

# Influence of perfusion and cyclic compression on proliferation and differentiation of bone marrow stromal cells in 3-dimensional culture

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## Abstract

Until now, there has been no *in vitro* model that duplicates the environment of bone marrow. The purpose of this study was to analyze proliferation and differentiation of human bone marrow stromal cells (hBMSC) under the influence of continuous perfusion and cyclic mechanical loading.

hBMSC of seven individuals were harvested, grown *in vitro*, and combined.  $10^6$  hBMSC were seeded on a bovine spongiosa disc and incubated in a bioreactor system. Cell culture was continued using three different conditions: Continuous perfusion (group A), 10% cyclic compression at 0.5 Hz (group B) and static controls (group C). After 24 h, 1, 2, and 3 weeks, we determined cell proliferation (MTS-assay) and osteogenic differentiation (osteocalcin ELISA, Runx2 mRNA). Tenascin-C mRNA was quantified to exclude fibroblastic differentiation.

In groups A and B, proliferation was enhanced after 2 weeks ( $48.6 \pm 19.6 \times 10^3$  (A) and  $44.6 \pm 14.3 \times 10^3$  cells (B)) and after 3 weeks ( $46.6 \pm 15.1 \times 10^3$  (A) and  $44.8 \pm 10.2 \times 10^3$  cells (B)) compared with controls ( $26.3 \pm 10.8 \times 10^3$  (2 weeks) and  $17.1 \pm 6.5 \times 10^3$  cells (3 weeks),  $p < 0.03$ ). Runx2 mRNA was upregulated in both stimulated groups after 1, 2, and 3 weeks compared to control (group A, 1 week: 5.2 ± 0.7-fold;  $p < 0.01$ , 2 weeks: 4.4 ± 1.9-fold;  $p < 0.01$ , 3 weeks: 3.8 ± 1.7-fold;  $p = 0.013$ ; group B, 1 week: 3.6 ± 1.1-fold,  $p < 0.01$ , 2 weeks: 4.2 ± 2.2-fold,  $p < 0.01$ ; 3 weeks: 5.3 ± 2.7-fold,  $p < 0.01$ ). hBMSC stimulated by cyclic compression expressed the highest amount of osteocalcin at all time points (1 week: 294.5 ± 88.4 mg/g protein, 2 weeks: 294.4 ± 73.3 mg/g protein, 3 weeks: 293.1 ± 83.6 mg/g protein,  $p \leq 0.03$ ).

The main stimulus for cell proliferation in a 3-dimensional culture of hBMSC is continuous perfusion whereas mechanical stimulation fosters osteogenic commitment of hBMSC. This study thereby contributes to the understanding of physical stimuli that influence hBMSC in a 3-dimensional cell culture system.

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**Keywords:** hBMSC; Bioreactor; Perfusion; Mechanical stimulation; Osteoblast differentiation

## 1. Introduction

Since Wolff, (1892) characterized the response of trabecular bone to mechanical strain, numerous authors have elucidated the significance of mechanical stimuli in

osteocytes and osteoblasts (Cheng et al., 2001; Kaspar et al., 2000; Tanaka, 1999; Meyer et al., 1999). The reported results are heterogeneous with regard to the effects of mechanical stimulation: *In vitro* experiments demonstrated that there is an increase in collagen I propeptide and a decrease in osteocalcin and alkaline phosphatase secretion following mechanical stress of 1  $\mu$  strain (Kaspar et al., 2000). These experiments have used osteoblastic cells in a 2-dimensional cell culture model. In contrast, Mauney et al. (2004) have observed osteogenic commitment of bone marrow stromal cells (BMSC) under

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the influence of mechanical stimulation. The results show a dexamethasone-dependent increase in proliferation, osteocalcin and alkaline phosphatase after 8 and 14 days following a continuous stimulation of 3–8%.

We observed an upregulation of Runx2 following stretching of BMSC in silicone dishes when 8% strain were applied (Jagodzinski et al., 2004). *In vivo*, high-frequency and low-amplitude stimulation has been described as efficient for bone formation (Wolf et al., 2001).

