simulate loading



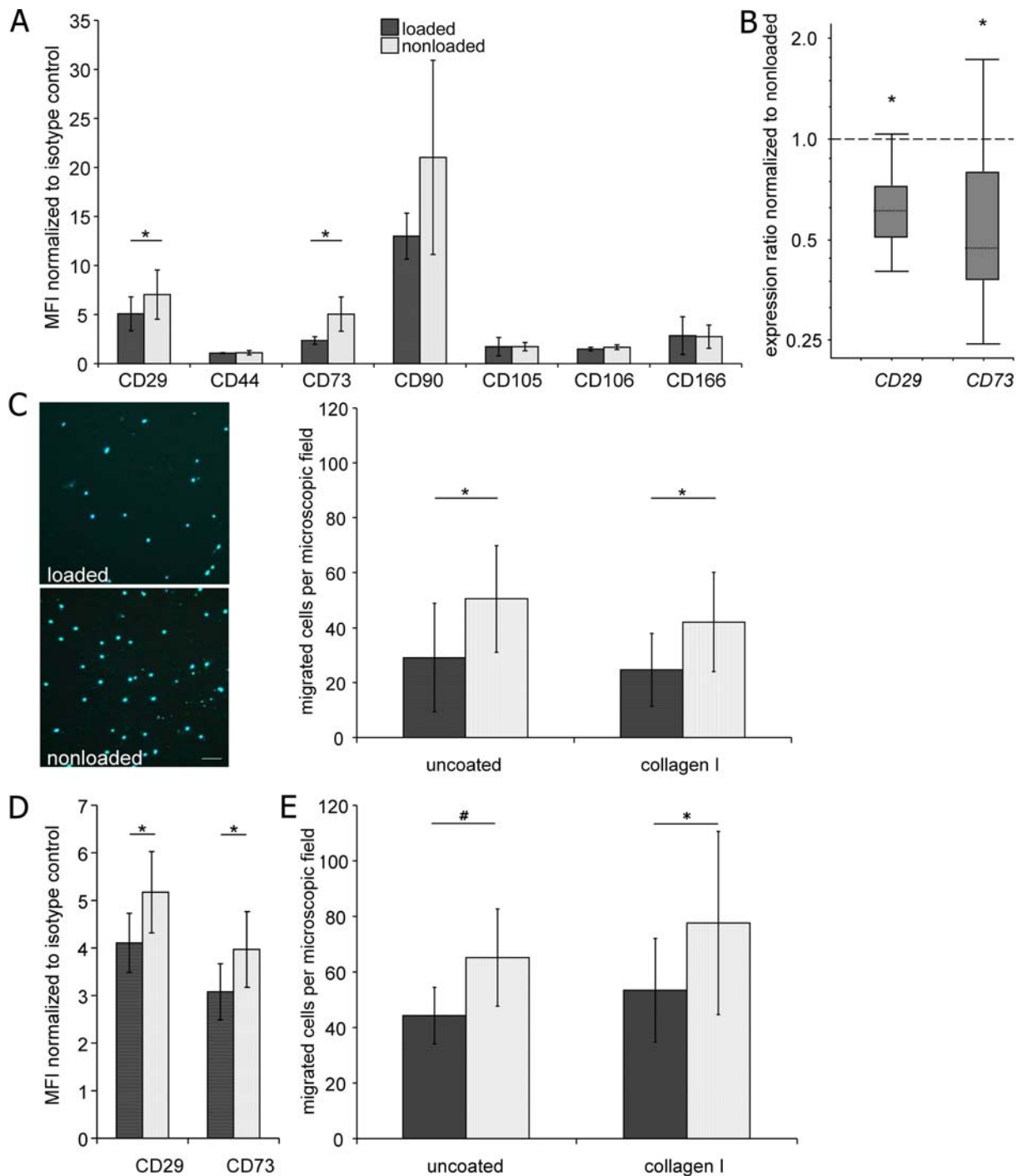
**Fig. 2. Mechanically loaded MSCs show molecular and phenotypic characteristics of MSCs.** After mechanical loading, the cells were isolated from the fibrin construct. **(A)** Cell viability was validated by means of CASY® Cell Counter Model DT. **(B, C)** Apoptotic cells were determined by double fluorescence staining with annexin V:FITC and propidium iodide and flow cytometry analysis. **(B)** Data analysis of flow cytometry data revealed no significant difference between loaded and non-loaded cells. **(C)** Shown are representative dot plots with relative fluorescence intensity of annexin V:FITC (x-axis) and relative fluorescence intensity of propidium iodide (y-axis) and appropriate gates determined with single colour staining. **(D)** Proliferation of loaded and non-loaded MSCs was determined by measuring cell activity on day one and six with the MTS test. The proliferation index was calculated by normalising day one to day six values. **(E)** MSCs could still differentiate into the adipogenic, chondrogenic and osteogenic lineage after loading. Bar indicates: 3 mm (osteogenic), 20  $\mu$ m (adipogenic), 100  $\mu$ m (chondrogenic). Abbreviations: FI, fluorescence intensity; FITC, fluorescein isothiocyanate ( $n = 5$ ).

conditions determined in the fracture gap *in vivo* (Fig. 1A). SEM analyses of non-loaded MSCs embedded in fibrin revealed a round and wrinkled morphology with pronounced cell-fibrin interactions compared to an oval and almost smooth morphology of loaded MSCs with low cell-fibrin interactions (Fig. 1B). TEM analyses further support these findings. In contrast to loaded MSCs, non-loaded MSCs seem to build more actin fibres (Fig. 1C). Cell activity analysis by MTS test revealed no significant difference between loaded and non-loaded MSCs (OD 490 nm: mean<sub>loaded</sub> = 1.359, mean<sub>non-loaded</sub> = 1.337;  $p = 0.963$ ). An equal number of viable cell in constructs exposed to mechanical loading and non-loaded controls was validated by means of CASY® Cell Counter Model DT (cell viability: mean<sub>loaded</sub> = 70.7 %, mean<sub>non-loaded</sub> = 71.8 %;  $p = 0.488$ ; Fig. 2A). Annexin V/PI staining revealed no significant difference in the number of apoptotic cells between loaded and non-loaded MSCs (percentage of apoptotic cells: mean<sub>loaded</sub> = 14.3 %, mean<sub>non-loaded</sub> = 13.9 %;  $p = 0.094$ ; Fig. 2B,C). Proliferation was unaffected by mechanical stimulation (cell activity day 7 normalised to day 1: mean<sub>loaded</sub> = 6.5, mean<sub>non-loaded</sub> = 5.6;  $p = 0.199$ ; Fig. 2D). Loaded and non-loaded MSCs were able to differentiate towards the adipogenic, chondrogenic and osteogenic lineage (Fig. 2E), and quantitative analysis revealed an

increase in chondrogenic, but no difference in adipogenic and osteogenic differentiation capacity after loading. After cultivation in appropriate differentiation media, chondrogenic differentiation ability was significantly increased after mechanical loading (area Alcian Blue: loaded = 53.0 %, unloaded = 39.4 %,  $p = 0.040$ ).

