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CD73/5'-ECTO-NUCLEOTIDASE ACTS AS A REGULATORY FACTOR IN OSTEO-/CHONDROGENIC DIFFERENTIATION OF MECHANICALLY STIMULATED MESENCHYMAL STROMAL CELLS

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Abstract

Bone regeneration is influenced by mesenchymal stromal cells (MSCs) and mechanical conditions. How healing outcome and mechanical stability are linked on the cellular level, however, remains elusive. Cyclic-compressive loading of MSCs affects the expression of molecules involved in angiogenesis and matrix assembly, but also reduces the expression of CD73, an ecto-5'-nucleotidase, which plays a crucial role in extracellular adenosine generation. Although, for almost 20 years, CD73 has been a major cell surface marker defining MSCs, little is known about its function in these cells. Therefore, the aim of this study was to determine the putative involvement of CD73 in MSC differentiation after cyclic-compressive loading. After cultivation in appropriate differentiation media, chondrogenic differentiation ability was significantly increased in loaded MSCs, hence following current models. Through treatment with the CD73 inhibitor adenosine 5'-(α, β-methylene) diphosphate, chondrogenic matrix deposition was further increased; in contrast, mineral matrix deposition and expression of osteogenic markers was reduced. One major signal transduction pathway, which is activated *via* CD73-mediated adenosine, is the adenosine receptor pathway. Thus, the adenosine receptor expression pattern was investigated. MSCs expressed the four known adenosine receptors at the mRNA level. After mechanical stimulation of MSCs, *Adora2a* was down-regulated. These data point towards a role of CD73 in MSC differentiation possibly *via* A2AR signalling, which is mutually regulated with CD73. In conclusion, the findings of this study suggest that CD73 is another regulatory factor in osteo-/chondrogenic differentiation of MSCs and may provide a – thus far underestimated – therapeutic target to guide bone regeneration.

Keywords: Mesenchymal stromal cells; mechanical stimulation; CD73/5'-ecto-nucleotidase; chondrogenic differentiation; osteogenic differentiation.

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Introduction

Bone healing is a complex, however well-orchestrated, multistage regenerative process. Bone fracture coincides with disruption of blood vessels resulting in activation of the coagulation cascade and formation of the haematoma, which encloses the fracture area. Inflammatory cells, fibroblasts, and mesenchymal stromal cells (MSCs) are recruited to the site. Once MSCs have reached the bone fracture site they are confronted with a challenging milieu characterised not only by inflammatory cytokines and low oxygen tension (hypoxia) but also by constant mechanical strain (Goodship and Kenwright, 1985; Komatsu and Hadjiargyrou, 2004). This condition is likely to affect 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.

Recently, we and others provided evidence that mechanical strain leads to reduced expression of the cell surface marker CD73 *in vitro* (Kang *et al*., 2011; Ode *et al*., 2011). In our study, bone marrow (BM)-MSCs underwent cyclic-compressive loading for three days. CD73 protein and mRNA expression were significantly reduced. Of note, this effect persisted for a week after removal of the loading stimulus (Ode *et al*., 2011). In another study, umbilical cord-derived MSCs underwent cyclic uniaxial stretching for ten days. Here, the expression of CD73 was also significantly decreased as strain increased (Kang *et al*., 2011). CD73 is an ecto-5'-nucleotidase that is attached to the outer plasma membrane by a glycosyl phosphatidylinositol (GPI)-anchor. It catalyses the hydrolysis of the phosphoric ester bond of 5'-ribo- and deoxyribonucleotides to the corresponding ribo- and deoxyribonucleoside, and phosphate (Hunsucker *et al*., 2005; Sträter, 2006), e.g. it generates extracellular adenosine by the dephosphorylation of adenosine 5'-monophosphate. Although CD73 is one major cell surface marker defining MSCs according to The International Society for Cellular Therapy (ISCT), it is surprising that little is known about the function of CD73 in these cells (Dominici *et al*., 2006). Interestingly, CD73 expression is regulated by Wnt-β-catenin signalling, one of the major pathways in bone homeostasis (Spychala and Kitajewski, 2004). Moreover, CD73 is also regulated by cytokines and growth factors, such as TGF-β, IL-1β and TNF-α, and hypoxia that are found in the haematoma during the early phase of bone healing (Hunsucker *et al*., 2005; Thompson *et al*., 2004), and recently, CD73 has

been reported to be involved in osteogenic differentiation: Lack of CD73 causes a lower bone mineral content in the trabecular bone of male mice, accompanied with decreased osteocalcin serum levels and reduced expression of osteogenic mRNA-markers in calvarial and femoral bone. Primary calvarial osteoblasts of CD73-deficient mice produce less alkaline phosphatase (ALP) and calcified bone nodules. In contrast, CD73-over expressing MC3T3-E1 cells express more osteocalcin (*Bglap*) and bone sialoprotein 2 (*Ibsp*) and produce more ALP and calcified bone nodules (Takedachi *et al*., 2012). Little is known about the role of CD73 in chondrogenesis of MSCs, except that CD73 is down-regulated during differentiation (Delorme *et al*., 2008; Song *et al*., 2006). One possible mechanism by which CD73 mediates the effects on osteogenic MSC differentiation could be *via* adenosine receptor signalling. In MC3T3-E1 cells, the expression of adenosine A2A receptors (A2AAR) and A2B receptors (A2BAR) increased during culture in osteoblastic mineralisation. In addition, enhanced expression of osteocalcin and bone sialoprotein in CD73-over expressing MC3T3-E1 cells was suppressed by treatment with an A2BAR antagonist but not with an A2AAR antagonist (Takedachi *et al*., 2012). In contrast, treatment with an A2AAR antagonist reduced the number of procollagen alpha2 type I-positive murine BM-MSCs in cultures (Katebi *et al*., 2009). These findings indicate that CD73, adenosine and adenosine receptors may also play a critical role in promoting differentiation of MSCs. Therefore, we hypothesise that CD73 plays a fundamental role in MSC-mediated bone fracture healing and may provide a thus far underestimated, therapeutic target to guide this regenerative process.