Various methods of stimulating cells have been investigated: In 2-dimensional cell cultures, mechanical stimulation includes: cyclic stretching (Zeichen et al., 2000; Neidlinger-Wilke et al., 2001), compression (Brown, 2000) and fluid shear stress (Allen et al., 2000). In the 3-dimensional setting, 4-point bending (Mauney et al., 2004) and hydrodynamic compression (Angele et al., 2003) and continuous perfusion (Bancroft et al., 2002) have been investigated. In summary, the experiments show that magnitude, frequency, duration and growth factors (Mauney et al., 2004) influence the cellular response. On the cellular level, fluid shear stress is found to play the key role inducing an osteogenic response (Allen et al., 2000). Up to the present point, no bioreactor system has been described that cultures bone marrow stromal cells on a 3-dimensional scaffold under the influence of both cyclic compression and constant perfusion.

Bone marrow stromal cells (BMSCs) are undifferentiated, pluripotent cells from the bone marrow, which are capable of forming different mesenchymal tissues such as bone, cartilage, fat, muscle, tendons and ligaments (Altman et al., 2002; Saito et al., 2003; Woodbury et al., 2002; Pittenger et al., 2000). Their high proliferation potential and simple acquisition by aspiration from the bone marrow is making them attractive for novel approaches in regenerative medicine (Caplan and Bruder, 2001; Pittenger et al., 2000).

Since mechanical stress fosters differentiation of BMSC into both osteogenic (Mauney et al., 2004) and fibroblast phenotype (Altman et al., 2002) under appropriate culture conditions, the motivation for this study was

to investigate an early (Runx2; Frank et al., 2002) and late (osteocalcin; Oliva et al., 2005) osteogenic marker. Tenascin-C expression was monitored as a control of fibroblast differentiation.

Mesenchymal precursor cells located within the bone marrow, at fracture sites or in bone defects are exposed to local physical stimuli such as fluid shear stress or cyclic axial compression. However, up to date, no appropriate *in vitro* system is available to study the influence of these stimuli on hBMSC differentiation. We therefore established a novel bioreactor system to subject hBMSC to continuous perfusion and cyclic compression and perfusion in a combined fashion to study the effect of both stimuli on the osteogenic differentiation of mesenchymal precursor cells.

## 2. Material and methods

### 2.1. Acquisition, isolation and cultivation of BMSC

Bone marrow aspirates from the iliac crest were collected from seven human donors undergoing iliac crest harvest of autologous cancellous bone. The donors were otherwise healthy and their age ranged from 23 to 52 years. The donors are described in detail in Table 1. One subject received dexamethasone at the time of cell aspiration. All procedures were approved by the institutional ethics committee, and informed consent was obtained from all donors.

hBMSC were harvested and cultured according to a modified standardized protocol (Hankemeier et al., 2005). Briefly, density gradient centrifugation was used to obtain a cell pellet that was resuspended in DMEM/Ham's F12 1:1 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 100 U/ml Penicillin/Streptomycin (Gibco, Karlsruhe, Germany), 0.5 µg/ml Amphotericin B (Biochrom, Berlin, Germany), 5 µg/ml ascorbic acid (Sigma, Dahlkirchen, Germany) supplemented with FGF-2 (3 ng/ml, Pepro Tech, Offenbach, Germany) buffered with Hepes buffer (Roth, Karlsruhe, Germany; pH 7.0).

Cells were plated in 75 cm<sup>2</sup> cell culture flasks (Nunc, Berlin, Germany) and incubated at 37 °C and 5% CO<sub>2</sub> in humidified atmosphere. The medium was changed 2 times a week. After reaching confluence between day 14 and 21, cells were released with 0.25% trypsin (Gibco, Karlsruhe, Germany) and combined. The cell pool was subcultured and cells of the third passage were used for experiments. At this point, cell phenotype was analyzed using the following protocol: 10<sup>6</sup> viable (trypan blue negative) bone marrow mononuclear cells were stained with the following

Table 1

Gender, age, diagnosis, operative procedure and drug administration at the time of bone marrow aspiration of the seven donors that contributed to the cell pool used for this study

