Mechanical Strain of Bone Marrow Stromal Cells Induces Proliferation and Differentiation into Osteoblast-Like Cells

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Summary

Mechanical loading is a prerequisite for proper fracture healing in patients. This loading is received by the cells and a cellular response is elicited. This consists of signal transduction, proliferation and mineralization. This fact is transferred to in vitro tissue engineering especially for bone. Possible cell sources are osteoblasts or stem cells. As the isolation of stem cells is not complex and yields high amounts of cells, these cells are favoured. In the current study, the effect of mechanical loading on the differentiation of stem cells into osteoblasts was investigated. Stem cells were isolated from bone marrow aspirates from patients needing bone grafts from the iliac crest. Cells were isolated using percoll gradient centrifugation. Cells were seeded on elastic silicon dishes. The cells were longitudinally strained (5%, 1 Hz) for 15' or 60'. As a control, no strain was applied. Also repetitive strain for 8 hours at three consecutive days was carried out. Parameters investigated included proliferation, apoptosis, and mineralization. 15 minutes strain resulted in increased proliferation. 60 minutes of strain showed hardly any effect. Repetitive strain application blocked proliferation in favour of differentiation as detected by increased mineralization. The investigation of signal transduction pathway after the repetitive long term strain did not reveal any phosphorylated kinases, probably due to the late time point. Thus application of mechanical strain to stem cells induces differentiation into osteoblasts.

In the future, mechanical conditioning of stem cells should be applied before seeding them on scaffolds. This could lead to an optimal engineered bone construct applicable for implantation purposes.

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Introduction

The effects of biophysical force on bone remodelling have become increasingly evident in recent years. It is well known that extended periods of immobilisation lead to bone loss. This is especially apparent in situations of weightlessness. Subjects exposed to weightlessness have shown diminished or arrested bone formation (1), reduced collagen production (2), increased osteoclast numbers (3), and consequently a decrease in mechanical properties of bone. Mechanical loading is one of the few positive stimuli for bone formation, and the use of suitable exercise regimes has been proposed as being potentially of significant benefit in maintaining bone mass in postmenopausal women and accelerating bone mass recovery after bed rest (4, 5). Mechanical load aligns collagen fibres and through this tissue reorganization increases functionality (6). Thus, mechanical loading is important for maintaining the physiological properties of mature bone. There have been several investigations dedicated to examine the influence of cyclic mechanical stretching upon osteoblasts obtained from cancellous bone chips (7, 8). Mechanical stress has been demonstrated to stimulate the secretion of osteogenic proteins (7). It could be demonstrated that cyclic stretching stimulates osteoblast proliferation and CICP (procollagen I propeptide) production but decreased the synthesis of alkaline phosphatase and osteocalcin (7).

This raised the question whether mechanical loading may also be able to enhance precursor cells to differentiate osteoblasts. This would be important in light of tissue engineering of bone constructs for regenerating bone tissue after trauma or osteoporosis linked complications. As an alternative to obtaining autologous bone from the iliac crest which is associated with considerable donor site morbidity, the field of tissue engineering promises the opportunity to develop a synthetic construct based on cells seeded onto an appropriate matrix. Bone marrow stromal cells (BMSCs) have been identified as an attractive cell source for a wide variety of tissue engineering strategies. Since even in older individuals, bone marrow stroma harvesting is a relatively easy

procedure, bone marrow contains a pluripotent population of cells capable of differentiating along multiple mesenchymal lineages (e.g. bone, ligament, adipose tissue, cartilage, muscle tissue), and can easily be expanded *ex vivo* utilizing routine cell culture techniques (9-15). Thus, BMSCs can serve as a basis for tissue engineering of autologous implants without concerns on transplant rejection.

Cyclic compressive loading of rabbit BMSC in agarose cultures stimulated chondrogenesis (16). Furthermore, mechanical strain has been shown to promote osteogenesis of BMSCs *in vitro*, verified by the upregulation of osteogenic marker proteins like alkaline phosphatase (17-19), osteocalcin (18, 19), osteopontin (20), and type I collagen (18, 21). These results, however, are dependent on the type and intensity of strain. Mechanical strain may act with different frequencies and strength, which appears to have relevance in regulating cell physiology (22, 23). *In vitro*, there are several devices for the application of strain. The three main systems are 1) circular membranes (Flexercell), 2) longitudinal strain (18, 24), and 3) 4-point bending (25). The disadvantage of the circular membranes is that the strain distribution across the membrane is heterogeneous. Therefore, in our studies we have used a longitudinal strain device.

