Title: Impact of substrate elasticity on human hematopoietic stem and progenitor cell adhesion and motility

Running title: Matrix elasticity modulates HSC motility

Authors: Cornelia Lee-Thedieck\textsuperscript{1,2}, Nicole Rauch\textsuperscript{3}, Roberto Fiammengo\textsuperscript{2}, Gerd Klein\textsuperscript{4,5}, Joachim P. Spatz\textsuperscript{2,5}

Affiliations: \textsuperscript{1} Institute of Functional Interfaces, Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Germany
\textsuperscript{2} Department of New Materials and Biosystems, Max Planck Institute for Intelligent Systems, Stuttgart, Germany & Department of Biophysical Chemistry, University of Heidelberg, Germany
\textsuperscript{3} South Westphalia University of Applied Sciences, Iserlohn, Germany
\textsuperscript{4} Section for Transplantation Immunology and Immunohematology, Center for Medical Research, University Medical Clinic, Tübingen, Germany

\textsuperscript{5} These authors (GK and JPS) have contributed equally to the presented work.

Key words: hematopoietic stem and progenitor cells, mechanotransduction, elasticity, hematopoietic stem cell niche

Corresponding authors: Prof. Dr. Gerd Klein Center for Medical Research, Section for Intelligent Transplantation Immunology and Immunohematology, University of Tübingen Waldhörnlestrasse 22 72072 Tübingen Germany phone: +49-7071-29 84465 fax: +49-7071-29 5567 email: gerd.klein@uni-tuebingen.de

Prof. Dr. Joachim Pius Spatz Director Max Planck Institute for Systems, Professor for Biophysical Chemistry, University of Heidelberg Heisenbergstr. 3 70569 Stuttgart Germany phone: +49-711-689 3610 fax: +49-711-689 3612 email: joachim.spatz@mf.mpg.de

Word counts: Total length: 7339 words (including figure legends)
Summary: 147 words
Summary

In the bone marrow, hematopoietic stem cells (HSCs) reside in endosteal and vascular niches. The interactions with the niches are essential for the maintenance of HSC number and properties. Although the molecular nature of these interactions is quite well understood, little is known about the role of physical parameters such as matrix elasticity. Osteoblasts, the major cellular component of the endosteal HSC niche, flatten during HSC mobilization. We show that this process is accompanied by osteoblast stiffening, demonstrating that not only biochemical signals but also mechanical properties of the niche are modulated. HSCs react to stiffer substrates with increased cell adhesion and migration, which could facilitate the exit of HSCs from the niche. These results indicate that matrix elasticity is an important factor in regulating the retention of HSCs in the endosteal niche and should be considered in attempts to propagate HSCs in vitro for clinical applications.
Introduction

Hematopoietic stem cells (HSCs) give rise to all types of blood cells. In the body they reside in the bone marrow in so-called stem cell niches, which provide all necessary signals for their maintenance and maturation (Schofield, 1978). The interaction of HSCs with their niches is complex and involves cell-cell and cell-matrix contacts as well as stimulation by soluble factors such as growth factors (Lymperi et al., 2010). Additionally, mechanical properties of the microenvironment, such as matrix elasticity play an important role in the differentiation of adult and embryonic stem cells (Engler et al., 2006; Evans et al., 2009; Gilbert et al., 2010; Holst et al., 2010; Huebsch et al., 2010). For instance, it has been demonstrated for mesenchymal stem cells that cell fate can be directed by adjusting the elastic properties of a compliant substrate (Engler et al., 2006). Recently it was shown that HSC expansion could be greatly enhanced on soft substrates (Holst et al., 2010). However, how matrix elasticity could play a role in the HSC niche itself, the place where HSCs naturally proliferate, remained an open question.

Two different HSC niches have been described: the vascular niche adjacent to bone marrow sinusoids (Kiel et al., 2005) and the endosteal niche at the interface between bone and bone marrow (Calvi et al., 2003; Zhang et al., 2003). Whether and how these niches are interconnected is still a matter of ongoing discussion and research (Garrett and Emerson, 2009; Kiel and Morrison, 2008; Lymperi et al., 2010). So far, the niche field is controversial without a clear-cut answer as to which niche is more important in different aspects of HSC function. The bone-lining osteoblasts, being the major cellular component of the endosteal HSC niche (Calvi et al., 2003; Zhang et al., 2003), are thought to form a cellular matrix for HSCs underlying them in the niche. In 2004 the Suda group showed that Angiopoietin-1⁺ osteoblasts and Tie2⁻ HSCs are in close contact. The Tie2–Angiopoietin-1 interaction induced HSC adhesion to the bone and enhanced their ability to become quiescent (Arai et al., 2004). However, osteoblasts do not act alone as niche cells. Therefore, a deeper understanding of
HSC niches and the contribution of different niche cell types to different HSC functions is needed (Ehninger and Trumpp, 2011; Lander et al. 2012).

HSCs constitutively leave the bone marrow and enter the blood circulation in low numbers (Bhattacharya et al., 2009). Trafficking is enhanced during infections or other diseases as part of tissue repair or host defense processes. In clinical settings, HSC mobilization is commonly achieved by treatment with the cytokine granulocyte colony-stimulating factor (G-CSF) (Spiegel et al., 2007). In 2006, Katayama et al. showed that during mobilization of HSCs with G-CSF in mice, osteoblasts are suppressed by signals from the sympathetic nervous system. A morphological feature of this suppression was a flattening of the bone-lining osteoblasts (Katayama et al., 2006). We therefore hypothesized (I) that a flat osteoblast during mobilization has different elastic properties from a “normal” osteoblast under steady-state conditions and (II) that the HSCs in contact with the osteoblasts can sense this change in elasticity and respond to it. By developing a highly simplified in vitro model for the endosteal stem cell niche and by modulating the substrate elasticity in HSC adhesion and migration experiments, data supporting both hypotheses have been obtained. Human osteoblast-like cells flatten upon adrenergic stimulation and concomitantly become stiffer. HSC-like cell lines and primary hematopoietic stem and progenitor cells (HSPCs) from umbilical cord blood are able to sense differences in the elasticity of their underlying substrate and respond to it with altered adhesion and migration.
Results

