A SMALL SCALE CELL CULTURE SYSTEM TO ANALYZE MECHANOBIOLOGY USING REPORTER GENE CONSTRUCTS AND POLYURETHANE DISHES

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Abstract

Mechanical forces are translated into biochemical signals and contribute to cell differentiation and phenotype maintenance. Mesenchymal stem cells and their tissue-specific offspring, as osteoblasts and chondrocytes, cells of cardiovascular tissues and lung cells are sensitive to mechanical loading but molecules and mechanisms involved have to be unraveled. It is well established that cellular mechanotransduction is mediated e.g. by activation of the transcription factor SP1 and by kinase signaling cascades resulting in the activation of the AP1 complex. To investigate cellular mechanisms involved in mechanotransduction and to analyze substances, which modulate cellular mechanosensitivity reporter gene constructs, which can be transfected into cells of interest might be helpful. Suitable small-scale bioreactor systems and mechanosensitive reporter gene constructs are lacking. To analyze the molecular mechanisms of mechano-transduction and its crosstalk with biochemically induced signal transduction, AP1 and SP1 luciferase reporter gene constructs were cloned and transfected into various cell lines and primary cells. A newly developed bioreactor and small-scale 24-well polyurethane dishes were used to apply cyclic stretching to the transfected cells. 1 Hz cyclic stretching for 30 min in this system resulted in a significant stimulation of AP1 and SP1 mediated luciferase activity compared to unstimulated cells. In summary we describe a small-scale cell culture/bioreactor system capable of analyzing subcellular crosstalk mechanisms in mechanotransduction, mechanosensitivity of primary cells and of screening the activity of putative mechanosensitizers as new targets, e.g. for the treatment of bone loss caused by both disuse and signal transduction related alterations of mechanotransduction.

Keywords: Mechanical strain, mechanosensitive reporter gene constructs, bioreactor.

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Introduction

Mechanical forces stimulate cell differentiation and control/maintain tissue function via membrane associated ”mechanoreceptor” mechanisms, activation of second messengers and downstream gene regulation in various mammalian systems like musculoskeletal and cardiovascular tissues (Hahn and Schwartz, 2009; Jani and Schock, 2009; Papachroni *et al.*, 2009). Cell adhesion molecules like integrins, membrane associated receptors and strain sensitive channels have been described to transmit mechanical forces to intracellular structures like the actin cytoskeleton and second messengers like calcium flux (Asparuhova *et al.*, 2009; Huveneers and Danen, 2009; Kiselyov and Patterson, 2009; Puklin-Faucher and Sheetz, 2009; Sharif-Naeini *et al.*, 2010). Mechano-biochemical transformation in single cells is elicited by the stimulation of ERK1/2 kinase phosphorylation cascades, activation and nuclear translocation of transcription factors and modulation of gene expression (Khatiwala *et al.*, 2009; Klossner *et al.*, 2009; Liu *et al.*, 2009; Rangaswami *et al.*, 2009; Young *et al.*, 2009).

As an immediate early effect c-fos is rapidly induced in many systems and probably contributes to steadily increasing signal intensity via activation of the c-fos/jun alias AP1 transcription factor complex. Promoters comprising AP1 response elements have been shown to respond to mechanical strain, as were elements comprising binding sites for SP1 or NFkB (Liedert *et al.*, 2009). A consensus shear stress responsive element SSRE (GAGACC) has been described to be activated especially in systems related to the vasculature (Inoue *et al.*, 2004; Kletsas *et al.*, 2002; Liedert *et al.*, 2006b; Liedert *et al.*, 2009; Miyagi *et al.*, 2005; Ogasawara *et al.*, 2001; Peverali *et al.*, 2001; Silberman *et al.*, 2009; Sumpio *et al.*, 1997).

Mechanotransduction contributes to physiology in that it mediates tissue modeling according to environmental and life style requirements and this applies especially to mesenchymal tissues. Deficient or enhanced mechanotransduction/mechanosensitivity may cause pathology. Osteoporosis is a disease, which is usually caused by a mixture of pathogenetic factors like disuse of the musculoskeletal system, altered bone quality and serious alteration of mechanotransduction processes, which stimulate bone modeling and remodeling according to mechanical loading. A series of genetic as well as age-related factors may translate this susceptibility into

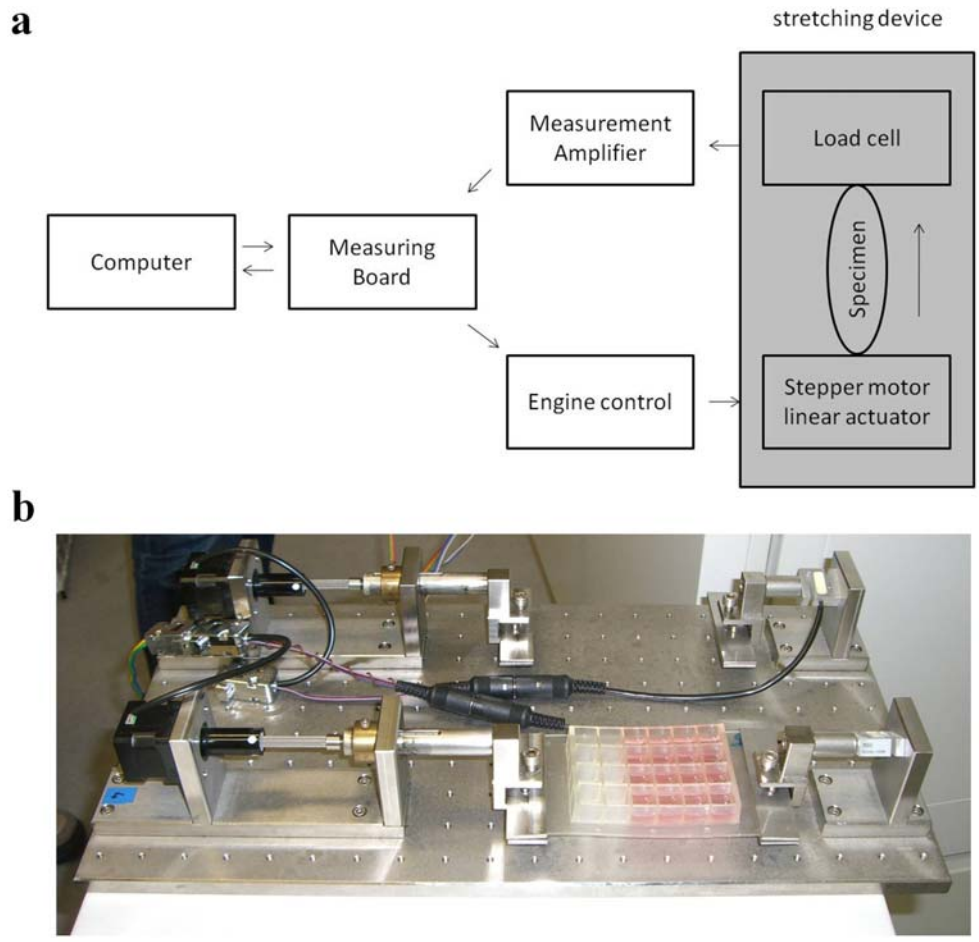

Fig. 1. (a) Schematic illustration of all bioreactor components. **(b)** Photograph of the newly developed bioreactor. The picture represents the grey shaded area of Fig. 1a.

inadequate bone loss and fragility fractures. The molecular mechanisms, which translate mechanical forces into biochemical signals and their alterations in disease are only partially known (Judex *et al.*, 2009). There are solid data to assume that loss of sex hormone production is a major contributor especially in postmenopausal osteoporosis. Estrogens according to epidemiological data are bone mechanosensitizers, since girls in puberty start to enhance bone mass albeit they do not increase muscle mass accordingly. The molecular basis of this interaction seems to be the interaction of estrogen signaling and the wnt/frz pathway (Armstrong *et al.*, 2007; Liedert *et al.*, 2010).