In the present study, we aimed to analyse the differentiation capacity of mechanically loaded MSCs and the putative involvement of CD73 under conditions relevant for bone healing. In order to do so, we mimicked the early phase fracture gap conditions by embedding the MSCs in fibrin, the major extracellular matrix of the haematoma, and by stimulating the cells with cyclic-compressive loading at a physiological amplitude and frequency.

Materials and Methods

Cell isolation, culture and characterisation

MSCs were isolated from bone marrow of 10-12 weeks old male Lewis rats and selected by plastic adherence (Dobson *et al*., 1999). Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10 % foetal calf serum (FCS) (Biochrom AG, Berlin, Germany) and 10 U/mL penicillin plus 100 µg/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).

Osteoblastic cells were isolated by the explant culture (Declercq *et al*., 2004). Briefly, skin, soft connective tissue, and periosteum were removed from tibiae and femora. Next, the diaphyses were cut free of epiphyseal cartilage. Bone was cut into pieces of approximately 2 x 2 mm² and washed by repeatedly vortexing in 20 mL phosphate-buffered saline (PBS) to remove blood and bone marrow. Approximately 20 bone pieces were incubated with culture medium in 175 cm² tissue culture flasks for 2 weeks with medium exchange every 3-4 d.

Bioreactor experiments

The bioreactor system used has been described previously (Ode *et al*., 2011). Briefly, MSCs from passages 2-4 and osteoblasts (OBs) from passage 1 were trypsinised, and 2 x 10^6 cells in 350 µL of bioreactor medium (culture medium containing 2.4 % Trasylol (Bayer, Leverkusen, Germany)) were mixed with 300 µL of fibrinogen/bioreactor medium (1:2) mixture and 50 µL of thrombin S/bioreactor medium (1:2) mixture (Tissucol; Baxter, Munich, Germany). This 700 µL cells/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. Non-loaded cells were kept in a second bioreactor under equal experimental conditions without any mechanical load. Afterwards, both loaded and non-loaded cells within the fibrin construct were either isolated by 225 U trypsin/1 mL PBS and further analysed by flow cytometry and differentiation assays or directly frozen in liquid nitrogen for RNA isolation. In order to reduce any bias through trypsin digestion in our experimental setting, each cell pair (loaded/non-loaded and MSCs/OBs) was treated in parallel under equal experimental conditions. Equal levels of cell viability and activity were validated by means of the electronic cell counter system CASY® DT (Schaerfe System, Reutlingen, Germany) and a CellTiter 96 AQueous test (MTS test; Promega, Mannheim, Germany), respectively.

Flow cytometry

For flow cytometry, cells within the fibrin construct were isolated by 225 U trypsin/1 mL PBS and stained with the following antibodies: 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, San Diego, CA, USA), rat (anti-mouse IgG):PE (BD Bioscience, Heidelberg, Germany) and donkey (anti-rabbit IgG):Cy5 (Dianova, Hamburg, Germany). Cells were analysed using FACSCalibur. 30,000 events were acquired and analysed using the FlowJo software.

Table 1. Primers used for quantitative PCR.

Protein	Gene	Primer Sequence (forward / reverse)	Reference
Adenosine receptor			
Adenosine A1 receptor	*Adora1*	5′ CCTTCTGCTTCATCGTGTCA 3′	
		5′ CTGGGTCACCACTGTCTTGT 3′	
Adenosine A2a receptor	*Adora2a*	5′ CCT CTT CTT CGC CTG TTT TG 3′	
		5′ GTT CCC GTC TTT CTG ACT GC 3′	
Adenosine A2b receptor	*Adora2b*	5′ GCTCCATCTTTAGCCTCTTG 3′	
		5′ AACCCAGGAAAGGAGTCAG 3′	
Adenosine A3 receptor	*Adora3*	5′ GGTCCACTGGCCCATACACA 3′	(Jenner and Rose'Meyer, 2006; Rose'Meyer *et al*., 2003)
		5′ TGTAGGTGATTTGCAACCACA 3′	
Cell surface marker			
ecto-5'-nucleotidase (CD73)	*Nt5e*	5′ GCAAGGAAGAACCCAACGTA 3′	
		5′ TCAGTCCTTCCACACCGTTA 3′	
Chondrogenic marker			
aggrecan	*Acan*	5′ CCACTG GAGAGGACTGCGTAG 3′	(Peng *et al*., 2008)
		5′ GGTCTGTGCAAGTGATTCGAG 3′	
collagen type 2 alpha 1	*Col2a1*	5′ GACCCTCTCTTAGCCACACACAC 3′	
		5′ AAGCTGCCCAGAATCCTAAATCT 3′	
fibromodulin	*Fmod*	5′ ACGTCTACACCGTCCCTGACA 3′	(Peng *et al*., 2008)
		5′ CCTGCAGCTTGGAGAAGTTCA 3′	
Sry-box containing gene 9	*Sox9*	5′ TCCTAACGCCATCTTCAAGG 3′	
		5′ CCCCTCTCGTTTCAGATCAA 3′	
Osteogenic marker			
alkaline phosphatase	*Alpl*	5′ CCTTGAAAAATGCCCTGAAA 3′	
		5′ CTTGGAGAGAGCCACAAAGG 3′	
osteocalcin	*Bglap*	5′ TGAGGACCCTCTCTCTGCTC 3′	
		5′ AGGTAGCGCCGGAGTCTATT 3′	
collagen type 1 alpha 1	*Col1a1*	5′ GGAGAGAGTGCCAACTCCAG 3′	(Peng *et al*., 2008)
		5′ CCACCCCAGGGATAAAAACT3′	
bone sialoprotein 2	*Ibsp*	5′ TCTGCATTTTGGGGATGG 3′	
		5′ CCGTTTCAGAGGAGGATAAAAG 3′	
runt-related transcription factor 2	*Runx2*	5′ GCCGGGAATGATGAGAACTA 3′	
		5′ GGACCGTCCACTGTCACTTT 3′	
osteopontin	*Spp1*	5′ GAGGAGAAGGCGCATTACAG 3′	
		5′ ATGGCTTTCATTGGAGTTGC 3′	
Housekeeping gene			
β-actin	*Actb*	5′ TGTCACCAACTGGGACGATA 3′	
		5′ GGGGTGTTGAAGGTCTCAAA 3′	
glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	5′ ATGGGAAGCTGGTCATCAAC 3′	
		5′ GTGGTTCACACCCATCACAA 3′	
elongation factor 1-alpha 1	*Eef1a*	5′ CCCTGTGGAAGTTTGAGACC 3′	
		5′ CTGCCCGTTCTTGGAGATAC 3′	