Highly simplified in vitro model of the endosteal niche

In order to test our first hypothesis that osteoblasts change their elasticity during mobilization, a highly simplified \textit{in vitro} model of the endosteal region of the bone was developed. Glass-bottomed culture dishes were coated with collagen type I to mimic hard collagenous bone. The osteosarcoma cell line CAL-72, which is widely used as a model for osteoblasts (Rochet et al., 1999), was seeded on top of it and grown to confluence to mimic the bone-lining osteoblasts. In the publication by Katayama and coworkers (Katayama et al., 2006) G-CSF was used to induce mobilization. However, in RT-PCR analysis osteoblasts turned out not to express G-CSF receptors (Supplemental Fig. S1), but \(\beta\)-adrenergic receptors (Supplemental Fig. S2). Therefore it was likely that the effects observed on the osteoblasts were mediated by signals of the sympathetic nervous system, namely the adrenergic stimulation. This stimulation by the nervous system was imitated by treatment with the adrenergic agonist clenbuterol (Fig. 1A). To determine whether the cells in this simplified \textit{in vitro} model change their dimensions, as described for murine cells during mobilization (Katayama et al., 2006), the cell monolayer was fluorescently labeled and the height was determined by confocal microscopy. Although in phase contrast microscopy no obvious changes in cell morphology could be observed (Fig. 1B), the monolayer and individual cells were shown by confocal microscopy to flatten significantly on clenbuterol treatment (Fig. 1C,D). A dose-response curve fit revealed an EC\(_{50}\) value of 28.44 nM for the monolayer (Fig. 1D), which is in accordance with EC\(_{50}\) values for clenbuterol described in other studies (Barnea et al., 2008; Iizuka et al., 1998; Kern et al., 2009). This change in cell height was accompanied by a remodeling of the actin cytoskeleton (Fig. 1E), as the ratio of F- to G-actin was decreased in clenbuterol-stimulated cells compared to the control cells (Fig. 1F). Similar observations were made for primary human osteoblasts (Supplemental Fig. S3).
Adrenergic stimulation of osteoblasts induces cell stiffening

To investigate whether osteoblasts change not only their shape but also their elasticity after adrenergic stimulation, the elastic modulus of CAL-72 cells was determined by atomic force microscopy (AFM). At each indentation depth (250, 500, 750 and 1000 nm) the clenbuterol-treated cells appeared significantly stiffer than the untreated ones (Fig. 2A,B). One explanation for this finding could be the effect of the underlying rigid substrate. A first hint was the increased measured elastic moduli at higher indentation depths (Fig. 2B,D). The smaller the distance between the probing AFM cantilever and the hard substrate (calculated as difference of the averaged cell height obtained from confocal microscopy experiments and the indentation depth) the higher appeared the respective elastic modulus (Fig. 2C).

Characterization of hydrogels for in vitro modulation of substrate elasticity

To test the second hypothesis that HSCs are able to sense elasticity changes, a substrate was developed in which the elasticity could be tuned. PEGDA was chosen as the material for hydrogels because of its biocompatibility and viscoelastic properties. The elasticity of the hydrogels was modulated by changing the length of UV-irradiation in the photo-polymerization process by which they were formed. This approach had the advantage that all the components of the PEGDA hydrogels were identical, because soft and hard hydrogels of one batch were formed from one single pre-polymer solution. Only the elastic properties were changed (Fig. 3A). The elastic properties of the PEGDA hydrogels were investigated in relation to UV-irradiation time, temperature and position relative to the UV lamp. With increasing UV irradiation time and therefore increasing cross-linking density, the elastic moduli of the hydrogels became higher (Fig. 3B). Temperature changes between 27 and 47°C had no influence on elasticity (Fig. 3C). The elasticity on the upper side of the hydrogel (close to the UV lamp) and on the bottom were the same within the limits of measurement accuracy. Thus, these hydrogels appeared to have uniform structure across their depth (Fig. 3D). The
elastic modulus was determined for each batch of hydrogels. Those with elastic moduli above 38 kPa were designated as hard and those with $E \leq 20$ kPa as soft (Fig. 3E).

**Mechanosensitivity of the hematopoietic model cell line KG-1a**

The hematopoietic cell line KG-1a was chosen as a model for HSCs due to its possession of a similar surface receptor constellation, as assessed by flow cytometry. KG-1a cells expressed hematopoietic stem cell markers like CD34 as well as the fibronectin receptors integrin $\alpha V \beta 3$ and integrin $\alpha 5 \beta 1$ (Fig. 4A). Their adhesion to a physically adsorbed spot of fibronectin could be inhibited by the addition of soluble RGD (Fig. 4B). These results indicated that KG-1a cells expressed two receptors capable of recognizing fibronectin, and that RGD is the recognition sequence for KG-1a cell adhesion to fibronectin.

To investigate whether KG-1a cells growing in suspension react to substrates of different elasticities in the same manner as adherent cells, they were seeded onto soft and hard fibronectin-functionalized PEGDA-hydrogels (Fig. 4C). The number of adhering cells was lower on the soft than on the hard hydrogels (Fig. 4D). In order to delineate the signaling cues for this mechanosensing process, we targeted phosphatidylinositol-3-kinase (PI3K), which is involved in the regulation of cell adhesion, migration and mechanotransduction processes (Chretien et al., 2010; Fuhler et al., 2008; Gayer et al., 2009; Giancotti and Ruoslahti, 1999; Melikova et al., 2004; Pardo et al., 2008; Zhang et al., 2001). Inhibition of PI3K with LY294002 impaired cell adhesion on both soft and hard gels. Even under these conditions, adhesion to hard hydrogels was favored indicating that the mechanosensitivity underlying the observed effect is partially but not exclusively dependent on PI3K-mediated signaling (Fig. 4D). Cell migration was assessed by tracking individual cells in time-lapse movies. KG-1a cells migrated much faster on the hard matrices than on the soft gels and accordingly the mean paused time was shorter on the hard gels (Fig. 4E left and middle). The tracked cells were classified into stationary cells (mean migration speed $\leq 1 \mu$m/min) which did not change
their position on the gel, slowly migrating (1 µm/min < mean migration speed ≤ 5 µm/min) and fast cells (mean migration speed > 5 µm/min). This classification showed that on the soft hydrogels the majority of the cells was stationary whereas on the hard gels the slowly migrating cells formed the most prominent group (Fig. 4E right). Inhibition of PI3K led to reduced migratory activity on hard hydrogels (Fig. 4F) although not to such a low level as observed on the soft gels. This finding underscored the role of PI3K in the mechanotransduction and migration machinery of KG-1a cells.