As shown above, mechanical strain can influence the differentiation of BMSC into osteoblasts. However, what is not clear are the pathways by which mechanical strain is transmitted into biological signals. In previous studies, in human patellar tendon fibroblasts, we have shown that cyclic longitudinal mechanical strain induces the secretion of Nitric oxide (NO) (26) and the activation of Jun-N-terminal-kinase/stress-activated protein kinase (JNK/SAPK) in a time dependent manner (27). Montaner *et al.* observed a link between JNK/SAPK and NFκB transduction pathways (28). Both JNK/SAPK and NF B are, among others, involved in proliferation and apoptosis (29). Indeed, cyclic longitudinal mechanical strain can modulate proliferation (30) and apoptosis (31) of patellar tendon fibroblasts. Also in bone cells, similar strain induced mechanotransduction pathways have been recognized. These include cAMP, cGMP (32, 33), cfos (34), IP3 (35), intracellular calcium (36), COX-2 & prostaglandins (34) and iNOS

& NO (34). Furthermore, cyclic longitudinal strain induced cbfa1 (18). Parts of these pathways are mediated via specific mechanosensitive calcium-channels (37) or integrins (38). The latter ones induce enhanced phosphorylation of cytoskeletally-anchored proteins such as MAPK (38).

In the present study, the influence of cyclic longitudinal mechanical strain was investigated in the context of differentiation of BMSC into osteoblast in association with signaltransduction pathways.

Materials & methods

BMSC isolation and cultivation

Human bone marrow aspirates were obtained during routine orthopaedic surgical procedures involving exposure of the iliac crest. The institute ethics committee approved all procedures, and informed consent was obtained from all donors.

For cell isolation, bone marrow aspirates were washed with cell culture medium (DMEM/Ham's F12 (1:1) (Biochrom, Berlin, Germany)) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 100 µg/ml penicillin/streptomycin (Biochrom, Berlin, Germany), 2.5 µg/ml amphotericin B (Biochrom, Berlin, Germany), 7.5% sodium hydrogen carbonate (Merck, Darmstadt, Germany), buffered with HEPES buffer (Roth, Karlsruhe, Germany; pH 7.0). The cell pellet was centrifuged over a percoll gradient (Amersham Biosciences, Buckenhamshire, UK; d=1.131 g/ml) for 15 min at 1750×g. The supernatant was washed again with cell culture medium and was then cultivated in standard cell culture flasks at 37°C and 5% CO_2 in humidified atmosphere for at least 5 days. The culture medium with non-adherent cells was removed. In order to obtain enough BMSC, cells of the third passage were used.

BMSCs were incubated with differentiation medium (culture medium as above supplemented with 50 µg/ml L-ascorbic acid, 10 mM β-glycerol phosphate and 10 nM dexamethasone 21-dihydrogen phosphate) for at least one week before the start of the experiments.