To further unravel the molecular mechanisms of mechanotransduction in health and disease, the subcellular level of alterations and the respective genomic and proteomic changes, adequate test systems are needed allowing for robust, sensitive, reproducible analyses, which can be performed in a higher throughput in comparison to presently available systems. Moreover experiments using primary cells require material sparing setups. We present here miniaturized 24-well polyurethane dishes with homogenous characteristics for cyclic strain application and a newly designed bioreactor system suitable for applying shear stress to cell cultures. As AP1 and SP1 response elements are mediating mechanical strain in signal transduction cascades these elements were chosen and cloned into luciferase reporter vectors, which can be transfected or lentivirally transduced into various cell lines and primary cells from patients.

Materials and Methods

Bioreactor design and production

A multifunctional bioreactor was constructed allowing for flexible resistance adopted cyclic stretching protocols of tissue samples/tissue engineering constructs in media flasks and flexible cell culture dishes (Fig. 1a). A variable fixation device allowed for appropriate anchorage of different specimen within the bioreactor (Fig. 1b). To ensure a high variability in applications, the whole system is build up modularly in a way that the different components of the reactor e.g. the driving module and the force measurement module can be positioned variably on a perforated board with a drilling hole distance of 25 mm. The propulsion module is equipped with an electric linear stepper motor (Series 35000, size 14) and the respective engine control (DCM 8028, both Haydon, Waterbury, CT, USA). The actuator can provide a maximum traction force of 100 N at a maximum speed of 12.2 mm/sec. The force measurement module consists of a load cell (KD 24S-100N) and a measurement amplifier (GSV 11H, both ME Messsysteme, Henningsdorf, Germany), which can detect forces up to 100 N with a resolution of ± 0.1 N. Force sensor and propulsion module / actuator are placed opposite to one another on the perforated plate. The reactor is connected to a computer, which drives the propulsion module and records the forces sensed by the force measurement module during the stretching process. Potential amplitude of the stepper motor ranges from 0 to

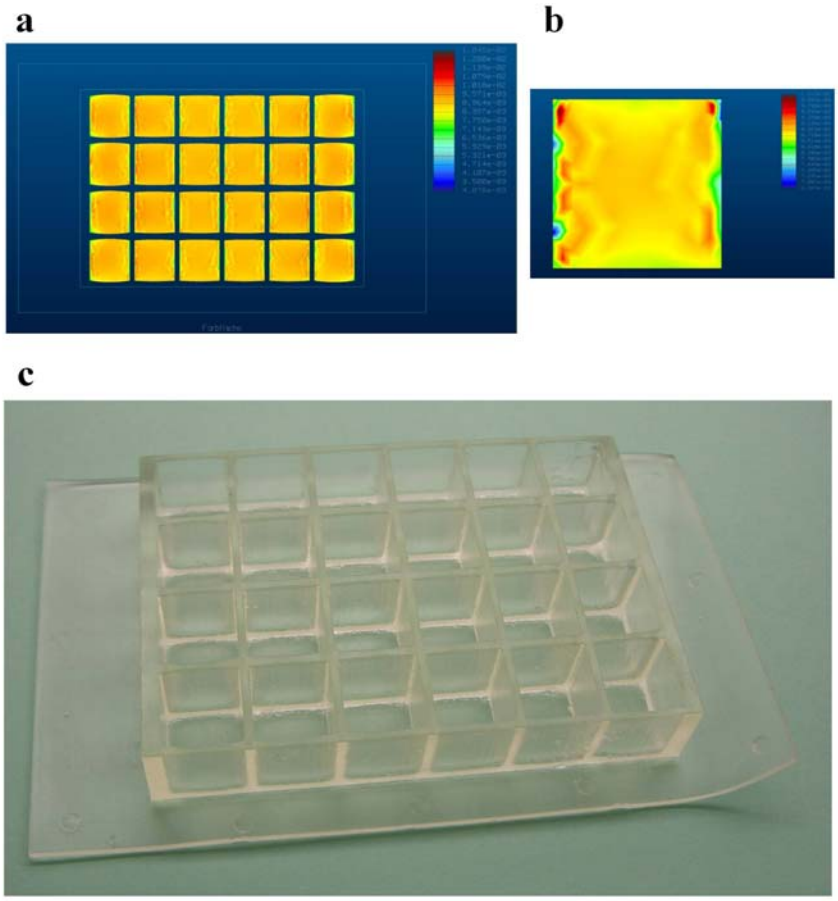

Fig. 2. Finite element analysis of **(a)** a complete 24-well PU dish and **(b)** one single well. Identical colors represent equal extension of the PU material after 1% cyclic strain application. Red color represents high extension while blue color represents low extension; **(c)** Photograph of one newly developed polyurethane dish.

38 mm. Velocity of the excursion can be modified continuously up to 12 mm/s. Considering these technical specifications, the reactor in combination with the flexible PU dishes described below theoretically enables mechanical stimulation with a maximum strain of about 25% (250,000 με) at 0.5 Hz and a maximum frequency of about 6 Hz with 0.5% (5000 με) strain. The modular configuration of the reactor together with the variable fixation device enables mechanical stimulation of specimen with a length from 15 to 150 mm and a width of up to 85 mm. For secure fixation of the flexible PU culture dishes, a chucking device with coplanar, roughened clamping plates, covering the whole breadth of the dishes was used (Fig. 1b). The bioreactor is designed to operate in a conventional incubator under cell culture conditions (37°C, 5 % CO_2, humidity 95 %). All metal parts can be sterilized by spray disinfection.