To exclude the possibility that the different cell densities (caused by the distinct adhesion behavior) on the hard and soft hydrogels were responsible for the observed effect on cell migration, KG-1a cells were seeded at various densities on hard hydrogels. The cell density had no obvious effect on the migration speed (Fig. 5). To ensure that the observed cell behavior was not elicited by different ligand densities on the different substrates, fibronectin was fluorescently stained after surface functionalization. The fibronectin density on soft and hard gels from individual batches was comparable (Fig. 3F,G). Thus, the differences in cell migration speed observed on the different substrates were due to different substrate elasticities. The cells were able to sense the difference between soft and hard hydrogels, but they did not react to variances in elasticity occurring from batch to batch within the two groups of hydrogels (Fig. 4G).

**Mechanosensing by primary human hematopoietic stem and progenitor cells (HSPCs)**

Primary human CD34⁺ HSPCs isolated from umbilical cord blood expressed CD34, integrin α5 and integrin β1. Integrin αVβ3 was only expressed at very low levels, if at all (Fig. 6A). HSPCs adhered better to hard than to soft hydrogels (Fig. 6B). In random migration only minor effects on the overall speed and velocity (= speed without pauses) could be observed. In terms of pauses during migration no differences could be detected (Fig. 6C). Addition of the strong chemoattractant SDF-1α increased the mean migration speed on the hard gels...
compared to the unstimulated migration, whereas the speed on the soft gels remained constant. This led to clearly faster migration and shorter pauses on the hard gels compared to soft substrates. The classification of the tracked cells revealed that less stationary and more fast cells were observed on the hard than on the soft gels (Fig. 6D). The inhibition of PI3K reduced adhesion and migratory ability of HSPCs on hard gels to an extent that resembled the observation made previously on soft gels (Fig. 6E,F).
Discussion

During the last few years, evidence has emerged that the nervous system regulates HSCs (Kalinkovich et al., 2009). Here, we present data suggesting that the nervous system uses matrix elasticity as a switch to modulate HSC behavior. We show that osteoblasts, which are the cellular support of HSCs in the endosteal niche, flatten after adrenergic stimulation and concomitantly appear stiffer. HSCs are able to sense differences in substrate elasticity and respond to it with altered adhesion and migration. These findings suggest that not only biochemical signals but also physical parameters such as substrate elasticity are important factors in the regulation of homeostasis.

In a recent report, Holst and coworkers demonstrated the importance of substrate elasticity for HSC expansion (Holst et al., 2010). However, they did not provide any data on the mechanisms responsible for elasticity playing a role in the HSC niche, the place where hematopoietic stem cells naturally proliferate. Our present study extends these previous observations and helps to provide a mechanistic basis for this finding. We propose a model as to how substrate elasticity is modulated in the HSC niche, thus providing evidence that the observed phenomena are not only in vitro effects but might also play a role in vivo.

During G-CSF-induced mobilization in a mouse model, osteoblasts, which are the cellular matrix underlying the HSCs in their niche, are suppressed by signals from the nervous system (Katayama et al., 2006). As part of this phenomenon, the osteoblasts flatten. To test our working hypothesis that flat and “normal” high osteoblasts differ not only in their cell shape but also in their elasticity, we developed a highly simplified in vitro model system of the human endosteal niche. Although this model reflects only certain aspects of the complex in vivo situation of the niche (e.g. only osteoblasts were used, while other niche cells were not included), the model was suitable to reproduce the findings made previously in a mouse model. As in the mouse, the human osteoblastic cells flattened upon stimulation with an adrenergic agonist, although to a lesser extent. The more pronounced flattening in the mouse
was most likely due to several signals acting in concert on the osteoblasts, whereas in our \textit{in vitro} model only adrenergic stimulation was applied.

The change in osteoblast height was accompanied by a remodeling of the actin cytoskeleton. Similar observations were described for keratinocytes (Pullar et al., 2006) and airway smooth muscle cells (Hirshman et al., 2001; Hirshman et al., 2005). Although it is well-known that osteoblasts express beta-adrenergic receptors (Huang et al., 2009) and that the nervous system is involved in the regulation of bone turnover (Togari et al., 2005), the activation of beta-adrenoreceptors in osteoblasts has not been previously linked to alteration of the actin cytoskeleton.

AFM studies revealed cell stiffnesses between ~0.1 and 0.6 kPa for untreated cells, which is similar to elastic moduli described for other osteoblast-like cell lines (Domke et al., 2000; Takai et al., 2005). After adrenergic stimulation with clenbuterol the osteoblast-like CAL-72 cells appeared much stiffer. This change could be caused by at least two effects: the first is the effect of the underlying rigid substrate. The shorter the distance between the probing AFM cantilever and the hard substrate (calculated as the difference of the averaged cell height obtained from confocal microscopy experiments and the indentation depth), the higher appeared the respective elastic modulus. Similar findings have been described for homogeneous synthetic materials (Dimitriadis et al., 2002). The thinner the measured sample, the more the hard substrate under the sample influenced the measured elasticity. Because the adrenergically-stimulated cells were thinner than untreated cells, the substrate effect could have caused their apparent stiffening. However, as a rule of thumb the substrate effect should not play a role at indentation depths below 10% of the sample height (Cai and Bangert, 1995), a condition that was given at indentation depths of 250 nm. Since already at this indentation depth the treated osteoblasts appeared significantly stiffer than the untreated cells, the substrate effect seems at least not to be solely responsible for the observed cell stiffening under clenbuterol treatment. The second effect is a change in the intrinsic elastic properties of
the cells due to cytoskeletal remodeling. A decreased F-actin content or disruption of the actin cytoskeleton with cytochalasin D have been linked to decreased elastic moduli (Cai et al., 2009; Janmey et al., 1991; Kasas et al., 2005). Adrenergic stimulation of osteoblasts led to a decreased F- to G-actin ratio compared to untreated cells. A disruption of the F-actin fiber network as described after cytochalasin D treatment was not observed. Together with the knowledge that the actin cytoskeleton determines the mechanical properties of cells (Byfield et al., 2009; Pourati et al., 1998) we conclude that the observed reorganization of the actin cytoskeleton upon adrenergic stimulation of osteoblasts with clenbuterol leads to the apparent cell stiffening.