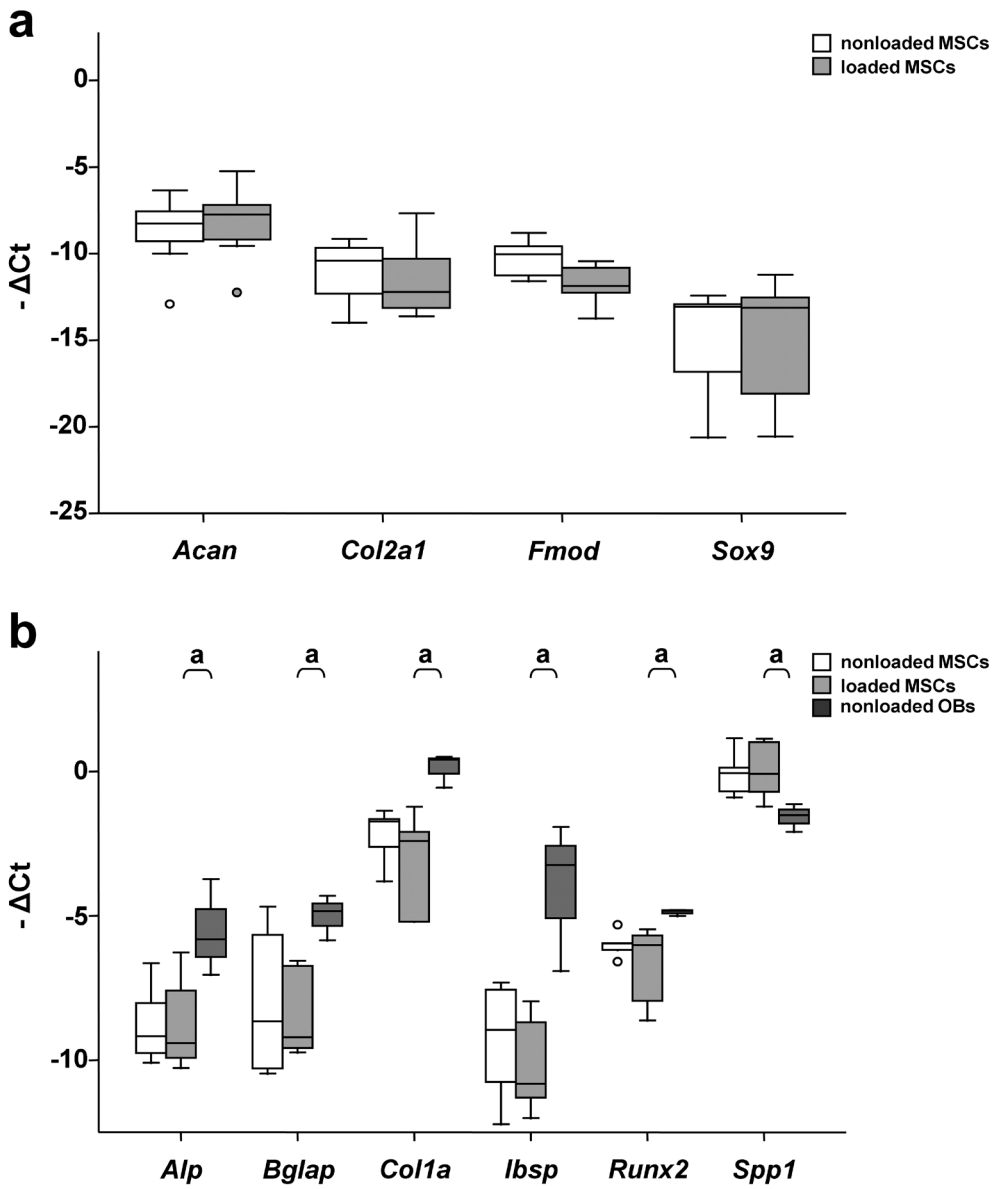

Fig. 1. MSCs neither increase their chondrogenic differentiation marker expression nor obtain an osteogenic phenotype by the sole stimulus of cyclic compression. The expression of mRNA specific for **(A)** chondrogenic, and **(B)** osteogenic differentiation markers of loaded MSCs, non-loaded MSCs and non-loaded osteoblasts (OBs) were evaluated by quantitative qRT-PCR and normalised for the housekeeping genes *Actb*, *Gapdh,* and *Eef1a*. (MSCs: $n = 6$; OBs: $n = 3$; °, outlier; a, $p < 0.05$).

RNA isolation, cDNA synthesis, and quantitative reverse transcription-polymerase chain reaction

Total RNA from frozen cell-fibrin constructs was extracted using Trizol® Reagent (Invitrogen, Karlsruhe, Germany) and reverse 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 differentiation markers were assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the iQTM SYBR® Green Supermix and the iQTM 5 Multicolor Real-time PCR Detection System and software (Bio-Rad, Munich, Germany) using the delta-delta-Ct-method. The transcript expression was normalised *versus* the housekeeping gene β-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, Karlsuhe, Germany; 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.

MSC differentiation

To induce osteogenic differentiation, 1.92 x 10^4 MSCs were cultured on 24-well plates and exposed to osteogenic differentiation media (200 µM ascorbic acid, 7 mM ß-glycerol phosphate, 0.01 µM dexamethasone; 21 d).