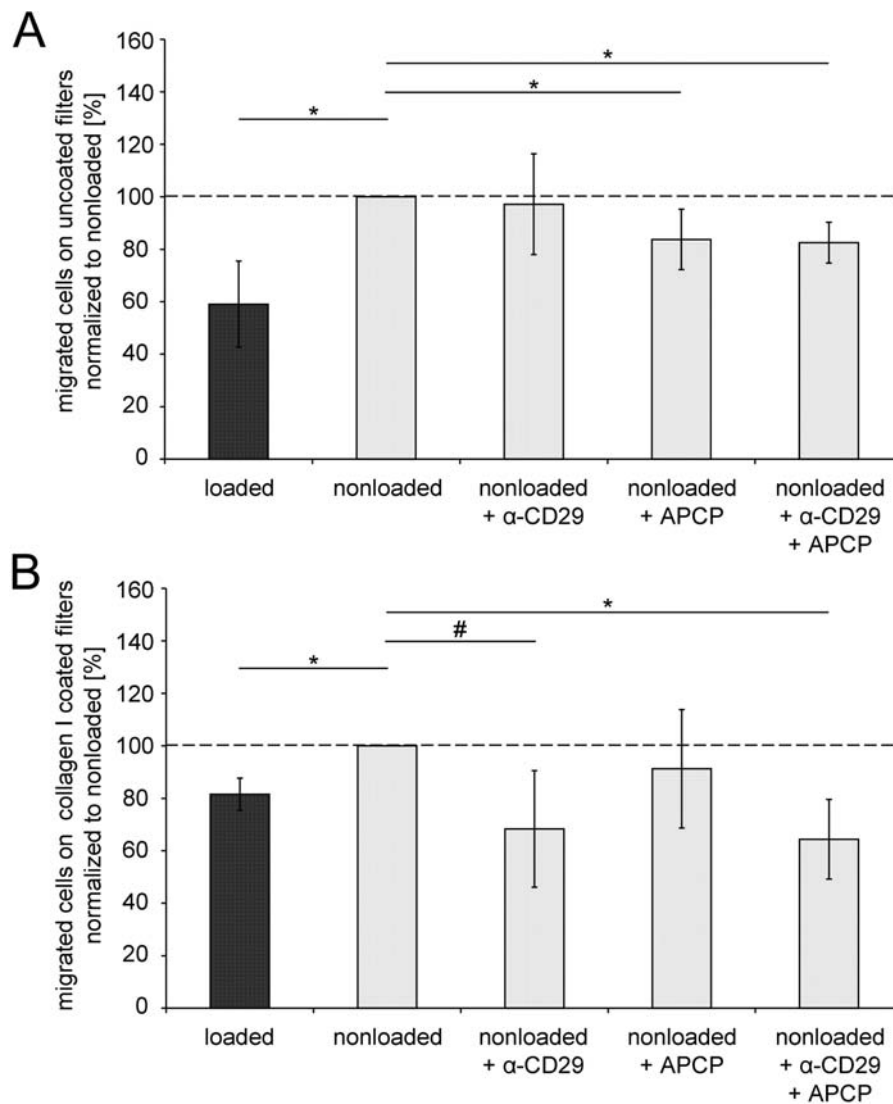
#### CD29 and CD73 are downregulated in response to mechanical loading

In order to investigate whether mechanical loading affects the MSC characteristics, their cell surface marker expression pattern was determined. Qualitatively, the markers CD29, CD44, CD73, CD90, CD105, CD106 and CD166 were still expressed on loaded MSCs. The analysed populations were homogenous with no subpopulations detectable. Quantitatively, cyclic-compressive loading led to a significant downregulation of CD29 (CD29 mean fluorescence intensity (MFI) normalised to the isotype control: ratio<sub>loaded</sub> = 5.09, ratio<sub>non-loaded</sub> = 7.03;  $p = 0.021$ ) and CD73 compared to the non-loaded controls (CD73 MFI normalised to the isotype control: ratio<sub>loaded</sub> = 2.35, ratio<sub>non-loaded</sub> = 5.04;  $p = 0.017$ ; (Fig. 3A). To analyse whether the apparent marker regulation also occurs on the mRNA level, quantitative RT-PCR analyses were performed. In accordance with flow cytometry data, also CD29 and



**Fig. 3. CD29 and CD73 are downregulated and migration is reduced in mechanically loaded and non-loaded MSCs** (A) The expression of MSC cell surface markers of loaded and non-loaded MSCs was investigated by flow cytometry. Shown is the mean fluorescence intensity (MFI) of loaded and non-loaded MSCs stained with specific Abs after normalisation to isotype controls. (B) The expression of mRNA specific for *CD29* and *CD73* of loaded and non-loaded MSCs was evaluated by quantitative reverse transcription-polymerase chain reaction and normalised for the housekeeping genes *Actb*, *Gapdh* and *Eef1a*. Shown is the gene expression of loaded MSCs normalised to non-loaded MSCs. Results are presented as medians with the whiskers indicating the min-max range. (C) MSC migration was investigated in a modified Boyden-Chamber assay on migration filters either uncoated or coated with collagen type I. The numbers of migrated cells from five microscopic fields per filter were analysed using NIH ImageJ software. Two representative pictures of migrated loaded and non-loaded cells and the average number of migrated cells per microscopic field (after five hours) are shown. Bar indicates 50  $\mu$ m. (D, E) To determine whether the observed effects are persistent results, the cells were re-embedded in fibrin and kept in non-loaded bioreactors for another six days. (D) The expression of CD73 and CD29 of loaded and non-loaded MSCs was investigated by flow cytometry. (E) MSC migration was investigated in a modified Boyden-Chamber assay on migration filters either uncoated or coated with collagen type I. ( $n = 5$ , #,  $p = 0.049$  without Bonferroni correction; \*,  $p < 0.05$ ).





**Fig. 4. Inhibition of CD29 and CD73 leads to reduced migration of MSCs on collagen type I and uncoated migration filters, respectively.** Migration of loaded and non-loaded MSCs on (A) uncoated and (B) collagen I coated filters was investigated in the presence of CD29 blocking antibody (10  $\mu$ g/mL) and 120  $\mu$ M APCP. Shown is the number of migrated cells after five hours normalised to non-loaded MSCs ( $n = 5$ , #,  $p = 0.051$  without Bonferroni correction; \*,  $p < 0.05$ ).

CD73 mRNA expression in MSCs was significantly downregulated in response to mechanical loading (relative gene expression normalised to the non-loaded control:  $E_{CD29} = 0.618$ ;  $p = 0.031$ ;  $E_{CD73} = 0.433$ ;  $p = 0.013$ ; Fig. 3B).