PEGDA hydrogels were chosen as a substrate to study the influence of substrate elasticity on HSCs uncoupled from other factors like surface coating, chemical composition of the substrate or biochemical signals secreted by other cells. PEG is a biocompatible material with viscoelastic properties. The elasticity of the hydrogels was tuned by altering the duration of the photo-polymerization process. This approach allowed us to form hard and soft gels in one batch from one identical pre-polymer solution. Also the ligand density after functionalization with fibronectin proved to be comparable on soft and hard gels. In the following cell experiments, only gels from the same batch were compared to each other. The hydrogels were classified into soft (E \leq 20 \text{kPa}) and hard (E > 38 \text{kPa}). This is much harder than the measured elasticity of the osteoblasts (\sim 0.1 – 1.0 \text{kPa}). However, since the osteoid matrix underlying the osteoblasts in the bone has an elastic modulus of \sim 35 \text{kPa} (Buxboim et al., 2010) and considering that the thinner the layer of osteoblasts becomes during mobilization, the closer the sensed elasticity approaches the elasticity of the underlying substrate, it follows that 35 kPa is the greatest degree of stiffness that the HSCs can theoretically experience in the endosteal niche. Thus, we are convinced that the hydrogels do reflect the elastic properties of the endosteal HSC niche \textit{in vivo}. 
Seeding the hematopoietic cell line KG-1a as well as primary HSPCs onto fibronectin-coated hydrogels revealed that these cells (which grow in suspension) were able to sense and to react to differences in matrix elasticity. Similar to obligate adherent cells like fibroblasts (Lo et al., 2000) hematopoietic cells adhered better and migrated faster on harder substrates. These findings indicate that HSCs are able to sense the mechanical properties of their substrate and might also be able to distinguish changes in elasticity occurring during mobilization as part of the osteoblast suppression and respond to them with increased adhesion and migration. Amoeboid migration over a surface as exhibited by leukocytes is adhesion-dependent (Friedl and Weigelin, 2008). Thus, the increased adhesive behavior of HSCs on harder substrates supports and allows increased migratory behavior.

Mechanosensitivity is a common phenomenon in embryonic and adult stem cells from different organs. Engler et al. were the first to report that the differentiation of mesenchymal stem cells was directed by matrix elasticity. They postulated that mesenchymal stem cells differentiate into that kind of tissue cell whose organ elasticity was matched by the substrate (Engler et al., 2006). Also in 3-dimensional systems, the fate of mesenchymal stem cells proved to be altered by matrix compliance (Huebsch et al., 2010). For muscle stem cells it was shown that their survival is dependent on the elastic properties of the microenvironment (Gilbert et al., 2010). Embryonic stem cells’ growth, spreading and differentiation were demonstrated to be sensitive to substrate stiffness (Evans et al., 2009). For HSCs, Holst et al. showed recently that HSC expansion is dependent on matrix elasticity (Holst et al., 2010). They coated tissue culture plates with tropoelastin (the most elastic biomaterial known). Tropoelastin formed a thin monolayer on top of the culture dishes, a substrate which was stretchable but not compressible. The PEGDA hydrogels used in the present study exhibited compressible elasticity. Taken together, the two studies demonstrate that HSCs are mechanosensitive and that cell growth, adhesion and migration are strongly influenced by the elasticity of the microenvironment. Also during embryonic development, mechanical signals
appear to have a profound impact on the hematopoietic system, which was found to be
dependent on the mechanical forces produced by blood flow (shear stress) (Adamo et al.,
2009; North et al., 2009). The NO-pathway was identified as being responsible for embryonic
HSC development in response to mechanical shear stress resulting from blood flow. Which
pathways are responsible for the elasticity-sensing and -responsiveness of HSCs on compliant
substrates remains to be elucidated. In inhibition experiments, we could identify PI3K as one
player in this process. PI3K was reported to be involved in the signaling cascade downstream
of integrins (Giancotti and Ruoslahti, 1999; Melikova et al., 2004), the inside-out activation
of integrins (Chretien et al., 2010), the regulation of growth factor-dependant migration of
cells (Fuhler et al., 2008; Zhang et al., 2001) and the mechanosensitive pathways induced by
strain in endothelial, epithelial and muscle cells (Chretien et al., 2010; Gayer et al., 2009;
Pardo et al., 2008). All these processes might play a role in the transduction of a mechanical
into a biochemical signal and the cell response with altered migration and adhesion.
Differentiation between the possible role of PI3K in the mechanosensing machinery, the
signal integration or cell response was not possible on the basis of the inhibition experiments
performed here. Nevertheless, we and others have found that adhesion of KG-1a cells and
HSPCs to fibronectin is mediated by integrins (Altrock et al., 2012; Franke et al., 2007;
Liesveld et al., 1993). Because adhesion is a prerequisite for cell migration over a surface, the
mechanosensitive migration behavior of the HSPCs on the applied fibronectin-functionalized
substrates with different stiffnesses is also dependent on the integrin-mediated adhesion to
fibronectin. Therefore, the observed effect of matrix elasticity on HSPCs is mediated by
integrins, which mechanically couple the cells to the extracellular matrix. It is well known for
other cell types that integrins are not only involved in sensing the chemical but also the
physical environment of a cell (Bershadsky et al., 2003; Schwarz et al., 2005; Zaidel-Bar and
Geiger, 2010; Zamir and Geiger, 2001). In conclusion, we think that HSPCs, similar to other
cell types, sense the chemical and physical properties (including substrate elasticity) of their
environment through integrin receptors and their associated proteins. The current finding that PI3K is involved in the mechanotransduction process suggests that the mechanical stimuli of the matrix received by integrins signal through known pathways, including PI3K. This leads to local changes of the actin cytoskeleton dynamics and therefore the observed altered adhesion and migration behavior. Non-muscle myosin II was reported to be a central player in sensing substrate rigidity (Moore et al., 2010) and is part of the mechanotransduction machinery of hematopoietic and mesenchymal stem cells (Engler et al., 2006; Holst et al., 2010). However, because myosin II has a fundamental role in cell adhesion and migration itself (Vicente-Manzanares et al., 2009), which were the readouts in our experimental system, inhibition of myosin II would not have allowed any conclusions as whether the observed effects were due to mechanotransduction or not.