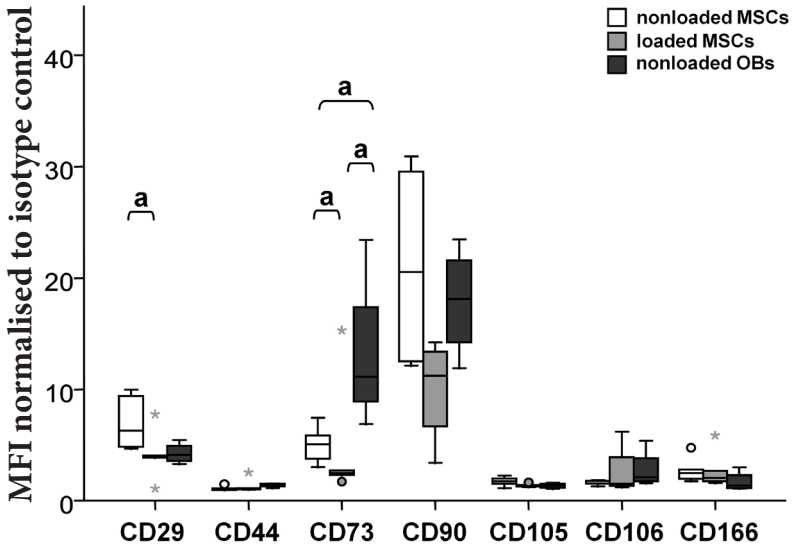

Fig. 2. Osteoblasts express more CD73 than MSCs. The expression of MSC cell surface markers of loaded and non-loaded MSCs, and non-loaded osteoblasts (OBs) was investigated by flow cytometry. Shown is the mean fluorescence intensity (MFI) of cells stained with specific antibodies after normalisation to isotype negative controls. (MSCs: $n = 5$; OBs: $n = 4$; °, outlier; *, extreme value; a, $p < 0.05$).

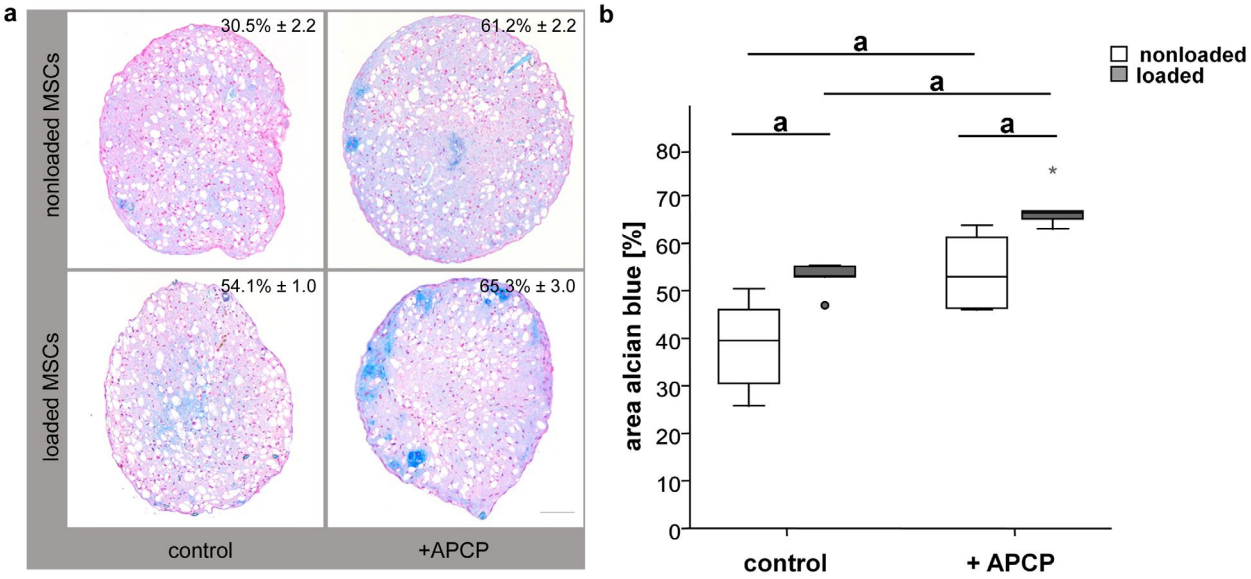

Fig. 3. Blocking of CD73 leads to increased chondrogenic differentiation of MSCs. MSCs were cultivated as pellet cultures in chondrogenic differentiation media supplemented with 120 µM APCP. After 28 d, matrix production of differentiated cells was detected with alcian blue. Chondrogenic matrix is stained in blue, cell nuclei in red. **(A)** Shown are representative stained pellet sections of differentiated non-loaded (top) and loaded MSCs (bottom) of one animal with the mean value of the alcian blue positive stained area ± SD. Bar indicates 100 µm. **(B)** The alcian blue positive stained area was quantified using the AxioVision software package 4.7 and normalised to total area. At least three different sections of one pellet were analysed. Abbreviation: APCP, CD73 inhibitor adenosine 5'-(α, β-methylene)diphosphate; ($n = 5$; °, outlier; *, extreme value; a, $p < 0.05$).

Osteogenic differentiation was detected by alizarin red (AR) staining. AR positively stained matrix was quantified after dissolving AR in cetylpyridinium chloride and then normalised to cell number, as determined using MTS-test (CellTiter 96 Aquaeous, Promega, Madison, WI, USA), according to the manufacturer's instructions. Each experiment was conducted in triplicate. ALP activity was measured at day 7, 11 and 14 according to a protocol described elsewhere (Krause *et al*., 2011). Briefly, all buffers and substrate were preheated to 37 °C. Cells were washed using 400 µL PBS and 500 µL AP-Buffer (100 mM NaCl, 100 mM Tris, 1 mM $MgCl_2$; pH 9.0). 250 µL AP-Buffer and an equal volume of p-nitrophenyl phosphate (pNPP, Sigma Aldrich, Munich, Germany) solution (1 mg/mL, 1 M diethanolamine (DEA), pH 9.8) were added. Cells were incubated for 5 min at 37 °C and reaction was stopped using 500 µL 1 M NaOH. Duplicates of 100 µL were transferred to a 96-well plate and absorption was measured at 405 nm at a plate reader. After blank value subtraction, pNPP consumption, i.e. 4-nitrophenolate accumulation, was calculated using a molar extinction coefficient of 18450 L x mol^{-1} x cm^{-1}. ALP activity was