Measurement and control program

The process of mechanical stimulation and data acquisition is controlled by an individually programmed graphical user interface based on the commercial software LabView (National Instruments Germany, München, Germany). Signals to the actuator and from the measurement module are integrated on a measuring board (cDAQ 9172, National Instruments Germany), which is physically connected to the computer with a conventional USB-cable. Thereby, the complete process of stimulation as well as data acquisition is accomplished by the software. The user interface based on the software allows for an independent controlling of multiple stretching devices. This setting enables application of preassigned stretching protocols with chancing parameters over time, e.g. variation of the oscillating stretching parameters such as amplitude and frequency and the setting of braking rates and pausing at the turning points of oscillation without human intervention. Implementation of a feed-back loop, which continuously integrates the information detected by the force sensor allows for technical surveillance of the actual forces exerted on the specimen, thereby permitting calibration of the system by definition of a reproducible construct preload as starting point for the experiments. Continuous automatic monitoring of the actual values additionally allows – if necessary – adaption of stimulation parameters by the computer in order to ensure constant application of the predefined values for stimulation. Based on data obtained from the sensor, actual forces and current elongation are continuously displayed by two graphs, permitting monitoring of process flow by the user. All data monitored are recorded and can be comprehensively analyzed after the experiment.

Design and fabrication of cell culture dishes

Design of the multi-well cell culture dishes for mechanical stimulation was aimed at equal strain distribution, both within each single well and between the different chambers across the whole culture dish (Fig. 2a and b). Therefore, construction including FEM calculations was performed with the software ProEngineer Wildfire 2.0 (Parametric Technology Corporation, PTC, Needham, MA, USA). Tetrahedrons and triangles were used as finite elements. Stimulation parameters used for FEM calculation were 1

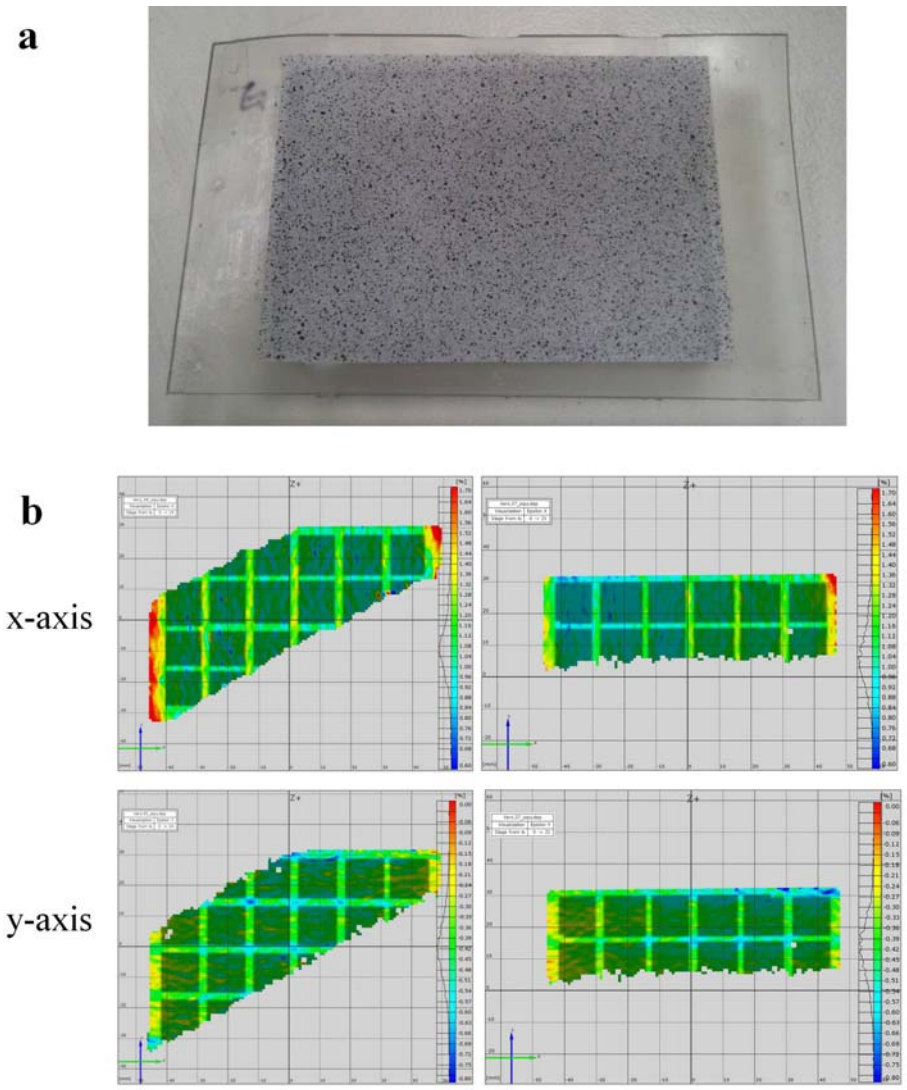

Fig. 3. (a) Stochastic surface pattern applied to the back side of a 24-well PU dish. A representative picture is shown, **(b)** Optical measurement of strain distribution along the x- (top) and the y-axis (bottom). Two representative experiments are shown. Identical colors represent equal extension of the PU material after 1% cyclic strain application.

Hz and 1% (10.000 με), respectively. Calculations were based on the material properties of elastic polyurethane as basic material for the culture dishes.

For production, the thickness and profile of interior and exterior walls was adapted according to previous FEM calculations. Length of the dishes is 130 mm, width 80 mm, surface area of each single tray is 1.7cm² (Fig. 2c). A 3D-CAD engineering drawing of the culture dish was used for production of a rapid prototyping model made of a photoreactive polymer (epoxide resin) by stereolithography (Raprotec, Remscheid, Germany). This model served as archetype to cast a silicon-based female mould, which allows for serial production of the final polyurethane-based dishes by a vacuum casting process. Production was realized with the vacuum casting device (MCP 4/01) and an elastic two-component polyurethane material (both MCP HEK Tooling, Lübeck, Germany; meanwhile MTT Technologies Ltd., Staffordshire, United Kingdom, Data sheets 8020-1, 8020-2) with a final tensile E-modulus of 6 MPa, a tensile strength of 8 MPa and a tear strength of 12 MPa. By this mode of production, in total 25 cell culture dishes can be produced from one female mould with a maximum deviation caused by production of 0.2 %. To prevent microbial colonization, cell cultures dishes were autoclaved before use and protected by a customized lid made of polystyrene.

Optical measurement of strain distribution

Optical analysis of strain distribution across the culture dishes was accomplished using the System Aramis 2M (GOM, Braunschweig, Germany) with an optical resolution of 1600 x 1200 pixels. For visualization of the relative movement at the ground of the culture dishes, a stochastic surface pattern was applied to the back side surface, using a matt white and a graphite spray (Fig. 3a). Measuring on the back side proved to be more accurate, since strain distribution and component characteristics on the front side are identical to those at the back side and there are no dividing walls between the trays that could impair the quality of optical measurements. The two cameras of the Aramis 2M system were calibrated by identifying 13 predefined positions on the calibration plate supplied by the manufacturer. During measurement, the cameras continuously took pictures at an image repeat rate of 12 Hz. To enable exact determination of the maximum strain, image repeat rate has to be 30 times higher than the oscillating frequency. Therefore, cyclic stretching for optical measurement was performed with 1% strain but

with at an oscillating frequency of 0.35 Hz. Comprehensive analyses of the pictures allowed for a 3-D view of the surface. All kinds of deformations could be seen as a displacement of the characteristic pattern. Digital image correlation via the Aramis software system allowed for computer-based analysis and quantification of the displacements. Pictures were separately analyzed for each axis (x and y) of the coordinate plane (Fig. 3b). Statistical analysis comparing independent tests as well as a possible influence of the spatial distribution of individual wells with regard to the 4 different rows and 6 columns of the dishes was performed using analysis of variance (ANOVA).