Cyclic longitudinal strain

The cell stretching system consisted of rectangular, elastic silicone dishes in which the whole dish, not only the cell culture surface, was deformable (Fig. 1). The dishes were designed for use in a stimulation apparatus driven by an eccentric motor that allowed variation in amplitude (0.5–10%) and frequency (0.5–2 Hz) of applied strain (Fig. 2). The dishes were moulded of a two component silicone elastomer (Silbione® RTV 71556 A+B, Rhône-Poulenc Silicon GmbH, Lübeck, Germany) at a ratio of 10:1 of silicone oil:crosslinker. The rectangular dishes were 8 cm long × 3 cm wide × 1 cm high, and the wells had a 5 cm × 2.3 cm cell culture surface. New dishes were autoclaved at 121°C and preconditioned for three days in culture medium before the cells were seeded.
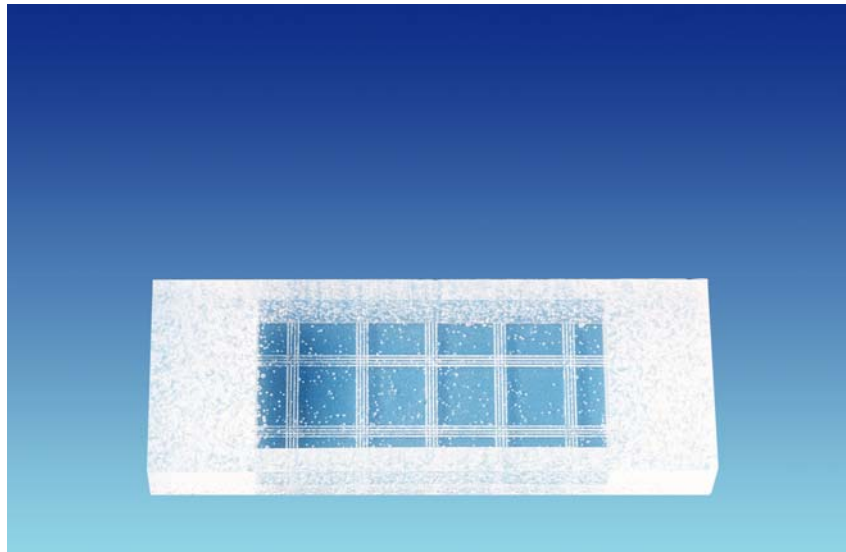
Fig. 1: Flexible silicone dishes used for cultivation and strain of BMSCs. The dishes were moulded of a two component silicone elastomer (Silbione® RTV 71556 A+B, Rhône-Poulenc Silicon GmbH, Lübeck, Germany) at a ratio of 10:1 of silicone oil-crosslinker. The rectangular dishes were 8 cm long × 3 cm wide × 1 cm high, and the wells had a 5 cm × 2.3 cm cell culture surface.
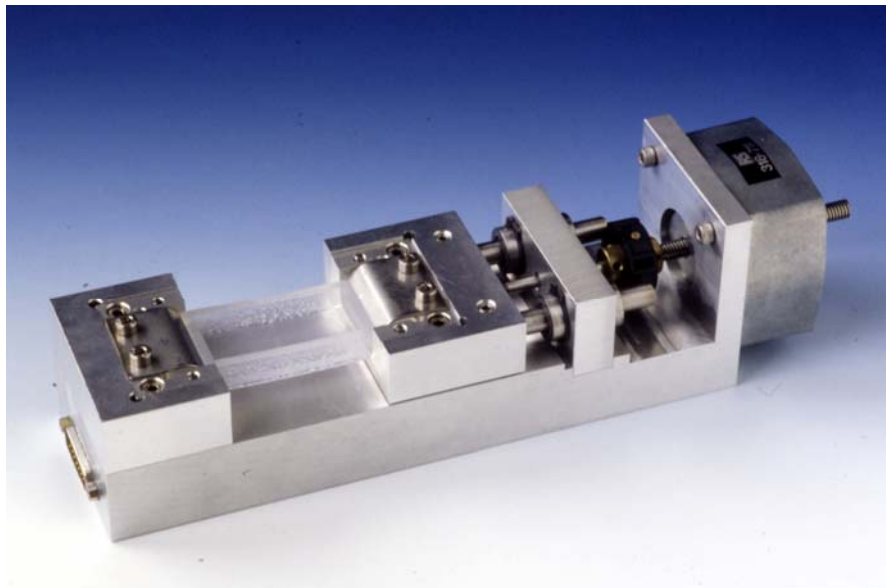

Fig. 2: Straining device for the application of cyclic longitudinal strain using an eccentric motor.

BMSCs of the second passage were harvested, counted and an overall viability of more than 90% was observed using trypan blue exclusion test. 1.5×10^5 cells were seeded on each silicone dish. After 24 hours of culture, the concentration of fetal calf serum was reduced to 1% for 24 hours in order to align most cells into the G0 phase of the cell cycle.

The cells in the silicone dishes were cyclic-longitudinally strained at a frequency of 1 Hz and an amplitude of 5%. Short time strain was applied for 15 and 60 min. The observation periods after cessation of strain were 6, 12, and 24 hours. Long time strain was applied for three times for 8 hours with pauses of 15 hours between the single strainings. As a control, cells were grown on silicone dishes, but did not receive any strain.

Proliferation

Cell proliferation was monitored using BrdU incorporation (Roche, Mannheim, Germany). BrdU was added to the cells on the silicone dishes directly before every stretching period of 8 hours. BrdU detection was performed according to the manufacturer's instructions. Relative proliferation rates were determined by comparing strained cells with static control cells.