The bone marrow is innervated by the nervous system, which regulates migration, mobilization and proliferation of HSCs (I) indirectly through its effect on osteoblasts and (II) directly through stimulation of adrenergic receptors expressed by CD34+ cells (Kalinkovich et al., 2009; Katayama et al., 2006; Mendez-Ferrer et al., 2010). Here, we suggest the following model for how the nervous system uses the elasticity of the osteoblasts in the endosteal HSC niche to modulate HSC adhesion and migration (Fig. 7): During mobilization, signals from the nervous system lead to osteoblast suppression. As part of this process the osteoblasts flatten and concomitantly appear stiffer. The HSCs in the endosteal niche respond to the change in elasticity of their cellular support with increased adhesion and migration, which could facilitate the egress of HSCs from their niche. We propose matrix elasticity as a newly identified, additional factor in the complex interplay of different processes that are involved in the multi-step process of mobilization.
Material and Methods

Cells

KG-1a cells were obtained from DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany) with 20% FBS (Invitrogen) under standard cell culture conditions. Human CD34+ HSPCs were isolated from umbilical cord blood using MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. CD34+ cells were kept in StemSpan® SFEM Medium (Stem Cell Technologies, Grenoble, France) supplemented with the StemSpan® CC-100 cytokine cocktail (Stem Cell Technologies) at 37°C, 5% CO2 for 16 to 48 hours before use. Umbilical cord blood was obtained from the DKMS cord blood bank (Dresden, Germany) after informed patient consent. The osteosarcoma cell line CAL-72, which was used as a model for osteoblasts (Rochet et al., 1999), was purchased from DSMZ and grown in DMEM (Invitrogen) with 10% FBS and 1% insulin-transferrin-sodium selenite medium supplement (Sigma-Aldrich, Taufkirchen, Germany).

Flow Cytometry

2 x 10^4 cells were used per sample. After washing with PBS/0.1% FBS, cells were stained with the following antibodies for 30 minutes on ice: anti-CD34-PC5 (clone 581, Beckman Coulter, Krefeld, Germany), anti-CD49e-FITC (clone SAM-1, Beckman Coulter), anti-CD29-RD1 (clone 4B4, Beckman Coulter). Incubation with anti-CD51/CD61-RPE (AbDSerotec, Düsseldorf, Germany) was performed at room temperature. IgG1-PC5, IgG2b-FITC and IgG1-PE (all from Beckman Coulter) were used as isotype controls. Samples were washed with PBS/0.1% FBS and either subjected directly to flow cytometry (FC 500, Beckman Coulter) or fixed with 1% formaldehyde and stored at 4°C for later analysis.
Adrenergic stimulation of CAL-72 cells

Cover-glasses suitable either for confocal microscopy or atomic force microscopy were coated with 0.01% (w/v) collagen type I (Sigma-Aldrich) for several hours at room temperature or overnight at 4°C. Excess fluid was removed from the coated surface. After drying, slides were rinsed with sterile tissue culture-grade water. CAL-72 cells were grown to confluence on these slides and then treated for 24 hours with clenbuterol hydrochloride (Sigma-Aldrich) in serum-free culture medium at the concentrations indicated in the respective figures. For determination of cell height by confocal microscopy, cells were labeled with 2 µM Vybrant® CFDA SE (Invitrogen) in PBS for 15 minutes at 37°C in the dark prior to clenbuterol treatment. The labeling solution was replaced by pre-warmed growth medium, which was exchanged after 30 minutes. After adrenergic stimulation, Z-stacks were acquired with a Perkin Elmer Ultra-VIEW ERS Spinning Disc confocal microscope using UltraVIEW ERS software (Perkin Elmer, Monza (Milano), Italy), minimally at 4 different positions per well and condition (experiment was run in quadruplicate).

The Z-stacks were used to examine the height of cell monolayers and of individual cells in the different treatment groups. The height of the monolayer was determined as the maximum height of the whole cell monolayer, visible in a microscopic view field. To identify the cell height of individual cells, the height of several cells per microscopic view field was measured (using Velocity software (Perkin Elmer)) over the cell nucleus, which is the highest point of the cell. Because cellular protrusions are underneath or on top of neighboring cells, the height of the whole monolayer is higher than the height of the individual cells forming the monolayer (Supplemental Fig. S4).

Cytoskeleton staining

Clenbuterol-treated and untreated cells were fixed with acetone for 5 minutes at -20°C and air-dried. After rehydration with PBS, non-specific binding was blocked for 30 minutes with
1% BSA in PBS. F-actin was stained with phalloidin labelled with Alexafluor 488 or Alexafluor 568 (both from Invitrogen) diluted 1:40 in the blocking solution. G-actin was visualized with Alexafluor 488-labelled DNase I (Invitrogen) diluted 1:500 in 1% BSA/PBS for 1 hour in the dark. After washing thrice with PBS, images were acquired using a Zeiss Axiovert 200M microscope (Zeiss, Göttingen, Germany) applying AxioVision Rel. 4.6 software (Zeiss) or a Perkin Elmer Ultra-VIEW ERS Spinning Disc confocal microscope using UltraVIEW or Velocity software (Perkin Elmer). The characteristics of the used objectives were: 40x LD-Plan-Neofluar/0.6/air (Zeiss) at the Axiovert 200M microscope; 40x C-Apo/1.1/water (Zeiss) or 63x Plan-Apo/1.4/oil (Zeiss) at the Spinning Disc confocal microscope. Quantification of the staining was performed with the help of Image J 1.38x (http://rsb.info.nih.gov/ij/). The stained area was determined by converting the images into binary ones and measuring the covered area with the “Analyze Particles” command. The mean staining intensity was determined in the unmodified images. The staining intensity per stained area was calculated for each condition and the ratio of F- over G-actin was compared between the treated and the untreated samples.