Donor number	Gender	Age	Diagnosis	Operative procedure	Drugs involved
1	Female	27	Th2, Th11-L4 fractures	Dorsal stabilization L2–L4	Fentanyl Sevoflurane Droperidole Fortecortin 8 mg
2	Female	43	Cartilage lesion medial femoral condyle	High tibial osteotomy	NSAID (COX-2 selective)
3	Male	50	Varus deformity	High tibial osteotomy	NSAID
4	Male	52	Varus deformity	High tibial osteotomy	NSAID
5	Male	29	Bone defect femur	Transplantation of tricortical bone grafts	NSAID
6	Male	23	Burst fracture T9	Dorsal stabilization T8–10	Fentanyl Sevoflurane Droperidole
7	Female	24	Graft failure following ACL reconstruction	Bone tunnel revision, ACL reconstruction	Acetaminophen

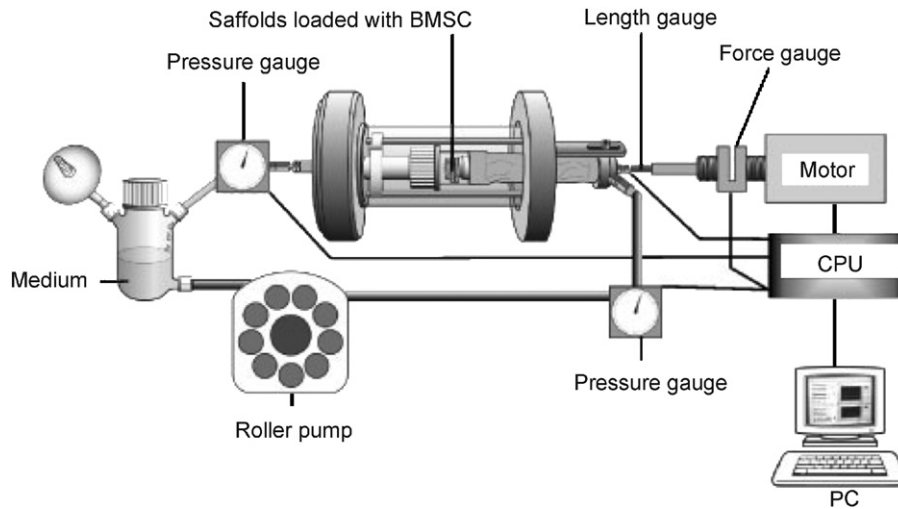


Fig. 1. Bioreactor system used for the stimulation experiments: a linear servomotor was used for compression of the scaffolds. Continuous perfusion was performed by a roller pump. All parameters were entered and monitored by a PC.

nondiluted antibodies (all obtained from BD, Franklin Lakes, USA): CD45-APC, CD235-APC, CD271-PE, CD73-PE (Trivedi and Hematti, 2008). The fraction of bone marrow mesenchymal stem cells (PE<sup>+</sup>, APC<sup>-</sup>) that were selected using a fluorometric cell sorter (BD FACSAria<sup>TM</sup>, BD, Franklin Lakes, USA) was  $89 \pm 6\%$ . Next,  $10^6$  hBMSC were resuspended in 250  $\mu$ l tissue culture medium and seeded on a bovine acellular matrix ( $4 \times 20$  mm Tutobone<sup>®</sup> discs, Tutogen Medical Inc., Neunkirchen a. Br., Germany). These scaffolds were demineralized using decal<sup>®</sup> (Decal Corporation, Tallman, NY, USA) for 20 min followed by thorough rinsing with PBS and culture media for 3 weeks. After 2 h of incubation at 37 °C, the matrix was placed into a 12-well tissue culture dish and overlaid with 2 ml tissue culture medium. Instead of FGF-2,  $10^{-7}$  M dexamethasone (Fortecortin, Merck AG, Darmstadt, Germany) was supplemented.

## 2.2. Bioreactor system

A modified, custom-made perfusion bioreactor system according to Bader (Jasmund and Bader, 2002) was equipped with a linear servomotor (Linmot<sup>®</sup> Inc., Delavan, WI). The system delivers a peak force of 135 lb, an acceleration of 45 Gs, and a maximum speed of 175 ips. The change of length of the system was monitored by an external length gauge. The scaffolds were held in a custom-made bearing located in the middle of the bioreactor. Continuous perfusion was applied by a roller pump (Ismatec IPC 16, ISMATEC SA, Glatfbrugg, Germany). The flow rate for groups A and B was maintained at 10 ml/min. Fifty percent of the tissue culture media was changed every other day and the system was kept in an incubator at 37 °C. The parameters: compression (0.2–25 mm, frequency (0.1–20 Hz), duration of stimulation (minutes), maximum force (25 N) could be adjusted for the experiments. Scaffolds of group B were subjected to a cyclic compression of 10% (0.4 mm for each 4 mm cubic scaffold). All data were transferred to a CPU at a 60 Hz frequency (Fig. 1).