Apoptosis

After the application of mechanical strain, cells on the silicone dishes were washed with PBS and then 1 ml FACS binding buffer were added. The cells were detached from the dishes using a cell scraper and spun down for 5 min at 1750×g and 4°C. Cell pellets were resuspended in 100 µl FACS binding buffer and incubated for 20 min in the dark with 5 µl FITC labelled Annexin V (Bender MedSystems Diagnostics GmbH, Vienna, Austria) to detect early apoptotic cells and 5 µl propidium iodide (Bender MedSystems Diagnostics GmbH, Vienna, Austria) to detect late apoptotic and dead cells. Cells were

washed and analyzed by flow-cytometry. Relative rates of apoptosis and cell death were calculated by comparing strained versus static control cells.

Mineralization

Mineralization was detected by using von Kossa staining. Cells on the silicon dishes were washed twice with phosphate buffered saline. Subsequently, cells were fixed for ten minutes using 3% phosphate buffered formaldehyde. A 3% silver nitrate solution was added to the cells for 30 minutes in the dark. Silver-calcium precipitation in the matrix was developed using 1% Pyrogallol for three minutes. Finally, surplus silver was removed and the precipitates were fixated using 5% sodium thiosulphate for three minutes. Lime precipitates were dark coloured. Nuclei were counterstained using nuclear fast red.

Western blotting

The influence of cyclic longitudinal mechanical load on expression and activation of signal transduction proteins (JNK, ERK, p38) was studied by western blot. Cells were washed with PBS and lysed using 100 µl Laemmli buffer (2.5% SDS, 12.5% glycerol, 0.025 M TRIS, 0.5 mM EDTA, 2.5% mercapto ethanol, 0.01% bromphenol blue) and vigorously detached from the dishes.

Gel electrophoresis and blotting was performed onto a nitrocellulose membrane. After incubation with the specific antibodies, bands were visualized using the ECL system (Amersham, Biosciences, Buckenhamshire, UK). Band intensity was analyzed densitometrically and semiquantified relating to the band intensity of β-actin. The amount of activated MAP kinase was related to the amount of total corresponding protein. Strained cells were compared with the respective static controls.

Statistical analysis

Comparisons between groups were performed using one-way analysis of variances (ANOVA) and a post-hoc t-test. A probability value less then 0.05 was considered statistically significant. Data are expressed as mean ± standard error of the mean (SEM).

Results

Proliferation

Six hours after 15 min of cyclic longitudinal strain, proliferation rates of BMSCs were significantly increased to 1.95±0.14 compared to static control cells (p<0.05). 12 and 24 hours after cessation of this 15 minutes' strain, no differences in proliferation from control cells could be detected. After 60 minutes of cyclic longitudinal strain a similar pattern was observed. However, no significant differences could be detected in comparison to static controls. Highest proliferation rates were observed six hours after cessation of the 60 minutes' strain (1.34±0.19). Again, proliferation rates returned tolevels as seen in static controls at 12 and 24 hours. Application of repetitive long time cyclic longitudinal strain resulted in lower proliferation rates compared to static control cells (Fig. 3).
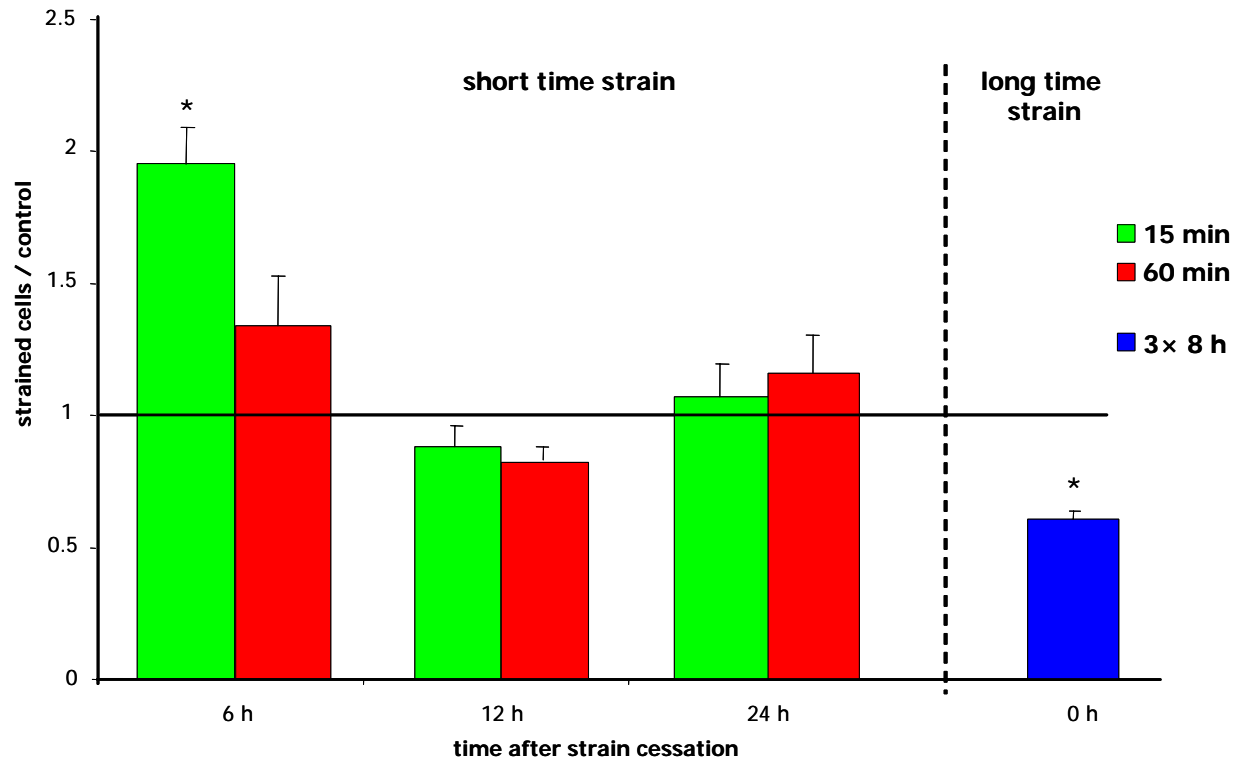
Fig. 3: Relative proliferation rates of strained cells vs. static control cells. The line at 1 depicts the static control cells. Proliferation was measured using BrdU incorporation. Strain was applied with a frequency of 1Hz, an amplitude of 5% and for the duration indicated. *p<0.05.