**Mechanical measurements of osteoblasts**

The AFM measurements of CAL-72 cells were carried out with the Nanowizard® II AFM (JPK Instruments, Berlin, Germany). Triangular tipless silicon nitride cantilevers (PNP-TR-TL-Au, Nanosensors) were armed with 8 µm microspheres (Supplemental Fig. S5A). The spring constant of the cantilever was determined using the thermal fluctuation method and the sensitivity was calibrated under medium at a place within the sample from which the cells were previously removed by scraping. The position of the cantilever in the sample was monitored by phase contrast microscopy (Supplemental Fig. S5B). For measuring the cells’ stiffness, the cantilever was positioned over the nucleus of single cells. For each condition and experiment, 25 to 30 cells were indented over the nuclei once with a loading rate of 1
µm/s until a constant force of 3 nN was reached (Supplemental Fig. S5C). From the resulting force-distance curves the elastic moduli of the cells were determined applying a Hertz model (Supplemental Fig. S5D) at different indentation depths by changing the fitting range using the SPM image processing v.3 software (JPK Instruments).

**Hydrogel production**

Polyethylene glycol (10,000 g/mol) diacrylate (PEGDA) was synthesized as described previously (Aydin et al., 2010). 600 mg/mL PEGDA and 96.5 mg/mL carboxyethyl acrylate were dissolved in water. 90 µL/mL photoinitiator [7.6 mg/mL 2-Hydroxy-4’(2’-hydroxyethoxy)-2-methylpropiophenone (98%, Sigma-Aldrich) in water] were added. The pre-polymer solution was degassed and put between quartz glass slides with a slit distance of 250 µm. Depending on the desired elasticity of the PEGDA hydrogels, they were polymerized between 30 seconds and 30 minutes with UV light. The obtained gels were washed three times with H2O_dil and/or 70% ethanol and stored at 4°C until use.

Before the experiments, gel pieces 14 mm in diameter were punched out, sterilized in 70% ethanol and washed with water. To promote cell adhesion, the hydrogels were functionalized with fibronectin isolated from human plasma as described elsewhere (Little et al., 2008). For this purpose the carboxylic acid groups of the gels were converted into active esters. The hydrogels were placed in a Petri dish on a 20 µl drop of 19.5 mg/mL N-hydroxysuccinimide (NHS, Sigma-Aldrich) and 78.0 mg/mL N-(3-Dimethylaminopropyl)-N’ethylcarbodiimide hydrochloride (EDC, Acros Organics/Fisher Scientific, Nidderau, Germany) in water. After 65 minutes at 4°C, the gels were drained and placed with the chemically-modified side on an 8 µl drop of 0.5 mg/mL fibronectin in 50 mM PBS and incubated for 50 minutes at 4°C. Finally, the gels were washed thrice for 10 minutes with 50 mM PBS and stored for 16 to 48 hours at 4°C until use.
Mechanical measurements of hydrogels

Initial mechanical characterization of the hydrogels was performed by atomic force microscopy (AFM) with a Molecular Force Probe Microscope (MFP-3D-CF, Asylum Research, Mannheim, Germany) which was mounted on an inverted microscope (Olympus IX 71, Olympus, Hamburg, Germany). V-shaped silicon nitride cantilevers (Novascan Technologies, Ames, IA, USA) with a polystyrene spherical tip of 6 µm were used. The macroscopic rheological properties were determined with a Piezo Axial Vibrator (PAV) (Kirschenmann and Pechhold, 2002) which was connected to a thermostat (Julabo, Seelbach, Germany) and the temperature was controlled with an accuracy of ± 0.02°C. The temperature was varied between 25°C and 47°C.

The routine characterization of each batch of hydrogels was carried out using a NanoWizard® II AFM (JPK Instruments) setup on a Leica DMI6000 CS microscope (Leica Microsystems, Wetzlar, Germany). 8 µm borosilicate glass microspheres (SPI supplies, Westchester, PA, USA) were glued to tipless silicon cantilevers (TL-CONT-10, Nanosensors, Neuchatel, Switzerland).

The cantilever’s spring constants were determined using the thermal fluctuation method on air. Prior to the hydrogel indentation experiments, which were performed in water at room temperature, cantilever sensitivity was calibrated in water on cleaned glass cover slides. In order to determine the elastic modulus E, the force indentation curves were fitted applying the Hertz-model.

Immuno fluorescent staining of fibronectin on top of hydrogels

Fibronectin functionalized gels were fixed with 4% PFA in PBS and then incubated with a rabbit anti-human fibronectin antibody (Sigma) diluted 1:450 in PBS for 1 hour at room temperature. After washing thrice with PBS, an anti-rabbit-Alexa Fluor 488 antibody (Invitrogen) diluted 1:200 in PBS was applied for 1 hour at room temperature. Samples were
washed thrice for 5 min at room temperature and once overnight at 4°C with PBS. The staining intensity was assessed with a microplate reader (Tecan Infinite M 200, Tecan, Crailsheim, Germany) and normalized to the fluorescence intensity measured on the hard hydrogels. As negative controls either unfunctionalized gels were used and/or the primary antibody was omitted.