## 2.3. Real-time PCR analysis

For RNA isolation, the tissue samples were chopped up and homogenized using 1 ml Trizol (Sigma, Deisenhofen, Germany) in a polytron homogenizer (Janke & Kunkel, Germany). Subsequently, 0.2 ml chloroform (Malinkrodt Baker, Deventer, Netherlands) was added. After centrifugation at 13,000g at 4 °C for 15 min, 500  $\mu$ l isopropanol (Merck, Darmstadt, Germany) was added to the supernatant. After an incubation period of 10 min at room temperature and another centrifugation step for

8 min at 13,000g, the pellet was washed with 500  $\mu$ l 75% ethanol and dried in a SpeedVac (Concentrator 5301, Eppendorf, Hamburg, Germany) for 10 min at room temperature. After addition of 10  $\mu$ l distilled water (Ampuwa), the pellet was dissolved for 10 min on ice. Amount and purity of total RNA were determined in a spectrophotometer (BioPhotometer 6131, Eppendorf) at 260 and 280 nm (Agilent 2100 bioanalyzer, Agilent Technologies, Waldbronn, Germany).

cDNA was synthesized according to a standardized protocol (Hankemeier et al., 2005). Primers for Runx2, Tenascin-C and PBGD were purchased from IBA (Göttingen, Germany).

Real-time PCR technology was used for quantification of Runx2 and Tenascin-C mRNA expression. Data were normalized to the reference gene porphobilinogen deaminase (PBGD).

The reaction mixture contained 5  $\mu$ l cDNA, 2  $\mu$ l of primer ( $10 \text{ pmol } \mu\text{l}^{-1}$ ) (see Table 2), 2  $\mu$ l FRET probes ( $4 \text{ pmol } \mu\text{l}^{-1}$ ) Runx2-FITC (cagtgatttagggcgcttctctcctccag-FITC), Runx2-Cy5.5 (Cy5.5-atgagtaggtgtccgcctcagaac), Tenascin-C-FITC (tgtcacagacaccactgcttgcacacctgg-FITC), Tenascin-C-Cy5.5 (Cy5.5-tcaagccctggctgagatcgatggcattga), PBGD-FITC (tcctctggcttcaccatcgaggc-FITC), PBGD-Cy5.5 (Cy5.5-tctgcaagcgggaaaccctcatg), (MWG), 2  $\mu$ l Fast Start DNA Master Hybridization Mix, 3.2  $\mu$ l MgCl<sub>2</sub> ( $25 \text{ mmol } \mu\text{l}^{-1}$ ) and 1  $\mu$ l DMSO (Roche Diagnostics). The mixture was adjusted to 20  $\mu$ l using DEPC-ddH<sub>2</sub>O. For denaturation, a temperature of 95 °C was maintained for 720 s. Amplification was performed at 95/60/72 °C for 15/12/10 s (45 cycles, slope  $20^\circ\text{C s}^{-1}$ ) (PBGD: annealing temperature 62 °C) without color compensation. After measurement of the relative fluorescence intensity (channel F3/F1) for each sample, a proportional baseline adjustment for sample quantification was carried out by second derivative maximum calculation. The resulting Ct values were used for expression analysis of Tenascin-C and Runx2 by relative quantification using the  $2^{-\Delta\Delta\text{Ct}}$  calculation method (Livak and Schmittgen, 2001) with PBGD as reference gene. Results are reported in percentages of gene expression of the non-stimulated controls (Fig. 4).

## 2.4. Cell proliferation

The MTS assay was used to investigate the cell adherence and proliferation of BMSC that were seeded on the scaffold. Cells were incubated with 0.5 mg/ml MTS for 2 h at 37 °C, the solution was then removed and formazan salt dissolved in dimethyl sulfoxide was added. The absorbance was determined at 490 nm using a plate reader (BP 800, Biohit, Finland). Cell number was determined from the calibration data (not shown linear fit, six points,  $n = 3$ ,  $R^2 = 0.97$ ), obtained using cells from the same culture.