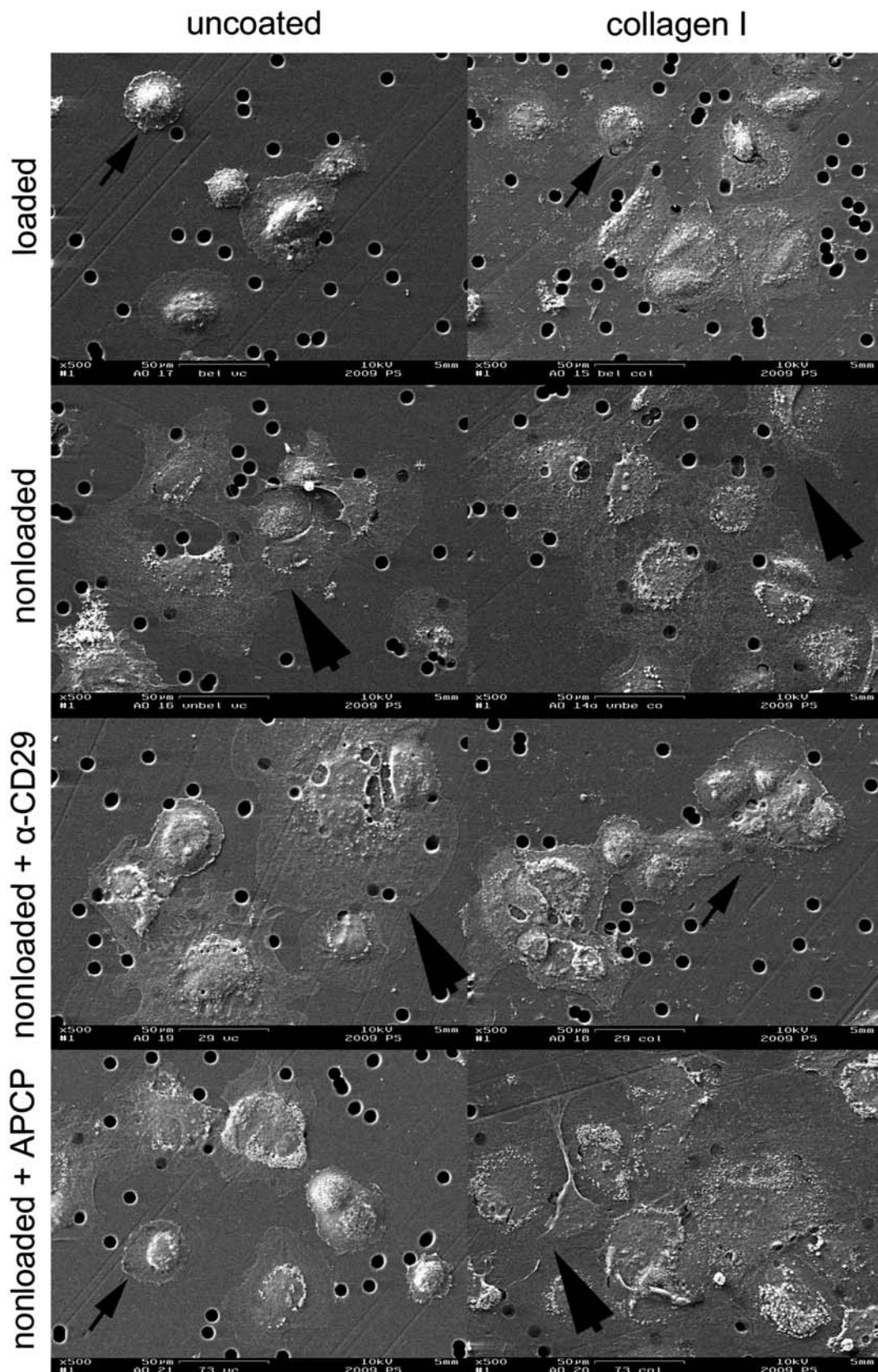
#### Migratory capacity is reduced after mechanical loading

Most cells, if not all, retain autochthonous cytoskeletal oscillation, polarity, and random migration in the absence of pro-migratory factors (Friedl, 2004). Random migration of MSCs can be stimulated by non-directional motogenic signals, such as the uniform application of the platelet-derived growth factor (PDGF) (Ozaki *et al.*, 2007). Since CD29 and CD73 are both associated with cell migration, the influence of cyclic-compressive loading on this MSC function was further investigated. MSC migration decreased significantly in response to loading, but was unaffected by coating (loading:  $p < 0.001$ ; coating:  $p =$

0.373; migrated cells per microscopic field: mean<sub>loaded, collagen type I</sub> = 24.7 cells, mean<sub>non-loaded, collagen type I</sub> = 42.0 cells,  $p < 0.001$ ; mean<sub>loaded, uncoated</sub> = 29.1 cells, mean<sub>non-loaded, uncoated</sub> = 50.46 cells,  $p < 0.001$ ) (Fig. 3C). These numbers of migrated cells correspond to a random migration rate of 4-11 %, which is in line with the literature (Rüster *et al.*, 2005; Thibault *et al.*, 2007).

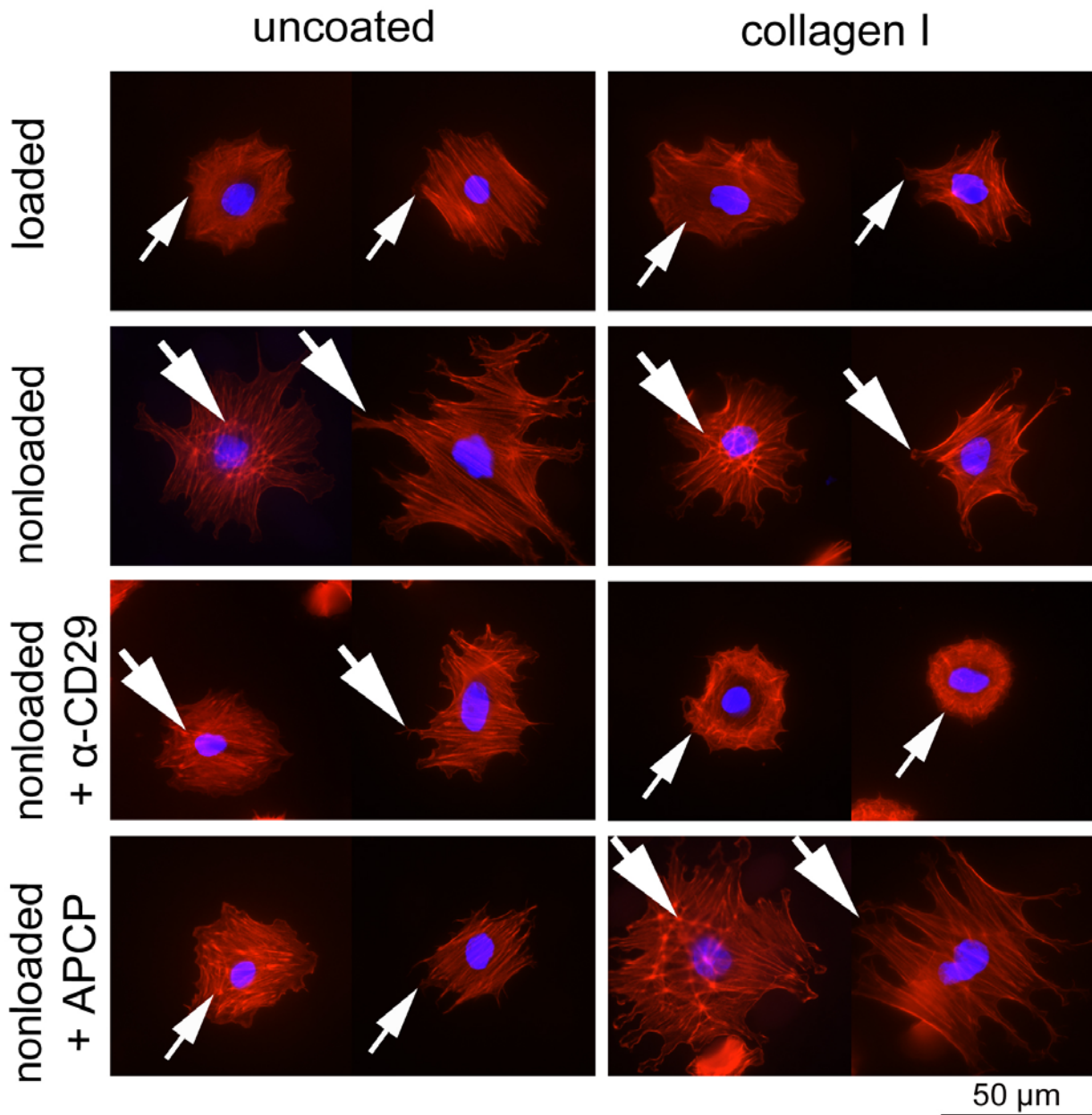
#### Reduced CD29 and CD73 expression and migratory capacity remain after removal of mechanical stimulus