Apoptosis

Apoptosis rates were slightly increased 6 hours after 15 minutes of strain application. 60 minutes of cyclic longitudinal strain induced apoptosis after 12 and 24 hours. Both apoptosis and cell death rates after long time strain decreased to 0.81±0.02 and 0.84±0.17 respectively compared to static control cells. The difference was statistically insignificant (Fig. 4).
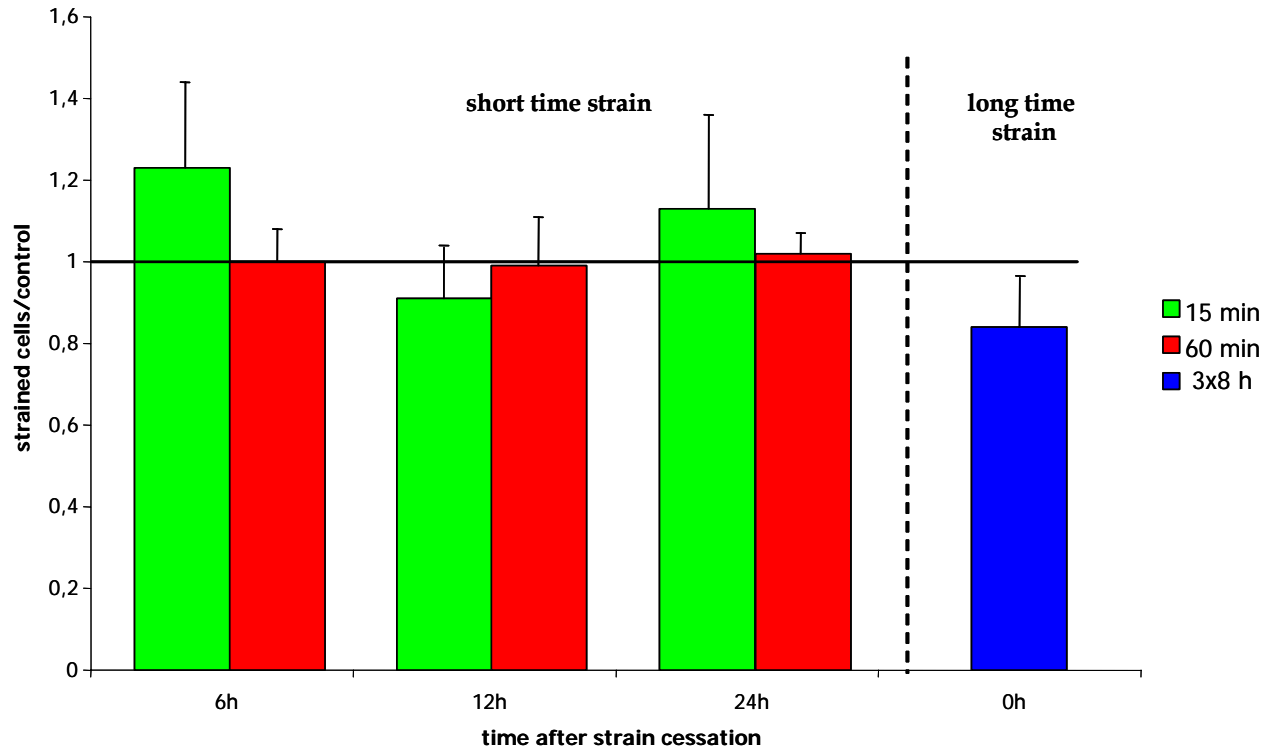
Fig. 4: Relative apoptosis rates of strained cells vs. static control cells. The line at 1 depicts the static control cells. Apoptosis was measured using Annexin V staining in flow cytometry. Strain was applied with a frequency of 1Hz, an amplitude of 5% and for the duration indicated.