Table 2

Primer names and sequences used for the quantification of Runx2, Tenascin-C and PBGD mRNA expression. Overview of annealing temperatures and product lengths

Sense primer	Sequence	Antisense primer	Sequence	T (°C)	Bp
Runx2-for	5'-gctacctatcacagagcaatta-3'	Runx2-rev	5'-cctgggtctgtaactga-3'	60	237
Tenascin-C-for	5'-tctctgcacatagtgaataacc-3'	Tenascin-C-rev	5'-cttcctctgtgagatc-3'	60	225
PBGD-for	5'-gggacaagattcttgatactgc-3'	PBGD-rev	5'-accacactctctggcag-3'	62	253

### 2.5. Osteocalcin

To determine the expression of osteocalcin, samples were incubated with 1 ml 0.01% Triton X-100 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and homogenized mechanically to disintegrate the cells, using a custom-made dispersing and mixing device (Institutional Central Research Laboratory). Samples were centrifuged (8.000g for 10 min) and the amount of total protein and the content of osteocalcin were determined. Total amount of protein was quantified using the BCA™-Protein Assay Kit (Pierce Inc., Rockford, IL, USA) at 562 nm. Osteocalcin was quantified using an ELISA kit (N-MID Osteocalcin One Step ELISA Kit, Nordic Bioscience Diagnostics A/S, Herlev, Denmark). The extinction was measured at 450 nm. The assay had a linear detection range between 0.5 and 100 ng/ml. Only values above the detection limit (0.5 ng/ml) were used and the values were normalized to the amount of protein.

### 2.6. Statistical analysis

Results were expressed as mean ± standard deviation. Statistical analysis to compare results between groups was carried out by multivariate analysis of variance (ANOVA) with a post-hoc Tukey test (SPSS 11.5; SPSS, Chicago, IL, USA). *F*-test was used in order to analyze between subject effects. A *p*-value ≤ 0.05 was considered to be statistically significant.

## 3. Results

Results of seven experiments are reported (*n* = 7).

### 3.1. Cell proliferation

Proliferation was increased in cell cultures that were only exposed to continuous perfusion or to a combination of cyclic compression and perfusion compared with static controls after two and three weeks (perfusion, mechanical stimulation, controls, respectively: 2nd week: 48.6 ± 19.6, 44.6 ± 14.3, 26.4 ± 10.8 × 10<sup>3</sup> cells; 3rd week: 46.6 ± 15.1, 44.8 ± 10.2, 17.1 ± 6.5 × 10<sup>3</sup> cells; *p* < 0.03). Factor analysis revealed that the type of stimulation rather than the time in culture had an effect on proliferation (*F* = 14.8, *p* < 0.01; Fig. 2).

### 3.2. Osteocalcin

The amount of osteocalcin decreased over time in controls (1st week: 190.6 ± 65.5, 2nd week: 98.2 ± 40.3, 3rd week: 69.1 ± 28.8 mg/g protein; *p* = 0.02). Under the influence of continuous perfusion, levels of osteocalcin were higher compared with controls (1st week: 228.9 ± 68.6, 2nd week: 188.1 ± 84.9, 3rd week: 161.8 ± 61.8 mg/g

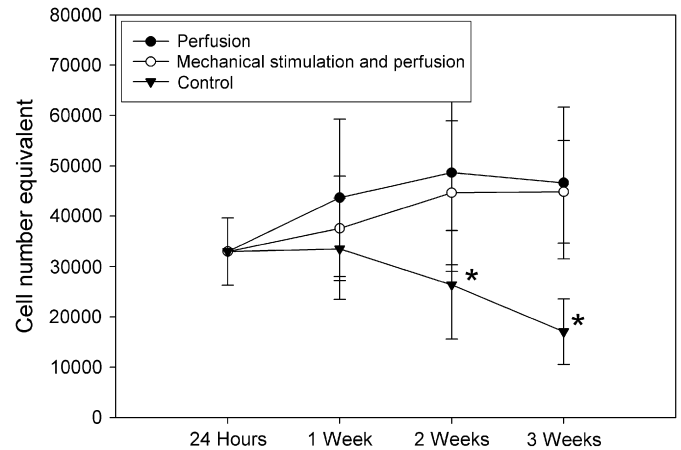


Fig. 2. Cell viability and proliferation were lower in controls than in constructs that were cultured under perfusion or mechanical stimulation and perfusion after 2 and 3 weeks (*n* = 7, \*ANOVA, Tukey-test: *p* < 0.03).