Subsequently, the question arose whether the downregulation of CD29 and CD73 as well as the reduction in the migratory capacity were persistent effects. To address that issue, MSCs were loaded for three days and kept non-loaded for another six days. CD29 and CD73 protein expression was still significantly downregulated in loaded compared to non-loaded MSCs (CD29 MFI normalised to the isotype



**Fig. 5. Inhibition of CD29 and CD73 leads to reduced cell spreading of MSCs on collagen type I and uncoated migration filters, respectively.** The cell morphology was analysed by SEM. Note the differences in cell spreading in loaded, non-loaded and treated MSCs as indicated by the arrows. Abbreviation: APCP, adenosine 5'-( $\alpha,\beta$ -methylene) diphosphate; thin arrows indicate decreased cell spreading; thick arrows indicate increased cell spreading.



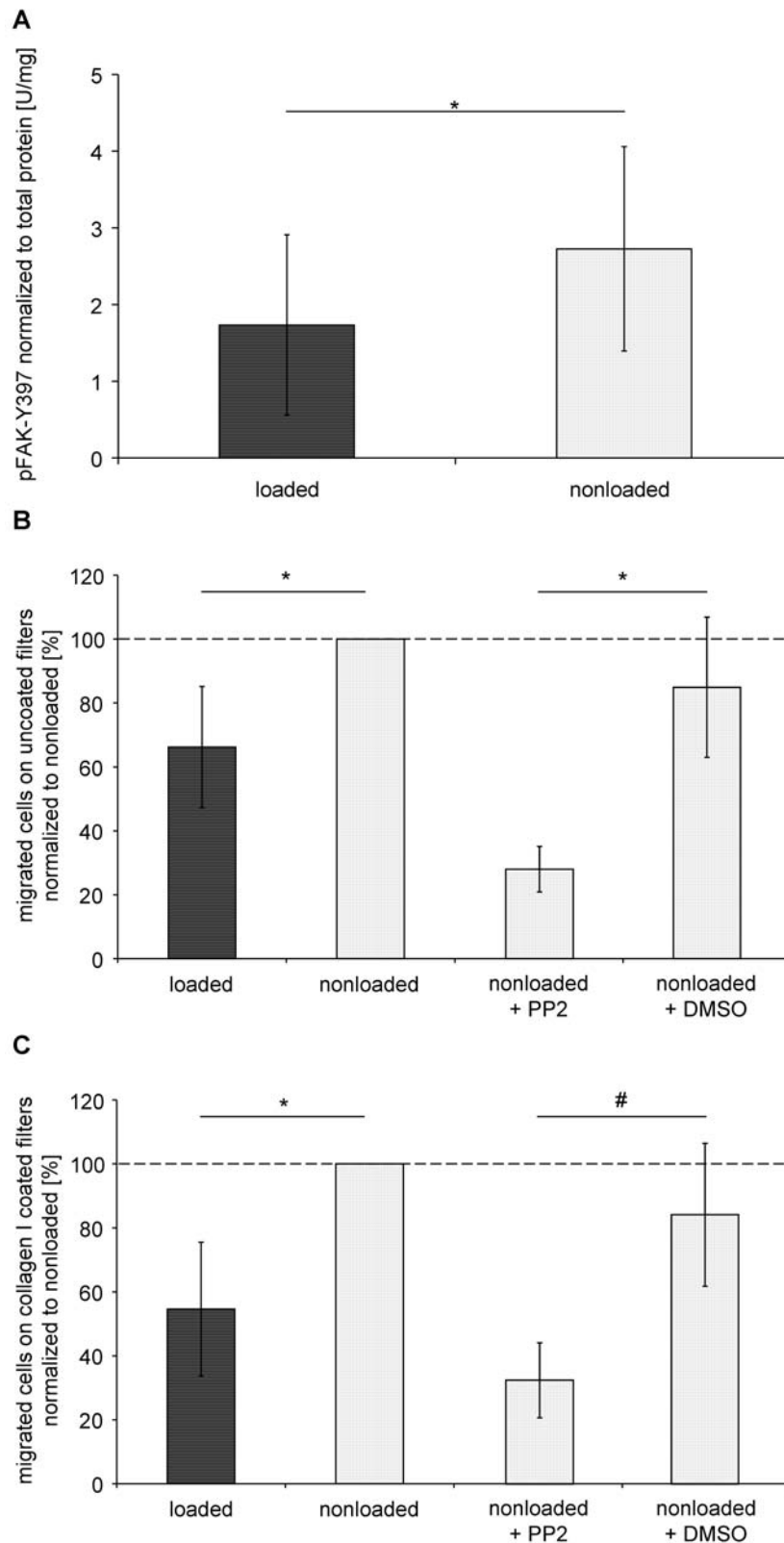


**Fig. 6. Inhibition of CD29 and CD73 leads to changes in the cytoskeleton of MSCs on collagen type I and uncoated migration filters, respectively.** The actin filaments and nuclei of migrated MSCs were stained with phalloidin dye-Alexa 594 and Hoechst-33258, respectively. Shown are representative pictures. Note the differences in lamellipodia (right pictures) and actin accumulation formation (left pictures) in loaded, non-loaded and non-loaded+treated MSCs as indicated by the arrows. Abbreviation: APCP, adenosine 5'-( $\alpha,\beta$ -methylene) diphosphate; thin arrows indicate decreased actin accumulation and lamellipodia formation; thick arrows indicate increased actin accumulation and lamellipodia formation.