Cell adhesion and migration experiments

Fibronectin-functionalized hydrogels in 24 well plates were equilibrated in adhesion medium [RPMI 1640, 1% FBS, 1% penicillin/streptomycin (Invitrogen), 1 mM MgCl$_2$ (Roth, Karlsruhe, Germany), 1 mM CaCl$_2$ (Merck, Darmstadt, Germany), 25 µM MnCl$_2$ (Roth)] for 30 to 60 minutes at 37°C, 5% CO$_2$. The medium over the gels was then exchanged. In the meantime, cells were harvested and resuspended in adhesion medium at concentrations of 5 x $10^6$ cells/mL for KG-1a cells and 2 to 5 x $10^5$ cells/mL for primary CD34$^+$ cells. 100 µL of this cell suspension were carefully added per well. After 1 hour at 37°C, 5% CO$_2$ unbound cells were removed by washing once with adhesion medium. Cell adhesion was assessed by taking pictures in 4-6 view fields around the center of the well with a Zeiss Axiovert 200M microscope [objective: 10x A-Plan/0.25/air (Zeiss)], equipped with a climate chamber, which was pre-equilibrated to 37°C, 5% CO$_2$ applying AxioVision Rel. 4.6 software (Zeiss). The number of cells per microscopic view field was determined with the help of ImageJ 1.38x software (http://rsb.info.nih.gov/ij/) by creating a binary image from the phase contrast micrographs and counting the cells with the command “Analyze Particles”. If cells were too dense or cell clusters could not be resolved by the software, cells were counted manually. Cell migration was followed by time lapse movies. 15-100 cells per movie were manually tracked with the ImageJ software using the Plugin “Manual Tracking”.

In some experiments, PI3K was inhibited by treating the cells with 50 µM LY294002 (Cell Signaling Technology, Beverly, MA, USA) 1 hour prior to the experiment, during adhesion
and migration. DMSO was used as a control. For chemotaxis studies, 100 ng/mL SDF-1 alpha (Peprotech, London, UK) was added to the medium before analysis by microscopy.

**Statistical analyses**

Statistical analyses were carried out with Microsoft Excel software. Statistical significance was determined by an unpaired, two-tailed student’s t-test. P ≤ 0.05 was rated as statistical significant. P-values are given in the figures or figure legends. Statistical analysis of the cytoskeleton remodeling was performed using Graphpad Prism 5.0 (www.graphpad.com) software and the statistical significance was evaluated by a two-tailed, paired Wilcoxon signed rank test.
Acknowledgements

We are indebted to Christine Mollenhauer (Max Planck Institute for Intelligent Systems) for excellent technical assistance. We thank Nadine Walter (Max Planck Institute for Intelligent Systems) for advice on AFM measurements as well as evaluation of AFM data, Sonja Hergeth for RT-PCR analysis and Graham Pawelec (University of Tübingen) for editorial assistance. The project was supported by contract research ‘Biomaterialien / Biokompatibilität’ of the Baden-Württemberg Stiftung (P-LS-Biomat/22).

Authorship Contributions

Cornelia Lee-Thedieck designed and performed research, analyzed and interpreted data and wrote the manuscript. Nicole Rauch and Roberto Fiammengo developed the PEGDA hydrogel system. Nicole Rauch performed initial mechanical characterization of hydrogels. Gerd Klein and Joachim P. Spatz contributed to study design and manuscript preparation.

Conflict of Interest Disclosures

The authors declare no competing financial interests.
References


Figure Legends

Figure 1: Human osteoblast-like cells flatten upon adrenergic stimulation with clenbuterol.

(A) CAL-72 cells were seeded onto collagen type I-coated dishes and grown to confluence. After fluorescently labeling the cells with Vybrant® CFDA SE, cells were treated for 24 hours with clenbuterol. Cell height of the monolayers and individual cells was assessed by confocal microscopy as described in the materials and methods section. (B) By phase contrast microscopy [Axiovert 40 CFL microscope (Zeiss), objective: A-Plan 10x/0.25/air (Zeiss), imaged at room temperature] no obvious influence of clenbuterol on cell morphology was observed. (C) In the cross section of a CAL72 monolayer obtained by image analysis of confocal Z-stacks using Velocity software, a reduction of the cell height could be detected upon clenbuterol treatment. (D) CAL72 cell height was plotted against the clenbuterol concentration. A dose-response curve fit (solid lines) was performed in order to determine the EC50 value for the effect of clenbuterol on cell height. Cell height decreased significantly from 8.51 to 6.54 µm for entire monolayers (squares) and from 6.17 to 4.95 µm for individual cells (filled circles). N = 4 independent experiments performed in quadruplicates; in each replicate, images were captured at 4 different positions; Mean ± s.e.m. ***: p ≤ 0.001 in student’s t-tests. (E) F- and G-actin of clenbuterol-treated and -untreated cells were stained with phalloidin-Alexafluor 488 or 568 and DNase I-Alexafluor 488 respectively. The representative micrographs shown here were recorded from a Perkin Elmer Ultra-VIEW ERS Spinning Disc confocal microscope using UltraVIEW or Velocity software (Perkin Elmer) with a 63x objective (Plan-Apo/1.4/oil, Zeiss) at 37°C, 5% CO2. (F) The ratio of F- to G-actin staining intensity per stained area (determined as stated in the material and methods section) decreased during clenbuterol treatment. N = 6 independent experiments; mean ratios of each experiments were plotted as dots; thinner lines indicate the mean; p-value according to Wilcoxon signed rank test.
Figure 2: Osteoblasts appear stiffer upon adrenergic stimulation.
(A) Two representative force curves of cells with and without adrenergic stimulation by clenbuterol are shown. (B) The clenbuterol-stimulated CAL-72 cells (orange) were significantly stiffer than the untreated cells (blue) at all indentation depths. N = 6 independent experiments. The mean ± s.e.m. is shown. (C) The mean elastic moduli of the different treatment groups were plotted against the averaged distance between the probing cantilever and the underlying substrate (= averaged substrate height). The data points were fitted linearly and the equation and coefficient of determination of the fitting are depicted in the diagram. Mean ± s.e.m.. (D) A representative force curve (red line) is shown. The curve was fitted applying a Hertz-model (trend line in blue) at three different indentation depths. For each indentation depth (indicated on the top right in each graph) one graph with the same force distance curve and the applying fitting line is presented.