protein; *p* = 0.02). Mechanically stimulated cultures contained the highest amounts of osteocalcin at all observed time points (1st week: 294.5 ± 88.4, 2nd week: 294.4 ± 73.3, 3rd week: 293.1 ± 83.6 mg/g protein). Levels were higher than in controls at all time intervals (*p* ≤ 0.03). After 2 and 3 weeks, there was more osteocalcin in cyclically compressed samples compared with the samples that were only perfused (*p* ≤ 0.03) (Fig. 3).

### 3.3. RT-PCR

Runx2 expression after 24 h was 1.9 ± 1.5-fold increased compared to control, indicating an early initiation of osteogenic differentiation. There was an upregulation in both stimulated groups after 1, 2, and 3 weeks (perfusion: 1 week: 5.2 ± 0.7; *p* < 0.01, 2 weeks: 4.4 ± 1.9; *p* < 0.01, 3 weeks: 3.8 ± 1.7; *p* = 0.013; cyclic compression: 1 week: 3.6 ± 1.1, *p* < 0.01, 2 weeks: 4.2 ± 2.2, *p* < 0.01; 3 weeks: 5.3 ± 2.7, *p* < 0.01). There was a difference in Runx2 expression between perfusion and mechanical stimulation after 3 weeks (*p* < 0.017). The effect of culture condition (mechanical stimulation and perfusion; *F* = 20.5) was five times greater than the effect of the time being in culture (*F* = 4.1, *p* < 0.01). The corresponding ΔΔCt-values are included in Fig. 4.

In only one out of seven experiments, very limited amounts of Tenascin-C expression were detected.

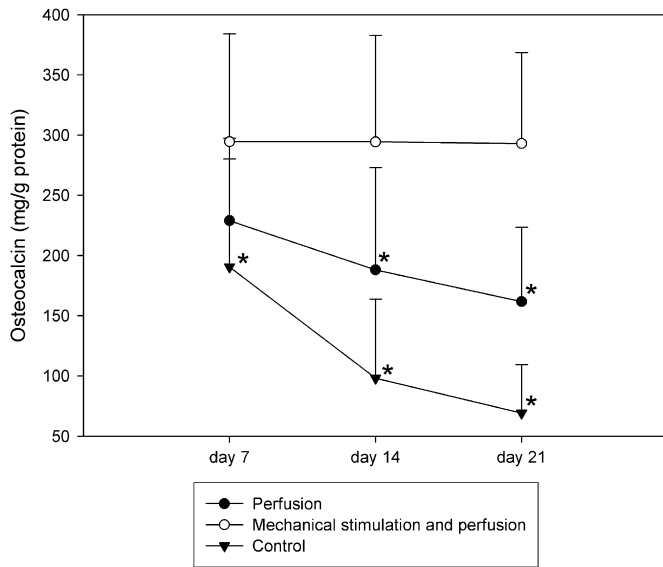


Fig. 3. Osteocalcin levels were decreased in controls and decreasing over time when continuous perfusion was applied ( $n = 7$ ): Significant differences on day 7: Mechanical stimulation–control ( $p = 0.03$ ), day 14: Mechanical stimulation–perfusion ( $p = 0.03$ ), Mechanical stimulation–control ( $p < 0.01$ ), day 21: Mechanical stimulation–perfusion ( $p < 0.01$ ), mechanical stimulation–control ( $p < 0.01$ ).