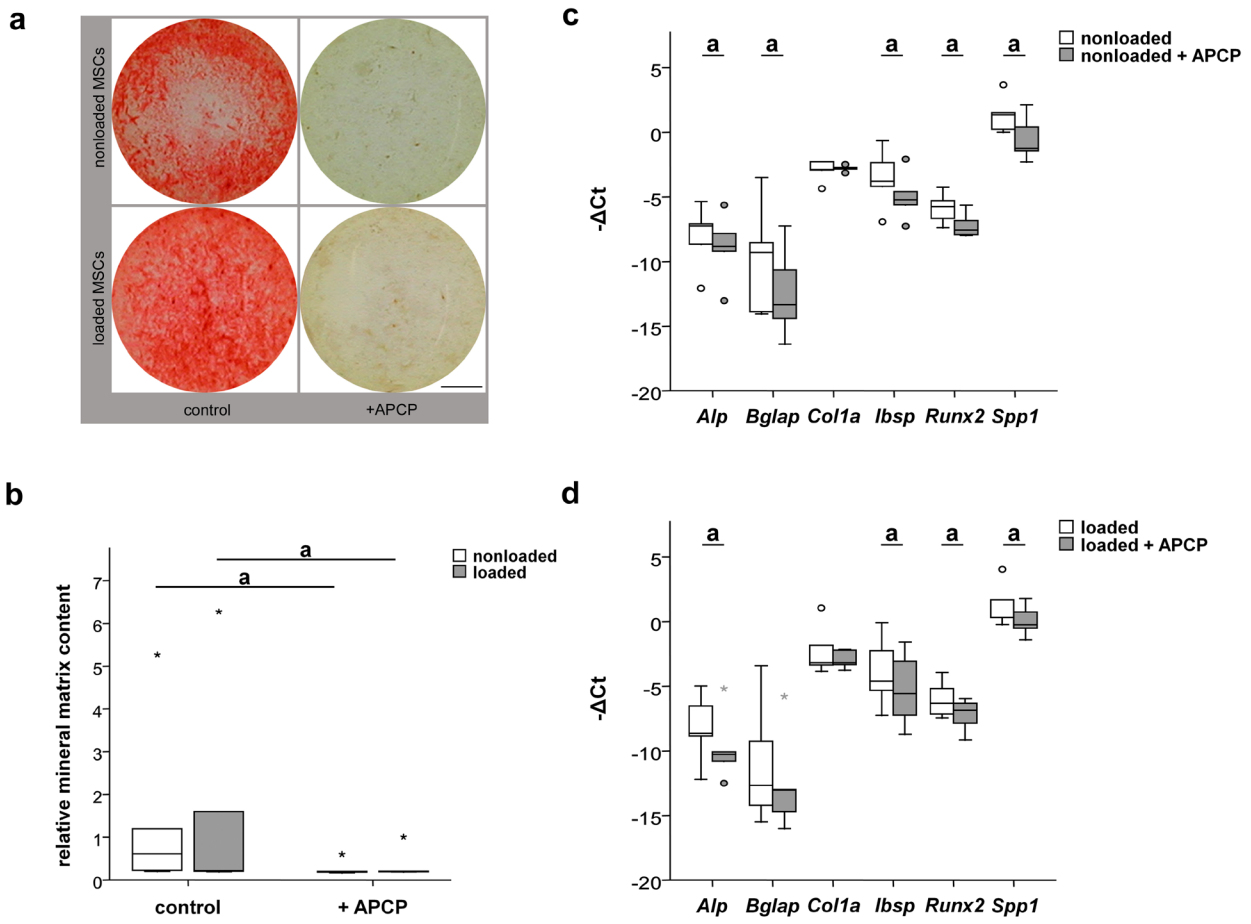

Fig. 4. Blocking of CD73 leads to decreased osteogenic differentiation of MSCs. MSCs were cultivated in osteogenic differentiation media supplemented with 120 µM APCP. **(A)** Shown are representative pictures of non-loaded (top) and loaded MSCs (bottom) of one animal. Bar indicates 3 mm. **(B)** Osteogenic differentiation was detected by alizarin red (AR) staining. AR positive stained matrix was quantified after dissolving of AR in cetylpyridinium chloride and then normalised to cell number, as determined using MTS-test. **(C and D)** The expression of mRNA specific for osteogenic differentiation markers of **(C)** loaded and **(D)** nonloaded MSCs was evaluated by quantitative qRT-PCR and normalised for the housekeeping genes *Actb*, *Gapdh,* and *Eef1a*. Abbreviation: APCP, CD73 inhibitor adenosine 5'-(α, β-methylene)diphosphate; (n = 5; °, outlier; *, extreme value; a, $p < 0.05$).

defined as nmol 4-nitrophenolate accumulation in 1 min at 37 °C. Values were normalised to cell number using Resazurin solution (Alamar Blue, Life Technologies, Darmstadt, Germany), according to the manufacturer's instructions. Each experiment was conducted in triplicate. For chondrogenic differentiation, a pellet culture in chondrogenic differentiation media (10 ng/mL TGF-β1, 10^{-7} M dexamethasone, 50 µg/mL ascorbic acid, 40 µg/mL proline, 100 µg/mL pyruvate, 6.25 µg/mL insulin-transferrin-sodium selenite media supplement (ITS), 1.25 mg/mL bovine serum albumin (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. A CD73 inhibitor, adenosine 5'-(α, β-methylene)diphosphate (APCP; 120 µM) was employed in differentiation assays. The alcian blue positive stained area was quantified using the AxioVision software package 4.7 and normalised to total area. At least three different sections of one pellet were analysed.

Statistics

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 (i.e. non-pooled MSCs or OBs from at least five different animals) were analysed for statistical significance either using Wilcoxon Test (related samples) or Mann-Whitney U Test (independent samples). Loaded/nonloaded and APCP-treated/non-treated control cells (MSCs and OBs) were always analysed pairwise originating from the same animal and under equal experimental conditions. MSCs and osteoblasts were non-related and therefore treated as independent samples in comparative analyses. Results are presented as median ± min/max. A significance level was set at $p < 0.05$.