Cell culture

Media for cell culture and FCS were obtained from PAA Laboratories, Linz, Austria. Telomerase immortalized human mesenchymal stem cells (hMSC-TERT) were cultivated as described (Simonsen *et al.*, 2002) in Earle's MEM containing 10 % FCS. Primary human mesenchymal stem cells (hMSC) were isolated from bone marrow from different donors and cultivated by a standardized protocol (Noth *et al.*, 2002). Bone marrows were recovered after informed consent from the explanted femoral heads of patients undergoing elective hip arthroplasty. The procedure was approved by the local Ethics Committee of the University of Würzburg. Briefly, bone marrow preparations were washed with Dulbecco's modified Eagle's medium, (DMEM/F12) supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 µg/ml ascorbate (Sigma-Aldrich, München, Germany), and centrifuged at 1200 rpm for 5 min. The pellet was reconstituted in medium and washed four times, and the supernatants of the washing steps containing the released cells were collected. Cells were centrifuged and cultivated at a density of 3 x 10^8 cells per 150 cm^2 culture flask. Adherent cells were washed after 2 days and cultivated until confluence. HEK293 cells (ATCC No. CRL-1573) were grown in Dulbecco's modified Eagle's medium (DMEM high glucose) containing 10 % FCS. All cells were grown at 37°C in a humidified atmosphere consisting of 5 % CO_2 and 95 % air.

Cloning of AP1 and SP1 responsive constructs

For functional analyses oligonucleotides containing AP1 and SP1 responsive elements (AP1: 5'-ATC **TGA CTC AG**C ATG CAT G**TG ACT CAG** CTA-3'; SP1: 5'-ATC **GCG GCG GGG GCG GGC GCC G**CA TGC ATG **GCG GCG GGG GCG GGC GCC G**CT A-3') were cloned in between the EcoRI sites of the pCRII vector (Invitrogen GmbH, Karlsruhe, Germany). Both, the AP1 and the SP1 sites were subcloned into the luciferase reporter vector pGL4.14[luc2/Hygro] (Promega, Mannheim, Germany) via SacI and XhoI restriction (all restriction endonucleases from New England Biolabs, Frankfurt am Main, Germany) to receive the pGL4.14-SP1 vector and via XhoI and BamHI restriction to receive the pGL4.14-AP1 vector and sequenced with the RVprimer3 (Promega) by dye terminator sequencing using the ABI 310 capillary sequencer as described previously. Constructs were used for transient transfection of HEK293 cells and for establishing stable hMSC-TERT cells lines.

Establishing stable hMSC-TERT-AP1 and hMSC-TERT-SP1 clones

The vectors pGL4.14-AP1, pGL4.14-SP1 and pGL4.14[luc2/Hygro] were linearised with SacII. 1 x 10^6 hMSC-TERT cells were mixed with 10 µg DNA in 400 µl serum-free medium in 2 mm wide cuvettes (Bio-Rad Laboratories, München, Germany) and transfected by electroporation at 120 V and 950 µF using a Gene Pulser 2 (Bio-Rad Laboratories GmbH). Cells were seeded into two wells of a 6-well plate and cultured overnight in serum-containing medium. 24 h post transfection 50 µg/ml hygromycin was added to select positive hMSC-TERT-AP1, hMSC-TERT-SP1 and hMSC-TERT clones containing the empty pGL4.14[luc2/Hygro] vector (hMSC-TERT-control). For cyclic stretching 3 x 10^4 cells were seeded per well on 24-well PU plates and cultivated for 48 h.

To control stabile integration genomic DNA was isolated from stable hMSC-TERT cells using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was performed with 50 ng genomic DNA using a PTC-200 Peltier thermal cycler (Bio-Rad Laboratories) in a volume of 50 µl (forward primer: RVprimer3 localized from base 5790 to base 5809; reverse primer 5'-TCATGGCTTTGTGC AGCTGC-3', localized from base 171 to base 190 of the pGL4.14[luc2/Hygro] vector). Primer oligonucleotides were purchased from Biomers (Ulm, Germany). Genomic DNA was amplified with Taq-polymerase purchased from Peqlab Biotechnologie (Erlangen, Germany) PCR bands were sequenced with the RVprimer3 as described above.

Cloning of the lentiviral luciferase reporter construct

As no suitable luciferase reporter vector for lentiviral transduction of primary cells was available on the market, we had to establish a construct, which fulfils our needs. First of all the SP1 and the AP1 responsive elements were cloned into pENTR™5'-TOPO, respectively, the coding sequence of the firefly luciferase was cloned into the KpnI and NotI sites of the pENTR™11 vector. The empty pENTR™5'-TOPO vector was used for generating the control plasmid. The pENTR™11-luciferase and the pENTR™5'-TOPO-SP1, pENTR™5'-TOPO-AP1 or the pENTR™5'-TOPO construct, respectively were recombined with the destination vector pLenti6/R4R2/V5-Dest (all vectors from Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions to receive the lentiviral luciferase SP1 reporter vector pLenti-SP1, the lentiviral luciferase AP1 reporter vector pLenti-AP1 and the control vector pLenti-0, which contains the luciferase coding sequence but no mechanoresponsive element.

Transient transfection of HEK293 cells

For transient transfection, HEK293 cells were seeded in the 24-well PU dishes (1.8 x 10^4 cells/well) and transfected at 50-70% confluence. Liposome-mediated transfection was performed using LipofectAMINE reagent (Invitrogen). 2 µl of LipofectAMINE and 0.9 µg of the pGL4.14-AP1 reporter construct and the empty pGL4.14[luc2/Hygro] vector, respectively were diluted in 100 µl serum-free medium and incubated for 30 min. 124

µl serum-free medium was added, and the solution was then carefully dripped onto the cells. HEK293 cells were incubated for 5 h in transfection solution. After removal of the medium cells were cultivated with serum-containing medium. After 24 h the cells were stimulated by cyclic stretching.

Lentiviral transduction of primary mesenchymal stem cells

After cotransfection of the lentiviral reporter constructs pLenti-SP1, pLenti-AP1 and pLenti-0 into 293FT cells by using the ViraPower™ Packaging Mix (all Invitrogen), 5 ml supernatant was mixed with 10 ml basal medium and used to transduce primary hMSC seeded in 75 cm^2 culture flasks at 50 % confluency. After 4 days 2 µg / ml blasticidin was added to select stably transduced cells. After 5 medium changes (2 – 3 weeks) cells were detached from the culture flasks, counted and 3 x 10^4 cells / 1.7 cm^2 well were seeded on the flexible PU dishes.