Mineralization

After long time straining, cells showed significant mineralization. Static control cells displayed no mineralization at all (Fig. 5). After short time strain, no mineralization was observed.
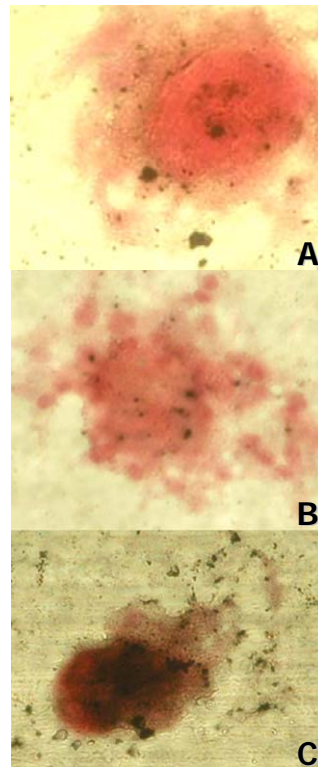
Fig. 5: Mineralization of the BMSC using von Kossa staining. A) static control BMSC cultivated in silicone dishes without any strain B) 60 minutes of cyclic longitudinal strain at 1Hz and an amplitude of 5% C) 3 times 8 hours of strain with 15 hours pause inbetween. The cyclic longitudinal strain imposed an amplitude of 5% at a frequency of 1Hz.

Western blotting

Activation of p38, ERK and JNK was determined only in long time strained cells. Phosphorylated p38 could not be detected. p38 was less expressed in strained cells compared to the static controls. ERK protein was increasingly expressed in strained cells compared to static control cells (Fig. 6). Phosphorylation rates of all detected proteins in strained cells were not significantly different from static control cells.
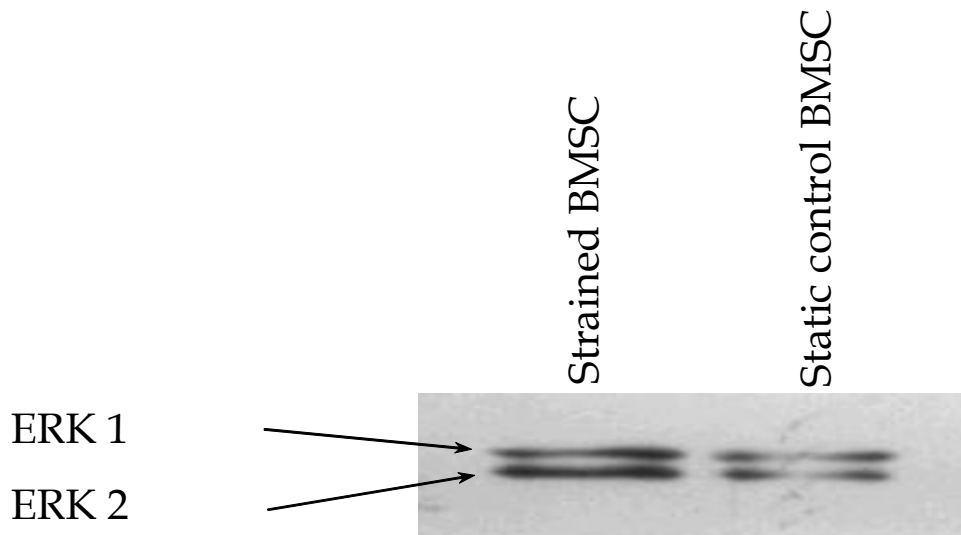
Fig. 6: Representative Western Blot of increased ERK 1 and 2 expression in cyclic longitudinally strained cells at a frequency of 1Hz with an amplitude of 5%.