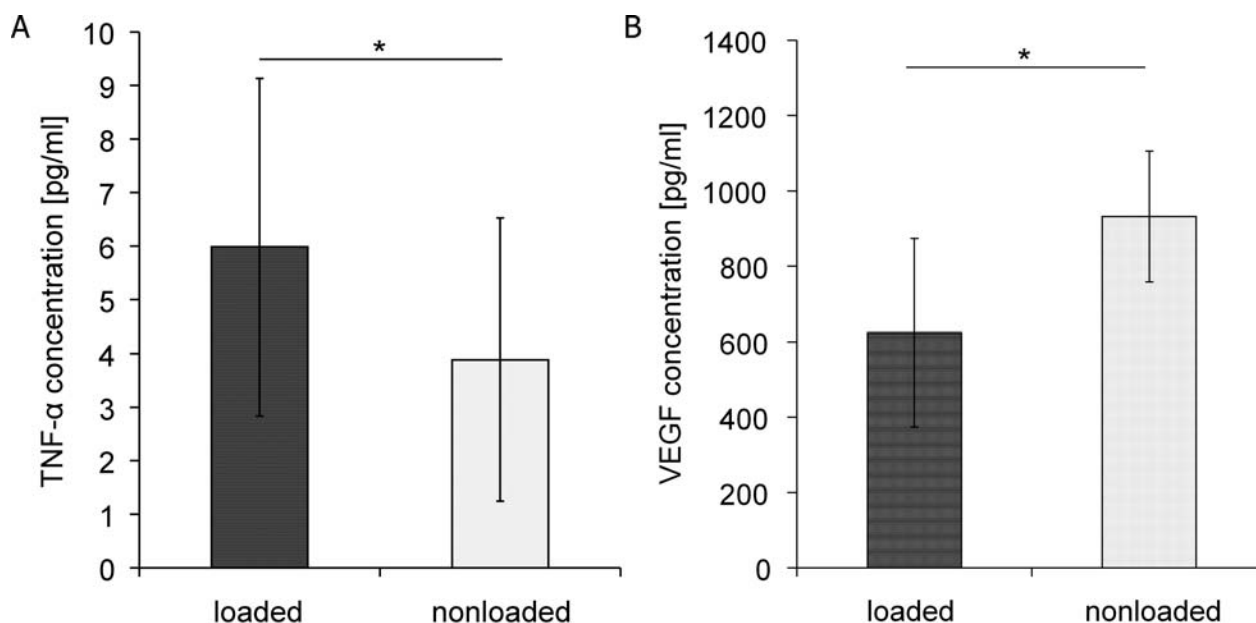
control: ratio<sub>loaded</sub> = 4.11, ratio<sub>non-loaded</sub> = 5.17;  $p = 0.001$ ; CD73 MFI normalised to the isotype control: ratio<sub>loaded</sub> = 3.08, ratio<sub>non-loaded</sub> = 3.97;  $p = 0.013$ ; Fig. 3D). On collagen type I, cell migration was significantly reduced in loaded MSCs (migrated cells per microscopic field: mean<sub>loaded, collagen type I</sub> = 53.4 cells, mean<sub>non-loaded, collagen type I</sub> = 77.6 cells,  $p < 0.05$ ). On uncoated filters, a lower migration capacity for loaded MSCs was observed after Student's  $t$  testing (migrated cells per microscopic field: mean<sub>loaded, uncoated</sub> = 44.3 cells, mean<sub>non-loaded, uncoated</sub> = 65.2 cells,  $p = 0.049$ ), but not when adjusted by Bonferroni's correction (Fig. 3E).

#### Lower migration potential depends on CD73 and CD29

Next, the putative involvement of CD73 and CD29 in the reduced migration capacity of loaded MSCs was analysed. On uncoated filters, blocking of CD73 by CD73 inhibitor adenosine 5'-( $\alpha,\beta$ -methylene) diphosphate (APCP) led to a statistically significant reduction in MSC migration compared to the control (migrated cells normalised to the non-loaded control: mean<sub>non-loaded, APCP, uncoated</sub> = 83.0 %,  $p < 0.05$ ), whereas blocking of CD29 by anti-CD29 had no significant effect (Fig. 4A). However, the reduction of cell migration was less pronounced after CD73 inhibition than



**Fig. 7. FAK is hypo-phosphorylated in loaded compared to non-loaded MSCs and Src family kinases are essential for MSC migration.** (A) The amount of pFAK (pTyr397) was measured with ELISA and normalised to total protein content. (B, C) Migration of loaded and non-loaded MSCs on (B) uncoated and (C) collagen I coated filters was investigated in the presence of 10  $\mu$ M SFK inhibitor PP2 (after five hours). Shown is the number of migrated cells after five hours normalised to non-loaded MSCs. Abbreviation: SFK, Src kinase family ( $n = 5$ , #,  $p = 0.023$  without Bonferroni correction; \*,  $p < 0.05$ ).



**Fig. 8. Expression of TNF- $\alpha$  and VEGF, regulators of CD73, are altered after mechanical loading.** ELISA of conditioned media of loaded and non-loaded MSCs were conducted. **(A)** TNF- $\alpha$ , a potential negative regulator of CD73 is upregulated after mechanical loading. **(B)** VEGF, a potential positive regulator of CD29 is downregulated after mechanical loading (Kasper *et al.*, 2010) ( $n = 5$ , \*,  $p < 0.05$ ).

after mechanical loading. On collagen type I coated filters, blocking of CD29 led to a statistically significant reduction in MSC migration compared to the control after Student's  $t$  testing (migrated cells normalised to the non-loaded control: mean<sub>non-loaded, anti-CD29, collagen type I</sub> = 69.2 %,  $p = 0.05$ ), but not when adjusted by Bonferroni's correction, whereas blocking of CD73 by APCP had no significant effect (Fig. 4B). Simultaneous blocking of CD29 and CD73 – on both coatings – led to a statistically significant reduction of MSC migration compared to the control (migrated cells normalised to the non-loaded control: mean<sub>non-loaded, anti-CD29, APCP, uncoated</sub> = 82.5 %,  $p < 0.05$ ; mean<sub>non-loaded, anti-CD29, APCP, collagen type I</sub> = 63 %,  $p < 0.05$ ).

#### Changes in the MSC cytoskeleton in response to mechanical loading and inhibition of CD73 and CD29

Changes in the migratory behaviour of cells are closely associated with alterations in cell spreading and cytoskeleton organisation. To address this issue, SEM analysis of loaded and non-loaded MSCs with and without treatment with CD29- and CD73-inhibitors were conducted and revealed less cell spreading in loaded and inhibitor-treated cells compared to non-loaded cells (Fig. 5). Moreover, migration depends on cytoskeleton remodelling, which is also sensitive to mechanical stress. Thus, the cytoskeletal structure was further investigated. As depicted in Fig. 6, there are less actin accumulations and lamellipodia formation detectable in loaded MSCs compared to non-loaded ones. Non-loaded MSCs treated with the CD29 blocking antibody and APCP resembled the loaded phenotype on collagen type I, and uncoated filters, respectively. Thus, these data further support the finding that mechanical loading inhibits the migratory capacity of MSCs.