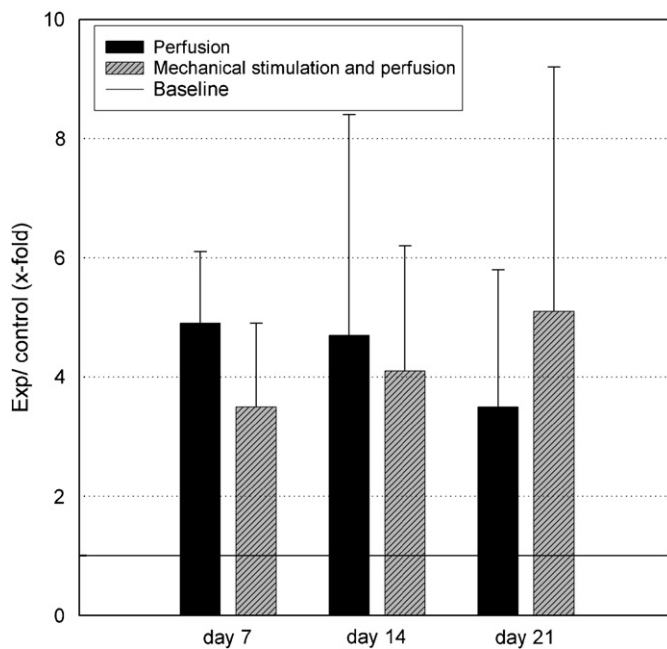


Fig. 4. Runx-2 expression was upregulated in stimulated groups compared with controls at all time intervals ( $p < 0.01$ ). After 3 weeks, there was higher expression of Runx-2 in mechanically stimulated samples compared with perfusion ( $p = 0.017$ ). Data is reported in  $x$ -fold expression of Runx-2 of static cultures, which is represented by the baseline ( $n = 7$ ).

#### 4. Discussion

Numerous studies have investigated the influence of hydrostatic pressure (Angele et al., 2003) or dynamic fluid flow (Bancroft et al., 2002; Mauney et al., 2004) on cell

cultures. The effect of mechanical stimulation has been investigated in 2-dimensional (Kaspar et al., 2000; Zeichen et al., 2000) and 3-dimensional (Mauney et al., 2004; Altman et al., 2002) *in vitro* environments. The specific aim of this study was to analyze the factors continuous fluid flow and cyclic mechanical compression on human bone marrow stromal cells (hBMSC) in a novel bioreactor that recapitulates the mechanical conditions in the intramedullary canal. To our knowledge, this is the first study that describes the effects of both stimuli on BMSC *in vitro*. Stimulation in a 4-point bending machine has been demonstrated to trigger osteogenic commitment of BMSC under the influence of 10 nM dexamethasone after 8 and 14 days (Mauney et al., 2004). This study confirms the findings of these authors in a context that uses axial compression instead of bending and continuous fluid of a media that contained the same amount of dexamethasone. BMSC have been driven into the fibroblast phenotype when a collagen gel and stretching and rotation were applied (Altman et al., 2002). Their culture media was supplemented with fibroblast growth factor (FGF-2). An upregulation of Tenascin-C and Collagen I and III could be observed after 14 days. Likewise, in a previous study, we could detect a change of BMSC phenotype into fibroblast-like cells, especially when a concentration of 3 ng/ml FGF-2 was supplemented: On days 14 and 28, collagen I, collagen III, fibronectin, and alpha-smooth muscle actin mRNA expression was significantly enhanced in the presence of low-dose FGF-2 (Hankemeier et al., 2005). These culture conditions were used in the current study to enhance cell proliferation in 2-dimensional cell culture. The study investigates the change of cell phenotype when BMSCs are transferred from 2-dimensional to 3-dimensional cell culture and dexamethasone, perfusion, and mechanical stimulation are applied.

Several strain rates, frequencies, exposure times and bioreactor systems have been used in order to mechanically stimulate cell cultures (Mauney et al., 2004; Kaspar et al., 2000; Zeichen et al., 2000; Neidlinger-Wilke et al., 2001). In this study, we used the same amount of compression, frequency and time of stimulation that we have identified in a 2-dimensional setup to be most effective for BMSC (Jagodzinski et al., 2004). However, different parameters may lead to different results. In appreciation of the results of other studies, one of the key elements of cell lineage differentiation is the specific environment (scaffold, growth factors) of the cells in order to achieve an osteogenic (Mauney et al., 2004; Jagodzinski et al., 2004), chondrogenic (Angele et al., 2003) or fibroblastic commitment (Altman et al., 2002).