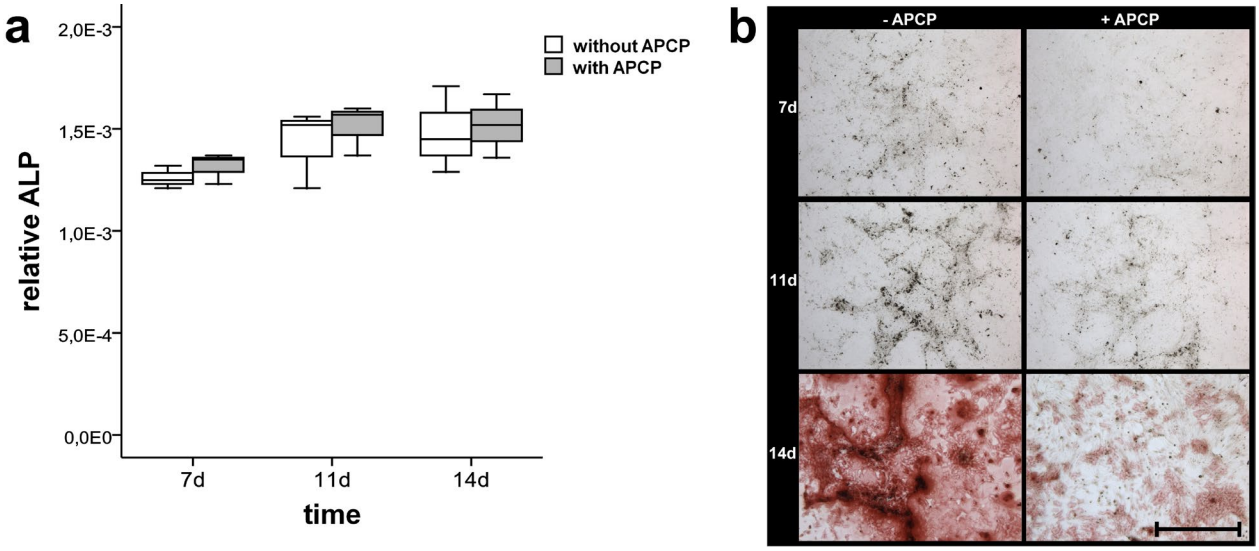

Fig. 5. Enzyme activity of ALP is not affected by blocking of CD73. MSCs were cultivated in osteogenic differentiation media supplemented with 120 µM APCP. **(A)** After 7, 11 and 14 d ALP activity, i.e. 4-nitrophenolate accumulation, was measured and normalised to cell number. **(B)** Shown are representative pictures of APCP-treated (right) and non-treated MSCs (left) of one animal after AR staining. Bar indicates 1000 µm. Abbreviations: APCP, CD73 inhibitor adenosine 5'-(α, β-methylene)diphosphate; AR, alizarin red (n = 3).

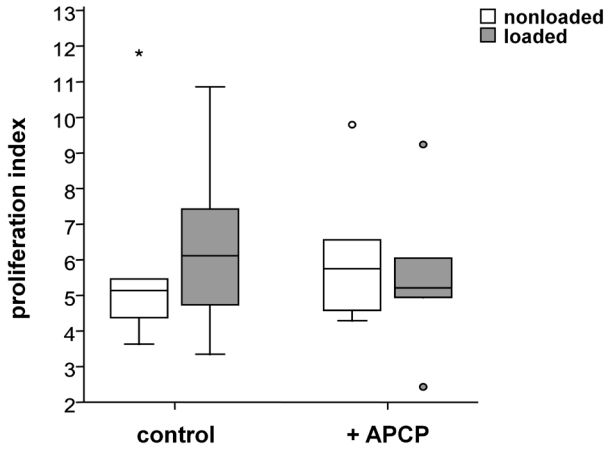

Fig. 6. APCP does not affect MSC proliferation. Proliferation of loaded and non-loaded MSCs was determined by measuring cell activity on day 1 and 6 by MTS-test. The proliferation index was calculated by normalising the day 1 to the day 6 values. Abbreviations: APCP, adenosine 5'-(α, β-methylene)diphosphate; w/o, without (n = 5; °, outlier; *, extreme value).

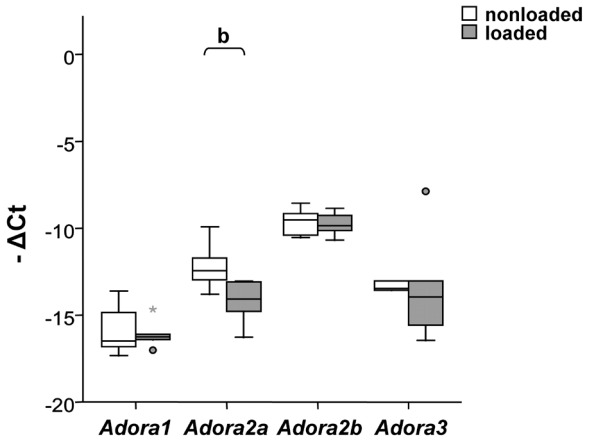

Fig. 7. Adenosine receptor Adora2a is down-regulated in MSCs after mechanical loading. The expression of mRNA specific for the four adenosine receptors of loaded and non-loaded MSCs was evaluated by qRT-PCR and normalised for the housekeeping genes *Actb*, *Gapdh,* and *Eef1a*. (n = 6; °, outlier; *, extreme value; b, p = 0.075).

Results

In accordance with previously published data, our first aim was to clarify whether mechanical stimulation alone leads to changes of MSCs differentiation marker expression, which was investigated by quantitative RT-PCR analysis directly after cyclic-compression. Among the chondrogenic (*Acan, Col2a1, Fmod, Sox9*) and osteogenic markers (*Alpl, Bglap, Col1a1, Ibsp, Runx2, Spp1*), none was significantly regulated (Fig. 1). To further control their stage of differentiation, loaded and non-loaded MSCs were compared to non-loaded osteoblasts with regard to their osteogenic differentiation markers. Gene expression analysis revealed that *Alpl, Bglap, Col1a1, Ibsp,* and, *Runx2* were significantly more highly expressed in non-loaded osteoblasts compared to MSCs. In contrast, the *Spp1* level was significantly lowered after loading. In addition, flow cytometry analysis of cell surface markers revealed that CD73 expression was significantly increased in non-loaded osteoblasts compared to loaded and non-loaded MSCs (Fig. 2). In line with these results, CD73 expression is also increased in MSCs during their

osteogenic differentiation (relative gene expression: $2^{-\Delta\Delta Ct}$ (CD73) = 2.69, n = 3, Student's t-test, p = 0.002). CD29 and CD73 were down-regulated in MSCs in response to loading and these observations remained persistent over a period of additional 6 d, where MSCs were kept non-loaded after 3 d of cyclic-compression, as described previously (Ode *et al*., 2011).