#### Candidate downstream targets of CD73 and CD29 contribute to the mechanically induced decrease in MSC migration

CD29 is known to activate FAK upon mechanical stimulation. Thus, the amount of pFAK (pTyr397) was measured and found to be reduced in loaded compared to non-loaded MSCs (mean<sub>loaded</sub> = 1.73 U/mg, mean<sub>non-loaded</sub> = 2.72 U/mg,  $p = 0.035$ ; Fig. 7A). Once activated, FAK allows interaction with a number of Src family kinases (SFKs). Moreover, CD73 is known to signal through SFKs. Therefore, it was investigated whether SFKs are involved in MSC migration. On uncoated filters, SFK inhibition by PP2 resulted in a significant reduction of MSC migration (migrated cells normalised to the non-loaded control: mean<sub>non-loaded, PP2, uncoated</sub> = 28.0 %, mean<sub>non-loaded, DMSO, uncoated</sub> = 90.4 %,  $p < 0.05$ ). On collagen type I coated filters, a lower migration capacity for PP2 treated MSCs was observed after Student's  $t$  testing (migrated cells normalised to the non-loaded control: mean<sub>non-loaded, PP2, collagen type I</sub> = 32.4 %, mean<sub>non-loaded, DMSO, collagen type I</sub> = 108.6 %,  $p = 0.023$ ) but not when adjusted by Bonferroni's correction (Fig. 7B,C).

#### TNF- $\alpha$ and VEGF might regulate CD73 and CD29 expression, respectively

A number of cytokines and growth factors are known to regulate CD73 and CD29 expression. To identify potential up-stream regulators of mechanoregulated CD73, the conditioned media (CM) of loaded and non-loaded cells were analysed. Among the investigated factors, the CD73 regulators Il-4 and INF- $\gamma$  were not detectable, whereas Il-1 $\beta$  was not regulated. In contrast, the CD73 regulator TNF- $\alpha$  was increased and the CD29 regulator VEGF was decreased in all three animals (Table 2). To statistically evaluate these data, ELISA analysis was performed with CM from MSCs from five animals (TNF- $\alpha$  concentration:



**Table 2:** Multiplex analysis of conditioned media [pg/mL]

	Loaded			Non-loaded		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
ACTH	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
RANKL	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Eotaxin	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
GCSF	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
GMCSF	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
GRO/KC	5680	142	2115	569	55.05	6955
IFNg	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
IL-10	150	n.e.	n.e.	n.e.	n.e.	67.8
IL-12p70	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
IL-13	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
IL-17	1.27	0.9235	1.0625	0.6485	0.958	0.9235
IL-18	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
IL-1a	4.385	n.e.	n.e.	2.925	n.e.	2.31
IL-1b	n.e.	6.34	n.e.	n.e.	2.945	n.e.
IL-2	n.e.	7.26	n.e.	n.e.	n.e.	n.e.
IL-4	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
IL-5	3.455	n.e.	2.165	n.e.	n.e.	4.98
IL-6	919	239	224	113	129.5	1765
IL-9	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
IP-10	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
MCP-1	1275	100.95	646	181	27.25	893
MIP-1a	244.5	28.85	90.05	29.05	n.e.	203
RANTES	845.5	65.6	652	385.5	31.65	701
TNFa*	8.445	31.45	4.645	n.e.	12	4.125
VEGF*	1740	963.5	1645	2230	1475	2295
Leptin	4.925	n.e.	4.715	4.24	n.e.	4.3

$N = 3$

Abbreviation: n.e. not expressed

\* - concurrently regulated in all three animals

mean<sub>loaded</sub> = 6.0 pg/mL, mean<sub>non-loaded</sub> = 3.9 pg/mL;  $p = 0.003$ ; Figure 8; VEGF concentration: mean<sub>loaded</sub> = 623.5 pg/mL, mean<sub>non-loaded</sub> = 931.8 pg/mL;  $p = 0.029$  (Kasper *et al.*, 2010)).

### Discussion

Interfragmentary movement – especially compression – is essential for successful fracture repair (Goodship and Kenwright, 1985). Hence, in the present study the influence of mechanical loading on the MSC characteristics and associated functions was investigated. Our results show a pivotal role for CD29 and CD73 in the migratory response of MSCs upon loading.

#### Mechanoregulation of CD73 and CD29 in MSCs

The results presented here demonstrate that mechanically loaded MSCs downregulate CD73 and CD29, but show molecular and phenotypic characteristics of MSCs (Dominici *et al.*, 2006). Our data are in contrast to previous studies reporting an upregulation of CD29 expression in other cell types of mesenchymal origin, namely chondrocytes and myoblasts. The response of those cells

was studied under cyclic hydrostatic pressure and tension, respectively (Lucchinetti *et al.*, 2004; Fan *et al.*, 2009). This discrepancy is likely due to not only their differentiated stage but also the different biomechanical loading regimes, since a strain specific regulation seems to be more the rule (Wong *et al.*, 2003). This is especially important, since most former expression analyses of CD29 in mesenchymal cells were not conducted using compression. Mechanoregulation of CD73 and CD29 might be mediated by TNF- $\alpha$  (Kalsi *et al.*, 2002; Hunsucker *et al.*, 2005) and VEGF (Lee *et al.*, 2006), respectively. Extracellular VEGF concentration is decreased in response to mechanical loading, possibly leading to reduced CD29 expression (Kasper *et al.*, 2010). In contrast, TNF- $\alpha$  expression was mechanically upregulated, potentially negatively regulating CD73 expression. The downregulation of CD73 and CD29 was observed on a transcription and protein level. This data corroborates that CD73 activity correlates with mRNA levels in most murine tissues and human cell lines (reviewed in Hunsucker *et al.*, 2005). However, also a direct regulation *via* mechanoresponsive promoter elements could be possible (Hansen *et al.*, 1995). Moreover, hypoxia is a major regulator of both CD73 and CD29 expression, possibly due to a HIF-1 $\alpha$  responsive

element in their promoter region (Thompson *et al.*, 2004; Keely *et al.*, 2009). However, this pathway seems to be unlikely in the present setting, since in a similar study using human MSCs, direct oxygen measurement confirmed non-hypoxic conditions and HIF-1 $\alpha$  expression levels were unaffected by mechanical loading of MSCs (Kasper *et al.*, 2007a).