Transfer of BMSC onto a 3-dimensional scaffolds leads to reduced proliferation compared with 2-dimensional cell cultures (Jagodzinski et al., 2004). Continuous perfusion has been addressed to increase proliferation (Bancroft et al., 2002). These findings are confirmed by this study, as perfusion had the strongest effect on proliferation

( $F = 14.8$ ). For static cultures, there was a decrease of proliferation over time (Fig. 2).

Many experiments have identified a positive effect of perfusion on cell proliferation (Bancroft et al., 2002; Datta et al., 2006; Gomes et al., 2003; Holtorf et al., 2005; Sikavitsas et al., 2005). A perfusion rate of 10 ml/min was being used in the current study and confirmed the findings. Factor analysis revealed that perfusion had the dominating effect on cell proliferation ( $F = 18.7$ ,  $p < 0.01$ , Fig. 2). Mechanical stimulation has been found to affect differentiation more than proliferation (Bancroft et al., 2002).

Runx2 is expressed early in the pathway of osteoblast differentiation (Yamaguchi et al., 2000). In this study, Runx2 was found to be upregulated early compared with non-stimulated controls and remained upregulated during the entire period of time. Mechanical compression lead to an elevated expression of Runx2 compared to a decrease in static or perfused cultures (after 21 days,  $p = 0.05$ , Fig. 4). These effects were also observed by analyzing osteocalcin, which was significantly increased in mechanically stimulated groups after 14 and 21 days. In contrast to human bone-derived cells that have shown to dedifferentiate under the influence of cyclic stretching (Kaspar et al., 2000; Neidlinger-Wilke et al., 2001), BMSC underwent an osteogenic differentiation under the influence of cyclic compression. This is in line with the findings reported by other authors (Mauney et al., 2004). Stanford et al. (1995) observed a dose-dependent suppression of bone specific mRNA levels in calvarial osteoblasts. Our protocol was optimized according to our previous experience and the reported protocols for osteogenic differentiation under the influence of mechanical stimulation (Jagodzinski et al., 2004, 2005; Mauney et al., 2004). Cell expansion under the influence of dexamethasone may have affected the primary phenotype of BMSC at the beginning of the experiment. The heterogeneity of human bone marrow stromal cells is a limiting factor and was compensated for in this study by pooling cells from seven donors (Kasten et al., 2006). Thus, standard deviations were within a closer range compared with previous experiments (Jagodzinski et al., 2004). Heterogeneous results may be caused by the variation of donor age, passage number, and culture conditions (Siddappa et al., 2007; Igarashi et al., 2007; Bertram et al., 2005). There is still controversy in the literature concerning the effects of culture time. Even though Caplan underscores the nearly unlimited proliferation capacity of BMSC (Caplan and Bruder, 2001), changes in telomerase activity and cellular ageing has been found with increasing cell culture passages (Banfi et al., 2002).

The variation of Runx-2 expression is a limitation of the results of this study and may have the following explanations:

1. Instead of a cell line we have used human donors varying in age and comorbidity (compare Table 1). This was partly compensated by pooling of the cells before conducting the experiments. However, there was still a

range of different cells (only  $89 \pm 6\%$  of the cells were detected exhibiting CD-73 and CD-271 antigens).

2. The bioreactor system used has been optimized for the setup used for this study (Runtemund et al., 2007). The xenogeneic scaffolds used may have caused a difference in resistance and may have affected the flow rate due to a variation of porosity between the experiments. The decalcification of the scaffolds may have caused a variation of stiffness and compressive forces. These factors may have affected especially the late time points 14 and 21 days.
3. Runx-2 is an early marker that is expressed to a lesser extent than other osteogenic markers. The RT-PCR used in this study is a very sensitive method and was used to detect potential early changes in cell differentiation. The results were confirmed by the analysis of protein secretion (osteocalcin, Fig. 3).

Further studies are necessary in order to determine the long-term viability and differentiation of BMSC *in vitro* and *in vivo*. Influences of compression, different mechanical and hydrostatic stress patterns over different periods have to be analyzed in more detail. The results of this study can be useful for comparing other stimuli and scaffolds in the same bioreactor system.

#### Conflict of interest

No author has any financial or personal relationship with other people or organizations that could have influenced this work inappropriately.

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