Cyclic stretching of HEK293, hMSC-TERT and primary mesenchymal stem cells and luciferase assay

Cyclic stretching was performed for 30 min at 0.05 to 2 Hz and 0.5% to 5% extension. To analyze AP1 signaling specificity, hMSC-TERT-AP1 cells were pre-treated before stretching for 2 days with or without 5 µM verapamil (Sigma Aldrich, Munich, Germany), an calcium channel blocker. After stretching and cultivating the cells for another 24 h, cells were harvested and lysed in 150 µl Reporter Lysis Buffer (Promega). 20 µl of each extract was analyzed for luciferase activity using the reporter gene assay provided by Promega GmbH in an Orion II Luminometer (Berthold Detection Systems, Pforzheim, Germany) in 96-well plates. Relative light units were normalized to protein content determined by using RotiQuant Protein Assay (Carl Roth, Karlsruhe, Germany) and the data from four wells were used to calculate the mean value.

Proliferation assay

3 x 10^4 hMSC-TERT-AP1 cells / well were seeded on the newly developed 24-well PU dishes and on conventional PS 24-well plates and the proliferation ratio was analyzed by using the WST-1 cell proliferation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 100 µl WST-1 reagent was added after 1, 3 and 6 days to 1 ml culture medium and incubated for 30 min in a cell incubator. 100 µl were transferred to a 96-well plate and absorption was analyzed at 450 nm (reference wavelength 620 nm) with a Tecan sunrise microplate reader and the Magellan V6.6 software (Tecan Group, Männedorf, Switzerland). Experiments were repeated three times and were performed in triplicates.

RT-PCR analysis of mechanoresponsive HB-GAM

HMSCs cells were seeded on PU dishes in a density of 3 x 10^4 cells / cm^2 and cyclic stretched for 30 min at 1 Hz and 1 % extension. Total RNA was immediately isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Two micrograms of total RNA were reverse-transcribed with MMLV reverse transcriptase (Promega GmbH) in a volume of 20 µl. For amplification of mechanoresponsive HB-GAM (primers HB-GAM sense (5'-GCAAACCATGAAGACCCAGA-3'), HB-GAM antisense (5'-GGCTTGGAGATGGTGACAGT-3'), annealing temperature 60°C) (Neidlinger-Wilke *et al.*, 2009) 1 µl of cDNA was used as a template in a volume of 50 µl. Taq DNA polymerase was obtained from Peqlab (Erlangen, Germany). PCR conditions were as follows: 30 seconds at 94°C, 30 seconds at annealing temperature, 60 seconds at 72°C. As a housekeeping gene EF1a was amplified (primers: EF1a sense (5'-AGGTGATTATC CTGAACCATCC-3'), EF1a antisense (5'-AAAGG TGGATAGTCTGAGAAG-3'), annealing temperature 54°C). Experiments were performed with three different preparations of primary hMSC. PCR bands were semiquantitatively analyzed by densitometry measurements using the Bio1D software (Vilber Lourmat, Eberhardzell, Germany).

Membrane staining with concanavalin A

HMSC-TERT-AP1 cells (3 x 10^4 cells/cm^2) were cultivated for 3 days on PU dishes. Then, the cells were fixed with 4% PFA and permeabilized with 0.2% Triton X-100. Membrane-staining dye, 15 µg/ml concanavalin A-Texas Red (Invitrogen GmbH) was applied in PBS for 30 min at 4°C. After washing with PBS, nuclear counter staining with DAPI (Invitrogen) was performed for 5 min at room temperature. Cells were photomicrographed with an AxioCam MRm camera on an Axiovert S100 microscope (Carl Zeiss). Three independent stainings were performed.

Results

Finite element analysis of polyurethane cell culture dishes

Different types of 24-well culture dishes regarding shape and assembly of the individual trays were planed and strain distribution analyzed by FEM calculation (Fig. 2a). Eventually, a construction of 24 contiguous, rectangular wells with selective modifications of the thickness and shape of distinct partition walls in conjunction with a chucking device covering the full breadth of the dishes proved to provide best possible approximation to a largely homogenous strain distribution within each tray as well as between the trays at different positions on the 24-well culture dish (Fig. 2a and b).

Optical measurement of strain distribution

Strain values and there spatial distribution throughout the PU culture dishes were measured optically employing the complete bioreactor system as used for cell culture experiments to allow for a comprehensive analysis of the actual strain values comprising all potential error sources. Only those areas of the dish, where the fineness of the stochastic pattern was sufficient to allow valid analysis were used for calculation of strain distribution (Fig. 3b). In order to allow unbiased evaluation, no technical measures for exponential smoothening were applied so