### Mechanoregulation of MSC migration

Here, we demonstrate for the first time that MSCs reduce their random migratory capacity, i.e., their basic motility machinery, after experiencing compressive loading and that this effect is mediated by CD73 and CD29. Cyclic strain has previously been shown to decrease cell migration of alveolar epithelial cells (Desai *et al.*, 2008) and gastric mucosal cells (Osada *et al.*, 1999), but to increase that of smooth muscle cells (Li *et al.*, 2003) and osteoblasts (Bhatt *et al.*, 2007), suggesting a cell-specific response. Direct comparison of these and our results is, however, difficult. So far, a concurrent investigation of mechanical loading and cell migration was analysed only under 2D conditions (e.g., wound scratch assay under the influence of cyclic strain applied with the Flexcell<sup>®</sup> systems). However, it is known that cell function and gene/protein expression may be altered substantially by a 2D monolayer culture as opposed to a 3D culture (Lee *et al.*, 2007; Hong and Stegemann, 2008). Because of this fact and the greater physiological relevance, in this study, mechanical loading was carried out under 3D conditions. Unfortunately, there is no method available to date to mechanically stimulate and measure cell migration concurrently in a 3D environment. Therefore, to evaluate MSC migration, the cells were removed from the 3D culture, potentially introducing a bias in interpretation of the results.

The process of cell migration can be viewed as a five-step cycle, which involves cell polarisation, extension of a protrusion, formation of stable adhesion near the leading edge of the protrusion, translocation of the cell body forward and release of adhesion and retraction at the cell rear. Integrins belong to a major family of promigratory receptors. They support adhesion to the ECM, stabilise the protrusion by means of structural connections to the cytoskeleton, and activate migration-related signalling molecules (Ridley *et al.*, 2003; Wang *et al.*, 2008). The involvement of CD29 in MSC migration has been demonstrated *in vivo*: e.g. lower numbers of MSCs engrafted and migrated into ischemic myocardium, if pre-treated with neutralising antibodies against CD29 (Ip *et al.*, 2007). An involvement of CD73 in cell migration was already suggested for other cell types. However, its role in cell migration is less clear. For example, CD73 expression is related to metastatic potential in human breast cancer cell lines (Zhou *et al.*, 2007). CD73 over expression increased migration of T-47D and of MB-MDA-231 *in vitro*, whereas APCP decreased both (Wang *et al.*, 2008). In this study, the inhibitory effect of APCP and a CD29-blocking antibody on MSC migration was observed only on uncoated and collagen type I coated filters, respectively. These data corroborate that CD73 possibly acts as an adhesion molecule for extracellular matrix components such as laminin and fibronectin (Zimmermann, 1992), which are

likely to be present on uncoated filters in culture medium containing serum proteins, but not collagen (Stochaj *et al.*, 1989). Another explanation could be that CD29 possibly dimerises favourably to the integrins  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$ . These integrins are the major collagen receptors whereas integrins  $\alpha 5\beta 1$  and  $\alpha 6\beta 1$  are the major fibronectin and laminin receptors (Chamberlain *et al.*, 2007). However, the treatment of MSCs with APCP did not reduce the migration capacity to the same degree, as did mechanical loading. A possible explanation could be that further adhesion molecules, chemokines, cytokines, proteases or growth factors receptor tyrosine kinases involved in cell migration might be affected by mechanical loading, such as CXCL8, CCR3, or CXCR4, and the growth factors receptor tyrosine kinases for HGF, IGF and PDGF (Ponte *et al.*, 2007).

Thus, CD73 and CD29 mediate the mechanically induced migration behaviour of MSCs depending on the underlying extracellular matrix, but further studies will need to identify potential additional players (Paulick and Bertozzi, 2008).

### Mechanoregulation of FAK in MSCs

In our study, the CD29 downstream target FAK-Y397 was reduced after three days of mechanical loading of MSCs. In short term mechanical stimulation experiments, however, cyclic strain (1 % elongation, 15 cycles/min and 4 h) caused increased phosphorylation of FAK-Y397 in osteoblastic MC3T3 cells and primary osteoblastic cells (Boutahar *et al.*, 2004). In another study, compressive stimulation (17.8N compressive load, 1 Hz, 30 min) of bone revealed a rapid load-induced activation of FAK-Y397 (Moalli *et al.*, 2001). One explanation for this discrepancy could be a proposed biphasic effect of FAK phosphorylation as suggested recently (Desai *et al.*, 2009); these authors observed that FAK-Y397 levels are transiently elevated after 30 min of cyclic strain, but significantly decrease by 6 h compared to unstimulated controls. Thus, they concluded that FAK phosphorylation increases over short time intervals of mechanical stimulation, but decreases after enhanced loading periods, which is further confirmed by our observations (Desai *et al.*, 2009). A second explanation could be a substrate specific regulation of FAK phosphorylation; e.g., in MSCs stimulated by cyclic strain, FAK-Y397 was increased on laminin and decreased on fibronectin coating (Huang *et al.*, 2009). However, fibrin has not been investigated thus far. Importantly, a direct CD29-FAK association was proposed based on results of *in vitro* peptide binding studies (Schaller *et al.*, 1995). Moreover, ablation of CD29 expression suppresses FAK-Y397 in mammary tumour cells (White *et al.*, 2004). Therefore, although we cannot rule out contrary short term effects, for long term mechanical stimulation, the reduced expression of CD29 is likely to cause the decrease of FAK-Y397.

### Actin cytoskeleton turnover in loaded MSCs

In this study, during migration, the formation of lamellipodia and actin accumulation and cell spreading were suppressed after mechanical loading, the first observation being in accordance with another study (Osada *et al.*, 1999). A similar phenotype was observed after inhibition of CD73

and CD29 in MSCs. A possible regulator involved in CD29-mediated cell migration is the FAK-Src family kinase (SFK)-complex (Sieg *et al.*, 1999). FAK-SFK signalling induces lamellipodia extensions and membrane protrusion and promotes focal adhesion turnover (Guo and Giancotti, 2004; Mitra *et al.*, 2005). Mutation of FAK-Y397 impairs the ability of FAK to promote cell spreading (Owen *et al.*, 1999). Cell migration in fibroblasts is decreased by SFK inhibition, which was also observed in MSCs in this study (Sieg *et al.*, 1999). Importantly, our data indicate that CD73 also links to the cytoskeleton. Indeed, it has been shown that filamentous actin accumulates under clusters of glycosylphosphatidylinositol (GPI)-anchored proteins (e.g., CD73) induced by receptor ligation and cross-linking (Harder and Simons, 1999; Paulick and Bertozzi, 2008). How GPI-anchored proteins, lacking an intracellular domain, transduce signals across the membrane remains puzzling. It has been hypothesised to occur by close association with transmembrane signalling molecules in lipid rafts (Paulick and Bertozzi, 2008). For example, antibody-mediated triggering of lymphocyte CD73 induces tyrosine phosphorylation of a ~26 kDa, ~28 kDa and ~50 kDa protein (Airas *et al.*, 1997). Furthermore, human and murine lymphocyte CD73 signalling seems to depend on Lck- and Fyn, two members of the SFK family and involved in cell migration, respectively (Löwenberg *et al.*, 2006; Resta *et al.*, 1994; Yamashita *et al.*, 1998). Thus, we conclude that the reduced migratory capacity of mechanically stimulated MSCs, associated with less formation of lamellipodia extensions and membrane protrusion, *via* downregulation of CD73 and CD29 might be due to impaired SFK and FAK-SFK signalling, respectively. Furthermore, a connection between the integrin  $\beta$ 2-subgroup LFA-1 and CD73 has been suggested (Airas *et al.*, 2000) and CD29-mediated cell adhesion was recently shown to be regulated by another GPI-linked cell surface protein, the urokinase receptor (Airas *et al.*, 1997). Thus, in future studies, it will be interesting to investigate a potential crosstalk between the two mechanoregulated cell surface proteins CD73 and CD29.