Subsequently, the putative involvement of CD73 in chondrogenic and osteogenic differentiation in MSCs was investigated. Mechanical stimulation and supplementation of chondrogenic media with APCP stimulated chondrogenic differentiation of MSCs (Fig. 3). However, the overall chondrogenic response of rat MSCs is less strong compared with human MSCs under almost identical conditions, which could not be improved by the additional treatment with BMP-2 in the differentiation media (data not shown).

Supplementation of osteogenic media with APCP inhibited osteogenic differentiation of loaded and non-loaded MSCs. In addition, osteogenic differentiation marker expression (*Alpl, Bglap, Col1a1, Ibsp, Runx2, Spp1*) was significantly reduced in APCP-treated loaded and non-loaded MSCs (Fig. 4). As ALP is a key regulator of osteogenesis, ALP activity of MSCs was analysed under the direct influence of APCP and found to be unaffected (Fig. 5). Cell viability was also unaffected by APCP (Fig. 6).

One major signal transduction pathway, which is activated *via* CD73 mediated adenosine, is the adenosine receptor pathway, possibly being involved in regulating MSC differentiation. Thus, the adenosine receptor expression pattern was investigated. MSCs expressed the four known adenosine receptors (*Adora1, Adora2a, Adora2b, Adora3*) at the mRNA level. After mechanical stimulation of MSCs, *Adora2a* was down-regulated by a mean factor of 0.37 (Fig. 7).

Discussion

The stem cell character of MSCs depends not only on the expression of specific cell surface markers, but also on the ability to differentiate into the osteogenic, chondrogenic, and adipogenic lineage. Therefore, the differentiation potential of MSCs after mechanical loading was investigated. In this study, loading conditions similar to the early phase of fracture healing resulted in enhanced chondrogenic differentiation potential of MSCs. Hence, the response of MSCs to mechanical loading followed current models that suggest that compressive load supports chondrogenic MSC differentiation (Table 2) (Campbell *et al*., 2006; Mauck *et al*., 2007; Pelaez *et al*., 2009). In contrast, osteogenic differentiation of MSCs was unaffected by cyclic compression (3D) adding weight to the notion that osteogenic differentiation requires a different mechanical stimulus, such as cyclic equibiaxial/uniaxial strain (2D) or four-point bending (Table 2) (David *et al*., 2007; Jagodzinski *et al*., 2004; Koike *et al*., 2005; Mauney *et al*., 2004). Little is known about the function of CD73 in MSCs thus far. Previously, CD73 was shown to be down-regulated in response to mechanical loading (Kang *et al*., 2011; Ode *et al*., 2011). Therefore, the putative involvement of CD73 in the enhanced chondrogenic differentiation of loaded MSCs was investigated. Here, through CD73 antagonist treatment, MSC chondrogenic differentiation was increased similar to the effect observed after loading. Chondrogenesis of MSCs is typically induced when the cells are exposed to TGF-β and hypoxia (Chamberlain *et al*., 2007). Both parameters also influence the expression and activity of CD73: TGF-β induces CD73 expression on activated CD4+ and CD8+ T-cells, dendritic cells and macrophages (Regateiro *et al*., 2011); and, hypoxia is a major inducer of CD73 expression, e.g. in intestinal epithelia, which is likely due to a hypoxia inducible factor-1α (HIF-1α) responsive element in its promoter region (Hunsucker *et al*., 2005; Thompson *et al*., 2004). These data might suggest that a higher CD73 expression correlates with an increased chondrogenesis. However, it is well known that CD73 expression is regulated by a number of cytokines in a cell-type specific manner: for example, interferon (INF)-α and -γ up-regulate CD73 levels in human endothelial cells, but not in lymphocytes and monocytes (Hunsucker *et al*., 2005; Niemela *et al*., 2004). TNF-α and IL-1β induce CD73 expression and activity in blood mononuclear cells and rat glomerular mesangial cells (Hunsucker *et al*., 2005), whereas TNF-α decreases it in human endothelial cells (Kalsi *et al*., 2002). Thus, it may well be that in MSCs either CD73 is down-regulated by hypoxia and/or TGF-β or the regulation of this enzyme is even more complex. This hypothesis is supported by the fact that CD73 is down-regulated during chondrogenic differentiation of MSCs (Delorme *et al*., 2008; Song *et al*., 2006). Therefore, further research is needed to shed light on the influence of cytokines on CD73 expression in MSCs and other cell types present in the fracture haematoma *in vivo*.

Interestingly, CD73 also seems to be important in the osteogenic differentiation of MSCs. In our study, through CD73 antagonist treatment, the mineral matrix deposition of MSCs was almost completely abolished and osteogenic differentiation marker expression, such as *Bglap* and *Ibsp*, decreased. Thus, these results complement the findings for osteoblasts from CD73-deficient mice (Takedachi *et al*., 2012). Moreover, osteoblasts express more CD73 on their cell surface than their undifferentiated progenitor.

One possible mechanism by which CD73 mediates the effects on MSC differentiation could be *via* adenosine receptor signalling. CD73 dephosphorylates AMP to generate extracellular phosphate and adenosine, which can be further processed by adenosine receptors. Adenosine receptors contain seven transmembrane domains and couple to intracellular G proteins. They are divided into four subtypes – the adenosine A1, A2a, A2b and A3 receptor (R) – which vary in their affinities for adenosine (Resta and Thompson, 1997). In this study, the four adenosine receptors were expressed on MSCs as described previously (Katebi *et al*., 2009). Following mechanical stimulation, A2aR tends to be down-regulated. These data are in line with other studies that reported that CD73 and A2aR expression are mutually regulatory, i.e. A2aR expression increases CD73 expression and *vice versa* in murine MSCs and human B-cells (Katebi *et al*., 2009;

Table 2. Mechanobiological responses of MSCs.