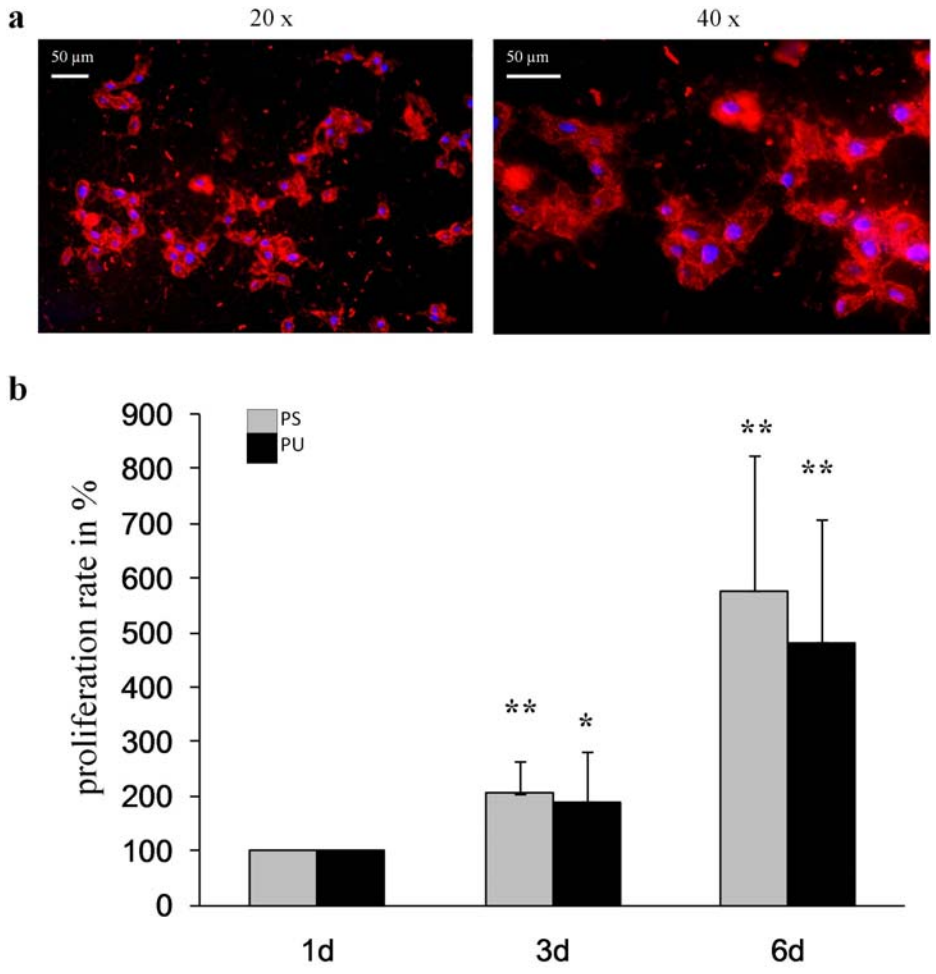

Fig. 4. (a) hMSC-TERT-AP1 cells grown on PU dishes and stained with concanavalin A (red) and counterstained with DAPI (blue). The bar represents 50 µm. **(b)** Proliferation rate of stable hMSC-TERT-AP1 cells grown on conventional polystyrene (PS, grey bars) and on polyurethane (PU, black bars) dishes for 1, 3 and 6 days without any mechanical stimulation. The data were obtained from three measure points from three independent experiments and are shown as means ± SEM. Statistics were calculated with the Student's *t*-test (**: $p<0.001$, *: $p<0.01$).

areas, where the stochastic pattern was too rough were strictly excluded. In single experiments, only partial aspects of the graphite spray pattern met these technical requirements and gave valid results. As described in the materials and methods section, the strain applied along the x-axis was 1%. Evaluation was focused on single trays. In total five independent experiments comprising 52 individual wells with 84 to 256 (average 207.6) measuring points per well were evaluated. The average strains of the single wells varied between 0.907 % and 1.132 % with the average value of the means being 1.043 %. Standard deviation of the means was calculated as 0.047 %. Mean standard deviation was 0.090 % (min 0.059 %, max 0.147 %). The minimum-values of all wells analyzed varied between 0.645 % and 0.945 %, respectively with a mean minimum at 0.791 %. Regarding all trays, average maximum was 1.32 % (min 1.146 %, max 1.524 %). The average range between maximum and minimum was 0.528 %. Analysis of variance of the independent experiments showed no significant difference with $p = 0.35$ and $F = 1.13$ (critical value 2.57). Statistical comparison (ANOVA) of the arithmetic means of the four different rows ($p = 0.26$; $F = 1.37$, critical value 2.80) and the six columns ($p = 0.87$; $F = 0.87$, critical value 2.42) also did not show any significant differences. Accordingly, visual analysis of the pattern of strain distribution did not show any significant pattern of areas with increased or alleviated strain. Deformation along the y-axis perpendicular to the intended loading direction is transverse contraction, thus strain values measured were negative. The arithmetic mean of transverse contraction over all wells was -0.416 %. Mean values of the individual wells ranged from -0.328 % to -0.474 %. Again, no obvious pattern of inhomogeneous strain distribution was observed and statistical comparison (ANOVA) of the arithmetic means of the four different rows ($p = 0.16$; $F = 1.79$; critical value 2.80) and the six columns ($p = 0.48$; $F = 0.91$, critical value 2.42) also did not show any significant differences. Thus strain can be supposed to be independent of the spatial distribution of the wells on the culture dish.

Cell growth and adhesion characteristics of cells in PU dishes

Since the PU material is not translucent and did not allow for direct microscopic control of cell cultures, populations of cells were stained with concanavalin A and counterstained with DAPI. Sufficient growth and spreading was observed during day 1-6 (Fig. 4a) until after seven days in culture the cell layer started to detach. In consequence, any stretching experiment was designed for culture in PU dishes of less than 7 days and the number of cells seeded was 3 x 10^4 / well to achieve sufficient cell-cell contacts and to get the cells as closely as possible to confluence by the start of stretching experiments. To exclude any influence of the PU material on cellular growth, hMSC-TERT-AP1 cells were seeded on