In summary, mechanical loading of MSCs leads to decreased expression of CD73 and CD29, possibly *via* altered expression of TNF- $\alpha$  and VEGF, reduced activation of FAK and, potentially SFKs, less actin accumulations and lamellipodia formation and thereby reduced migration.

### Conclusion

Data from this study provide evidence that migration of MSCs is strongly influenced by mechanical stimulation equivalent to conditions of the early bone-healing phase. We therefore speculate that MSCs migrate into the early fracture haematoma, become entrapped by mechanical stimulation, and thereby accumulate in the haematoma in order to fulfil their biological role to contribute to bone regeneration. Future studies will need to investigate the concurrent influence of mechanical loading and inflammatory chemokines/cytokines (chemotaxis) and/or biomechanical properties of the underlying ECM (durotaxis) on MSC migration *in vitro*.

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## Discussion with Reviewers

**Reviewer I:** I would recommend the inclusion of FACS histograms in Fig. 3 to provide a more clear view of difference in surface marker expression.

**Authors:** A reduction of protein expression and thereby a reduction of fluorescence intensity by e.g., 40 % does not result in an extreme shift of the histogram, but is still biologically relevant. To clearly illustrate the difference between loaded and non-loaded MSCs, we therefore chose to display our data in bar charts and further validated our results on mRNA level. From our point of view, histograms, showing the differences less clear, will not add much value to the manuscript and were therefore not included.

**Reviewer I:** Please comment on the expression of VEGF and TNF- $\alpha$  and the role in the described result of the minor down regulation of the two surface markers. Especially the absolute amount of TNF- $\alpha$  is very low and there are only minor differences in the expression. Why do the authors check the effect of TNF- $\alpha$  in a more than 100 fold over

dosage. What is the functional link between VEGF/TNF- $\alpha$  and the observed results of decreased migratory capacity?

**Authors:** In this study the results demonstrated a reduced expression of MSC surface markers CD73 (ecto-5'-nucleotidase) and CD29 (integrin  $\beta$ 1) after mechanical loading by 57 % and 38 %, respectively. A downregulation of proteins within this range was proven by different studies to be biologically relevant. For example, an upregulation of the matrix metalloprotease-2 (MMP-2) in MSCs by 40-50 % led to a paracrine stimulation of angiogenesis, which was hindered by inhibition of MMP-2 in the presence of recombinant TIMP-2 (biological MMP-2 antagonist) and a pro-MMP-2 blocking antibody (a-pro-MMP-2) (Kasper *et al.*, 2007; Glaeser *et al.*, 2010, text references). Thus, we believe that the downregulation of the two surface markers in our study is also biologically relevant.

A number of cytokines and growth factors are known to regulate CD73 and CD29 expression, e.g. TNF- $\alpha$  and VEGF (Kalsi *et al.*, 2002; Hunsucker *et al.*, 2005; Lee *et al.*, 2006, text references). It is important to note that the expression of TNF- $\alpha$  (a CD73 negative regulator (Kalsi *et al.*, 2002; Hunsucker *et al.*, 2005)) and the expression of VEGF (a CD29 positive regulator (Lee *et al.*, 2006)) were increased by 54 % and decreased by 33 %, respectively. Thus, the range of alteration is comparable to the change of expression of the cell surface markers after loading. Therefore, we hypothesise that the mechanically induced decrease of the migratory capacity of MSCs via CD73 and CD29 occurs possibly via altered expression of TNF- $\alpha$  and VEGF.

In order to get a first idea whether TNF- $\alpha$  indeed influences the expression of CD73, we simplified the experimental set-up and treated 2D-cultured MSCs with high dose of TNF- $\alpha$  and found the expression of CD73 mRNA to be downregulated (preliminary data not included in the manuscript). However, to fully reveal this mechanism in 3D and after loading, extensive analyses (dose- and time-dependent; protein- and mRNA-level) are needed which, we believe, is beyond the scope of this study and will therefore be part of future studies.

Furthermore, the set of marker molecules defined to be expressed on MSC is more or less described for *in vitro* cultivated cells. The set of *in vivo* markers for MSCs is object of intense discussion (Augello *et al.*, 2010, additional reference). Since marker molecule expression can differ in the *in vivo* and the *in vitro* situation, and isolated MSCs will probably change the set of surface proteins upon *in vitro* cultivation, it is questionable if the observed results can be translated in the *in vivo* situation.

Augello *et al.* (2010) hypothesise in their review that the possibility exists that the MSC phenotype varies between *in vitro* and *in vivo* mainly because of the removal from their natural environment and the use of artificial culture conditions. This hypothesis is based on publications by Jones *et al.* (2002, 2006) (additional references) that describe MSCs losing the expression of

some surface markers (Stro-1, LNGFR, and HLA-DR) while also acquiring new ones (CD106, CD146) during *in vitro* cultivation. However, it is important to note that neither CD73 nor CD29 were shown to change during *in vitro* cultivation in these studies. In fact, Jones *et al.* (2006) provided evidence that the expression level of CD73 remained the same between freshly isolated and culture-expanded MSCs. Moreover, CD29 is the "cluster of differentiation" for integrin  $\beta$ 1. MSCs express several integrins, such as  $\alpha$ 1 $\beta$ 1,  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1,  $\alpha$ 4 $\beta$ 1,  $\alpha$ 5 $\beta$ 1,  $\alpha$ 6 $\beta$ 1,  $\alpha$ 6 $\beta$ 4  $\alpha$ V $\beta$ 3 and  $\alpha$ V $\beta$ 5 with different ligand binding properties, suggesting a key functional role for integrin  $\beta$ 1 (CD29) in MSCs (Ode *et al.*, 2010, additional reference). In addition, the involvement of CD29 in MSC migration has been demonstrated *in vivo*: e.g., lower numbers of MSCs engrafted and migrated into ischemic myocardium, if pre-treated with neutralising antibodies against CD29 (Ip *et al.*, 2007, text reference). Thus, it is very unlikely that this key cell surface protein will be absent *in vivo*.

In summary, these data indicate that the cell surface proteins CD73 and CD29 are not *in vitro* artefacts. Therefore, it is reasonable that the observed results could be translated in the *in vivo* situation. However, we totally agree that conclusive evidence needs to be obtained from appropriate *in vivo* studies.

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