Discussion

The significance of mechanical loading for bone metabolism has been demonstrated extensively in various studies. Mechanical passive states of the skeletal system due to weightlessness, functional immobilisation or prolonged post-operative bed rest have been shown to result in decreased bone formation and mineralization as well as reduced protein synthesis (1, 2). On the other hand, bone mass increases with increased skeletal loading (4, 5). In the mean time, the application of physical forces has entered the field of tissue engineering using electromagnetic fields, ultrasound or mechanical loading including pressure, fluid flow, torsion and tension. For bone tissue engineering, mechanical forces like linear stretching or pressure correlate most closely with the

physiological conditions and, therefore, are most widely used in connection with bone cells or bone like tissue. However, the ways of strain application vary widely according to substrate material and geometry as well as physical parameters like strain duration, elongation and frequency.

In order to differentiate BMSCs into osteblasts and additionally precondition them, BMSCs were strained on flexible silicone dishes in a cyclic longitudinal way. The applied frequency (1 Hz) and elongation (5%) were chosen in accordance with other studies that implied that these values are optimal (17, 39, 40). Since it has not yet been described what is the optimal strain duration, at first short time strain (15 or 60 min) was applied and the strained cells were tested for proliferation rates and mineralization.
15 min strain gave rise to a significant twofold increase of the proliferation rate 6 h after strain ended but 6 h later (12 h after strain end) the proliferation rate was back to normal. 60 min strain, however, did not yield an increased proliferation rate. Overall, 15 min short time strain appears to be the better strain duration yielding increased proliferation. However, all observed effects were only short-lasting and cell metabolism seemed to be back to pre-strain levels 12 – 24 h after the cessation of strain application. Application of repetitive strain regimens to fibroblasts are known to induce sustained increased proliferation rates with hardly any apoptosis (41). This protective effect was accounted far by the induction of HSP72 (41). Therefore, in the present study, repeated long time strain (3×8 h) was also applied.

Long time strained BMSCs showed a 40% reduction of proliferation rate that could be a sign for an advanced differentiation status. Proliferation rates are decreased during differentiation and terminal by differentiated cells often do not proliferate at all. Furthermore, FACS analysis after repeated long time strain, no increase in the percentage of either early apoptotic or of the late apoptotic cells. As a matter of fact, proliferation rates were even lower than those observed in static controls. In fact, earlier studies indicate an association between apoptosis rates and differentiation status. Weyts *et al.* induced apoptosis in osteoblasts by mechanical strain but observed decreasing

apoptosis rates with longer cultivation in osteogenic medium (42). Therefore, low apoptosis rate after repeated long time strain observed in this study may not only indicate the development of strain tolerance but also an advanced cell differentiation status. The increased differentiation of the BMSCs is strongly supported by the pronounced mineralization as detected by von Kossa staining.

We were not able to relate MAP kinase phosphorylation to any cellular reaction after repeated long time strain. Obviously BMSCs do not activate MAP kinases permanently but only for a short time to induce the subsequent reactions. Interestingly, low MAP kinase levels after repeated long time strain suggest increased MAP kinase degradation after numerous activations due to the applied strain. Thus, it is very well possible that the MAP kinases have been phosphorylated extensively. Further experiments will be carried out in order to investigate early MAP kinase activation. The increased expression of ERK in strained cells without phosphorylation should also be investigated.

In conclusion, this study shows that short time strain of up to 1 h does not lead to persistent induction of human BMSCs osteogenic differentiation. Thus, longer and/or repeated strain seems to be necessary in order to maintain a continuous differentiation stimulus. Moreover, this accustoms the cells to a mechanical active environment. Mechanical strain is a useful tool to help differentiate BMSCs to osteoblasts. Such differentiated cells can be seeded onto scaffolds and implanted to treat slow defects.

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