Cell type (Species)	Loading conditions		Matrix	Cell response		Reference
BM-MSC (Rabbit)	Cyclic compression* 10 %, 1 Hz, 4 h/d over 2 d, 3 d, 7 d, 14 d	3D	Agarose	Increase in aggrecan, collagen type II, c-Jun, Sox9, TGF-β receptor mRNA Decrease in collagen type I mRNA	C ↑	(Huang *et al*., 2004) (Huang *et al*., 2005)
BM-MSC (Human)	Cyclic compression* 10 %, 1 Hz, 4 h/d over 3 d	3D	Fibrin	Increase in aggrecan, collagen type II mRNA	C ↑	(Pelaez *et al*., 2009)
AT-MSC (Goat)	Pulsating fluid flow 0.6 Pa, 5 Hz, 1 h	2D	Polylysine	Decrease in Alpl mRNA	O ↓	(Knippenberg *et al*., 2006)
BM-MSC (Mouse ST2 cell line)	Cyclic equibiaxial strain 10-15 %, 1 Hz, 2 d	2D	Collagen type I	Increase in proliferation; collagen type I mRNA Decrease in AP activity; Runx2, osteocalcin mRNA	O ↑ P ↑	(Koike *et al*., 2005)
BM-MSC (Human)	4-point bending 3 %, 0.2 Hz, 20 min	3D	DMB	Increase in AP activity and ALPL mRNA	O ↑	(Mauney *et al*., 2004)
BM-MSC (Rat)	Cyclic equibiaxial strain 0.3 %, 0.5 Hz, 30 min, 5 d static culture	2D	Silicone	Increase in AP activity; DNA content; osteocalcin protein	O ↑ P ↑	(Yoshikawa *et al*., 1997)
BM-MSC (Human)	Cyclic uniaxial strain 8 %, 1 Hz, 3x2 h/d over 4 d	2D	Silicone	Increase in Runx2, Alpl, collagen type I and III, osteocalcin mRNA	O ↑	(Jagodzinski *et al*., 2004)
BM-MSC (Bovine)	Cyclic compression* 10 %, 1 Hz, 3 h/d over 5d	3D	Agarose	Increase in proteoglycan deposition	C ↑	(Mauck *et al*., 2007)
BM-MSC (Human)	Cyclic compression* 15 %, 1 Hz, 1.5 h/d over 8 d	3D	Alginate	Increase in collagen X mRNA	C ↑	(Campbell *et al*., 2006)
ST-MSC (Bovine)	Cyclic equibiaxial strain 0.4 %, 1 Hz, 300 cycles/d	2D	Collagen type I	Increase in AP positive cells; Runx2 and osteocalcin protein; Runx2, osteocalcin, and osterix mRNA Decrease in Oil-red-O positive cells; Pparγ protein; Fabp4 and Pparγ mRNA	O ↑ A ↓	(David *et al*., 2007)
BM-MSC (Rabbit)	Cyclic compression* 5 %, 0.1 Hz, 10 d	3D	PLCL, fibrin	Increase in aggrecan, collagen type II protein and mRNA; GAG content	C ↑	(Jung *et al*., 2009)
BM-MSC (Rat)	4-point bending 0.2 %, 0.5 Hz, 40 min, 6 h static culture	2D	Silicone	Increase in Runx2, Alpl mRNA; AP protein; cell number	O ↑ P ↑	(Qi *et al*., 2008)
AT-MSCs (Human)	Cyclic uniaxial strain 10 %, 1 Hz, 7 d	2D	Collagen type I	Decrease in cell number	P ↓	(Lee *et al*., 2007b)

Identification: *, unconfined compression; A, adipogenic differentiation; AT, adipose tissue; BM, bone marrow; C, chondrogenic differentiation; D, dimensional; DBM, demineralised bone; P, proliferation; PLCL, poly(lactide-co-caprolactone); O, osteogenic differentiation; ST, sternum.

Napieralski *et al*., 2003). Moreover, MSCs from A2aR knockout mice express less CD73 (Katebi *et al*., 2009). Whether A2aR is also involved in MSC differentiation needs to be further elucidated.

Taken together, this study provides evidence that CD73 has a regulatory function in controlling the chondrogenic and osteogenic differentiation of MSCs, possibly acting as a switch by stimulating one lineage at the expense of the other. These results support earlier findings that proposed CD73 as a molecular coordinator of bone metabolism.

Compromised bone healing is hypothesised to be associated with limitation in MSC number and/or function.

Thus, *in vivo* stimulation of MSC function holds great promise as a tool for cell-based therapy approaches to enhance bone repair. A prerequisite for this approach, however, is a profound understanding of the molecular mechanisms during fracture repair. Our data suggest that CD73 is a, thus far underestimated, therapeutic target to guide this regenerative process. Once, the mechanism of CD73-mediated differentiation of MSCs in the interplay with mechanical (e.g. compression) and biological (e.g. cytokines and hypoxia) stimulation is understood, specific manipulation of chondrogenesis or osteogenesis – and thereby improved bone regeneration – might be possible.

Conclusion

In conclusion, the altered CD73 expression in mechanically stimulated MSCs might link the mechanical boundary conditions to the cellular response and thereby control the bone fracture-healing outcome. Conversely, CD73 is also regulated by cytokines and growth factors, such as TGF-β, IL-1β and TNF-α – as well as conditions such as hypoxia – that are found in the haematoma during the early phase of bone healing (Cervella *et al*., 1993; Hunsucker *et al*., 2005; Thompson *et al*., 2004). Thus, in addition to the mechanical microenvironment, alterations in the biological microenvironment during bone healing might also account for CD73-mediated MSC behaviour. Future studies need to investigate this mechanism in biologically challenging situations, such as (1) aged patients, whose MSCs seem to harbour a lower responsiveness to mechanical stimulation, and (2) patients with pro-longed or excessive inflammatory responses, which is likely to affect CD73 expression and function and thereby MSC differentiation.

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