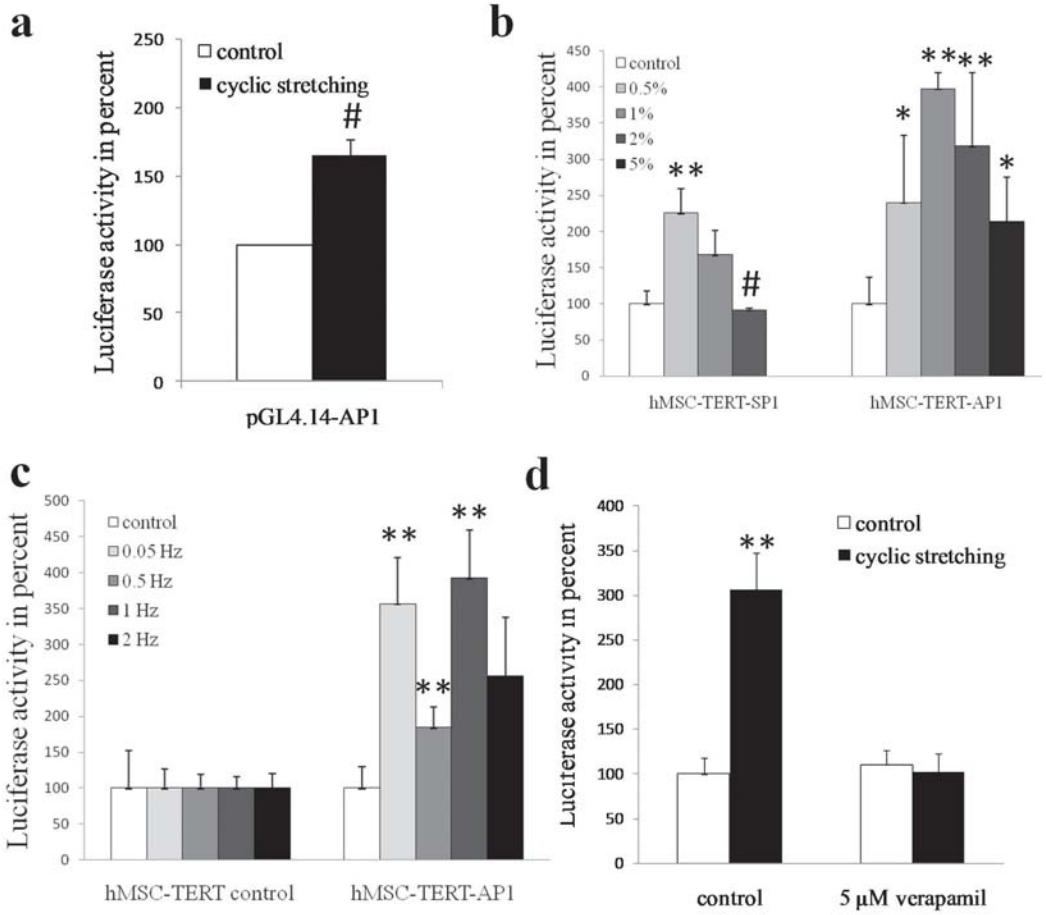

Fig. 5. Luciferase activity of strain responsive reporter constructs. **(a)** Transient transfection of the pGL4.14-AP1 reporter construct into HEK293 cells and stimulation of cells by cyclic stretching with 1% extension and 1 Hz (black bar). The white bar represents the unstimulated control. **(b)** Stable hMSC-TERT-AP1 and hMSC-SP1 cells were stimulated with variable extensions but a constant frequency of 1 Hz. **(c)** hMSC-TERT-AP1 cells were stimulated with variable frequencies but a constant extension of 1%. **(d)** hMSC-TERT-AP1 cells were treated with the calcium channel blocker verapamil and stimulated by cyclic stretching with 1% extension and 1 Hz (black bars). The white bars represent the non-stretched control. All data were obtained from three independent experiments with four measure points each and are shown as means ± SEM. Statistics were calculated with the Student´s *t*-test (**: $p<0.001$, *: $p<0.01$, #: $p<0.05$).

conventional PS 24-well plates and on the here described 24-well PU dishes and proliferation was determined with the WST-1 assay after 1, 3 and 6 days in comparison without any mechanical stimulations (Fig. 4b). Proliferation increased in cells grown on either surface, PS (grey bars) or PU (black bars), from day 1 to day 6, which excluded any unfavorable impact of the PU material on cellular viability and growth although the proliferation rate was lower on PU compared to PS after 6 days.

Establishing of mechanoresponsive AP1 luciferase reporter constructs

To test if the newly established PU dishes are suitable for applying mechanical strain to cells and if the strain responsive luciferase reporter constructs are responding to mechanical stimuli HEK293 cells were exemplarily transiently transfected with the pGL4.14-AP1 construct and the pGL4.14[luc2/Hygro] vector as a control. After cyclic stretching luciferase activity was induced nearly 2-fold in pGL4.14-AP1 transfected cells (Fig. 5a, black bar) compared to control cells (white bar). The pGL4.14[luc2/Hygro] transfected control cells did not respond to cyclic stretching (data not shown).

Characterization of stable mechanoresponsive AP1 and SP1 cell lines

After we received the proof-of-principle that the construct as well as the PU dishes are suitable for analyzing mechanical strain applied to cell monolayers we established stable hMSC-TERT-AP1 and hMSC-TERT-SP1 cells, which were stimulated with variable extensions from 0.5% to 2% (SP1 clone) and 0.5% to 5% (AP1 clone), respectively. The SP1 construct showed the highest responsiveness at an extension of 0.5%, a declining activity at 1% extension and no effect at 2% strain (Fig. 5b, left panel), while the AP1 construct was most responsive at 1% extension and showed a lower induction when amplitudes of 0.5%, 2% and 5% were applied (Fig. 5b, right panel). The hMSC-TERT-control cells were not responsive to mechanical strain (data not shown).

Additionally, different frequencies starting from 0.05 Hz to 2 Hz were applied to hMSC-TERT-AP1 cells and luciferase activity was determined. The AP1 responsive element was strongly induced at 0.05 Hz and 1 Hz, while other applied frequencies displayed a lower induction of luciferase activity (Fig. 5c). To validate the specificity of AP1 signaling, cells were pre-treated for 2 days with the

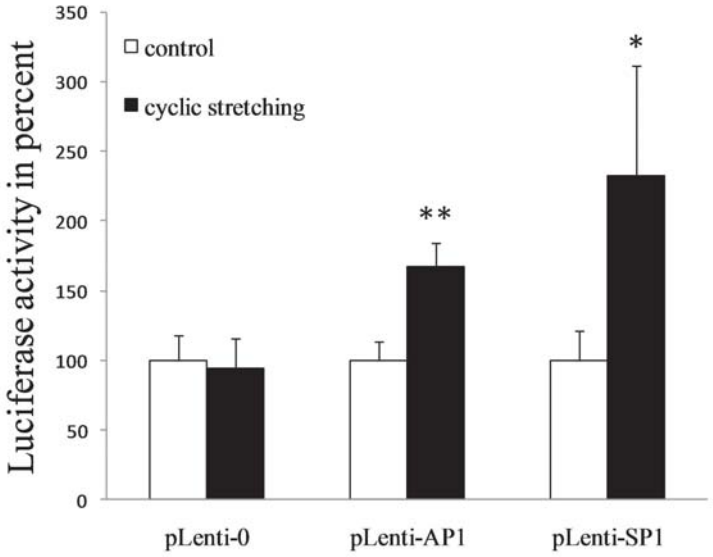

Fig. 6. Lentiviral transduction of primary mesenchymal stem cells with the newly developed luciferase reporter vectors. pLenti-AP1, pLenti-SP1 and pLenti-0 luciferase constructs were transduced into hMSC and cells were stimulated by 1% and 1 Hz cyclic stretching (black bars). White bars represent unstimulated controls. The data were obtained from 4 different measure points from four independent experiments and are shown as means ± SEM. Statistics were calculated with the Student's *t*-test (**: $p<0.001$, *: $p<0.01$).

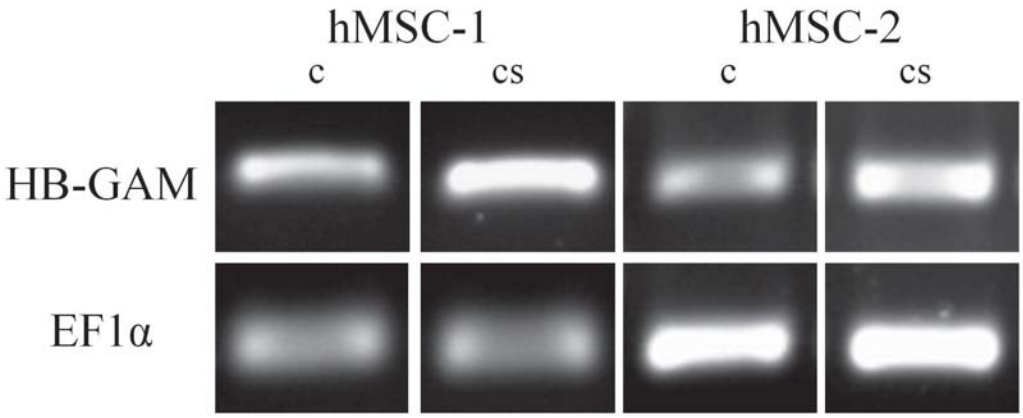

Fig. 7. Expression of the mechanoresponsive gene HB-GAM in hMSC after cyclic stretching. As a housekeeping gene EF1α was amplified. Two representative experiments are shown.

calcium channel blocker verapamil, which completely abolished the AP1 mediated mechanoresponsivity in hMSC-TERT-AP1 cells (Fig. 5d). No effect of verapamil was seen in hMSC-TERT control cells (data not shown).

Mechanosensitivity of primary human mesenchymal stem cells

The coding sequence of the firefly luciferase, the SP1 and the AP1 responsive elements were cloned into a lentiviral expression vector (pLenti-SP1 and pLenti-AP1) to have the opportunity to analyze mechanical strain in primary mesenchymal stem cells. After applying mechanical stimuli to pLenti-AP1 and pLenti-SP1 transduced primary hMSC luciferase activity was enhanced 1.6-fold and 2.3-fold, respectively, while cells transduced with the empty vector pLenti-0 did not respond to mechanical strain (Fig. 5).

Regulation of the mechanoresponsive gene HB-GAM

To further analyze if the here described bioreactor and the PU dishes are suitable for mechanical stimulation three different preparations of primary mesenchymal stem cells were stimulated with cyclic stretching and the expression of the mechanoresponsive gene HB-GAM was analyzed by RT-PCR in three different hMSC preparations. Bands were densitometrically evaluated. The expression of HB-GAM was significantly upregulated 1.7-fold (SEM ± 0.24; $p < 0.05$) by cyclic stretching although a high donor variability was observed (Fig. 7).

Discussion

In this work we describe a newly developed bioreactor suitable for applying 2-D mechanical strain to cellular monolayers grown in small scale formats on polyurethane surfaces. A variety of bioreactor systems capable of applying mechanical strain to 2-D and 3-D cell cultures were developed and are reviewed by Brown (Brown, 2000). Mechanical strain can be applied to cells by fluid shear stress (Wang *et al.*, 2010), by using compression systems (Bougault *et al.*, 2008) or by bending circular substrates (Cha and Purslow, 2010). One commercially available cellular strain device (FX-4000 T, Flexcell International Corporation, Hillsborough, NC, USA) was extensively characterized by the group of G. Duda (Bieler *et al.*, 2009) in terms of strain distribution across the surface

of the culture dishes by digital image correlation techniques (Cottrell *et al.*, 2009). The authors showed that only cells adhering to a central area 1.8 cm in diameter are subjected to homogenous mechanical strain while cells surrounding this area receive higher strain extensions. It would result in misleading impressions in terms of mechanically induced effects if cells were pooled across the whole well area. These findings demonstrate that there is a need for cell culture dishes, which have a homogenous strain distribution across the whole well surface when subjected to mechanical strain. Our newly developed PU dishes are intended to fulfill this criterion as we demonstrated by finite element analysis and optical measurements. We have previously described and applied a reactor system using 18 cm^2 silicone dishes (Liedert *et al.*, 2006a; Liedert *et al.*, 2009), which we now adapted for small scale applications. Moreover our 24-well format well-design combined with the sensitive luciferase readout allows for small volume analyses with comparably high throughput, which is of specific importance when precious primary cell cultures are analyzed. Additionally, the 24-well format of our PU dishes allows for stimulating multiple samples simultaneously, which is beneficial in terms of identical culture and stimulation conditions. Cells showed reasonable adherence to the newly developed PU dishes demonstrated by concanavalin A staining and proliferation rates comparable to rates obtained from cells grown on conventional PS.

The newly developed reactor and the PU dishes are suitable for stimulating primary cells and cell lines with mechanical load. In order to get a sensitive and reliable readout for the effects of strain application we tested a self-established reporter gene assay comprising known mechanosensitive response elements, which control luciferase expression. By using these luciferase-based mechanoresponsive reporter constructs we could demonstrate that AP1 and SP1 responsive elements were induced by cyclic strain applied to stable hMSC-TERT cells and cyclic strain induced lentivirally transduced SP1 and AP1 responsive elements in primary hMSC. The specificity of the AP1 activation was analyzed by co-treating stretched hMSC-TERT-AP1 cells with verapamil, a calcium channel blocker, which disrupts the characteristic signaling cascade initialized from the interaction of mechanical forces with membrane-associated proteins resulting in calcium influx, ERK1/2 activation and transcriptional induction of e.g. c-fos proteins (Li *et al.*, 2002). As previously demonstrated the promoter of the mechanosensitive gene HB-GAM comprises an AP1 site, which probably mediates mechanotransduction (Liedert *et al.*, 2009).

The established hMSC-TERT-AP1 and hMSC-TERT-SP1 cell lines are a suitable additional tool and screening system for testing putative mechanosensitizers / inhibitors of mechanotransduction in a small scale format. As hMSC-TERT cells are mesenchymal stem cells immortalized with telomerase they are a stable and represent a versatile system for the here described screening procedures. They can be used over a long time period without the risk of proliferation retardation or spontaneous differentiation induction. As we could previously show they can be differentiated into osteogenic, adipogenic and chondrogenic precursors to a certain extend to scan also differential mechanotransduction-related effects along mesenchymal differentiation pathways (Abdallah *et al.*, 2005).

After we adduced the proof-of-principle that our established luciferase reporter constructs respond do mechanical load, we cloned the responsive elements together with the coding sequence for firefly luciferase into a lentiviral vector system to have the opportunity to transduce primary cells for maximal flexibility. We could show that the constructs are suitable for investigating mechanical load on primary cells and provide a versatile tool for analyzing mechanical effects on cells isolated from patients with impaired mechanotransduction, e.g. MSC from osteoporotic subjects.

In summary we describe a complete and comprehensive solution for a small scale cell culture/bioreactor system including sensitive readout systems and clonal cell populations capable of analyzing subcellular crosstalk mechanisms in mechanotransduction, mechanosensitivity of primary cells and of screening the activity of putative mechanosensitizers as new targets. This will be relevant for the treatment of e.g. bone loss caused by both disuse and signal transduction related alterations of mechanotransduction in degenerative diseases.

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Discussion with Reviewer

Reviewer I: How readily can the strain system be reproduced and used as a high throughput system. Is there enough cell material in each well that permits analyses (or do wells need to be pooled) – the next question is relevant to this one. What was the variability in biological measures from well to well and by spatial well position?

Authors: One well contains sufficient cells for measuring luciferase activity comparable to the cells grown in a conventional 24-well PS plate, so pooling is not necessary. Strain distribution was analyzed optically both within single trays as well as in comparison of different trays considering their spatial distribution / position on the dish. These experiments were performed with our bioreactor in conjunction with our PU-dishes thus depicting the reproducibility of stimulation parameters with the overall-system in a setting identical to that used for biological investigations.

Reviewer I: While PU apparently permits cell attachment with substrate coating, it may be desirable or important to coat the PU with collagen, fibronectin or some other substrate as it will likely affect mechanotransduction as well as phenotypic development in the cells. Can such coating be done relatively easily?

Authors: Fibronectin coating can be done easily but was not performed in the here described experiments, since protein/peptide coating itself is known to elicit specific cellular responses which we intended to avoid.