Figure 3: Production and mechanical characterization of PEGDA hydrogels.
(A) PEGDA hydrogels with different elastic properties were produced by varying the duration of UV light irradiation for the photo-polymerization of the gels. Shorter irradiation times yielded partially cross-linked hydrogels which were softer than the fully cross-linked hydrogels obtained after longer irradiation. (B) The change in UV irradiation time influenced the elasticity of PEGDA hydrogels as determined by AFM. (C) The stability of the elastic properties of PEGDA hydrogels from 27 to 47°C was investigated by Piezo Axial Vibrator (PAV) measurements. (D) The mean elasticity of the top and the bottom of the hydrogels was determined by AFM. (B-D) Mean values ± s.d. are depicted; N = 10 - 15 independent experiments. (E) The PEGDA hydrogels were classified into hard and soft substrates according to their elastic moduli E. Data are presented as box-and-whisker plots; N = 7. (F) After biofunctionalization, the fibronectin on top of soft and hard hydrogels was fluorescently stained using specific antibodies and relatively quantified. N = 3 independent experiments,
mean ± s.d. of the mean values are shown. (G) Exemplary micrographs of fibronectin stainings on soft and hard hydrogels are presented.

Figure 4: Mechanosensitivity of the hematopoietic cell line KG-1a.

(A) The expression of the integrins α5, β1 and αvβ3 as well as CD34 by KG-1a cells was verified by flow cytometry. (B) The adhesion of KG-1a cells to a spot of fibronectin was inhibited by addition of linear RGD in solution. The border of the fibronectin spot is indicated by a dotted line. Micrographs were taken at room temperature using an Axiovert 40 CFL microscope (Zeiss) with a 10x-objective (A-Plan 10x/0.25/air, Zeiss) applying Axiovision Rel. 4.6 software (Zeiss). (C) From left to right: PEGDA hydrogels (depicted as light blue discs) were functionalized with fibronectin (green), equilibrated with medium in a cell culture dish and then cells (yellow) were added. After one hour of incubation cell adhesion and migration were assessed. (D) More KG-1a cells adhered to hard (blue) than to soft (orange) hydrogels. Adhesion was dependent on PI3K as demonstrated by addition of PI3K inhibitor (filled columns: DMSO control, striped columns: PI3K inhibitor). N = 4 independent experiments. (E) Speed and velocity (= speed without pauses) of KG-1a cells was higher and the paused time lower on hard (blue) than on soft (orange) gels. N = 3 independent experiments. Cells were classified into the categories fast (white, overall speed > 5 µm/min), slow (grey, overall speed between 1 and 5 µm/min) and stationary (black, overall speed ≤ 1 µm/min). (F) Overall speed and velocity on the hard substrates were decreased, and the paused time increased upon PI3K inhibition (striped) as compared to the control (filled). N = 3 independent experiments. Cells were classified as described above. DMSO labels the control, LY the PI3K inhibitor. (G) The mean migration speed of KG-1a cells was plotted against the elastic moduli E of the respective hydrogels. Open squares represent hydrogels designated as soft and closed squares represent hard hydrogels. Although the range of elasticity covered by the hard gels was relatively wide, the migration speed was not obviously
influenced by this variable occurring from batch to batch. The migration speed on the soft gels was much lower compared to the speed on hard ones. (D-F) Mean values ± s.e.m. are depicted, (G) mean ± s.d..

**Figure 5: Influence of cell density on migration speed.**

(A) 10,000 to 250,000 KG-1α cells were seeded onto hard PEGDA hydrogels functionalized with fibronectin. After 1 hour, 6 pictures were taken in the center of each sample (Axiovert 200M microscope, Zeiss; objective: A-Plan 10×/0.25/air, Zeiss) and the number of cells per field-of-view was plotted against the number of cells seeded in order to determine how dense the cells appeared in microscopy. The data were subjected to linear fitting and the equation and coefficient of determination of the fitting are depicted in the diagram. (B) Comparison of the migration speed at different cell densities revealed that the overall speed was independent of the cell densities observed in the experiments of the present study. The result of one representative experiment out of 4 is shown. Mean ± s.d..

**Figure 6: Mechanosensing by primary human CD34^+ HSPCs isolated from umbilical cord blood.**

(A) The expression of CD34 and the integrins α5, β1 and αVβ3 by HSPCs was assessed by flow cytometry. (B) HSPCs adhered better to hard (blue) than to soft (orange) substrates. N = 6 independent experiments. (C) Substrate elasticity had only a minor effect on the random migration of HSPCs. Concerning the paused time no differences could be detected. The tracked cells were classified into fast (white, overall speed > 5 µm/min), slow (grey, overall speed between 1 and 5 µm/min) and stationary (black, overall speed ≤ 1 µm/min) cells depending on their migratory ability. (D) In the presence of SDF-1 alpha HSPCs migrated faster and paused less on hard than on soft gels. The tracked cells were classified as stated above. (E) HSPC adhesion to hard substrates was reduced upon PI3K-inhibition (striped)
compared to the control (filled). (F) PI3K inhibition in HSPCs yielded slower migration and an increased proportion of paused time. HSPCs were classified as described above. DMSO labels the control, LY the PI3K inhibitor. (C-F) N = 3 independent experiments; (B-F) Mean ± s.e.m..

**Figure 7: Elasticity modulation in the endosteal niche during HSC mobilization.**

During mobilization osteoblasts (OB) are suppressed in response to signals from the sympathetic nervous system. As part of this suppression, they become flatter and stiffer. The HSCs in contact with the osteoblasts in the endosteal niche sense the change in elasticity of the underlying cells and respond to it with increased adhesion and migration, which could support the egress of HSCs from the endosteal niche.
Figure 1
Figure 2

A

B

C

D

vertical deflection: extend [nN]

0 μM clenbuterol

10 μM clenbuterol

vertical deflection: extend [nN]

P-values: 2.0x10^{-3} 4.0x10^{-3} 7.6x10^{-3} 1.1x10^{-5}

elastic modulus [Pa]

indenteration depth [nm]

10 μM 0 μM clenbuterol

elastic modulus [Pa]

average substrate height [μm]

y = -245.5x + 1.642.7

R^2 = 0.9395

vertical deflection: extend [nN]

250 nm

500 nm

750 nm

tip-sample separation [μm]

Journal of Cell Science Accepted manuscript

Lee-Thedieck et al.

MATRIX ELASTICITY MODULATES HSC MOTILITY
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Supplemental Material and Methods

Cells used for supplemental experiments

TF-1 cells were obtained from Dr. T. Kitamura (University of Tokyo, Japan) and maintained in RPMI 1640 (Invitrogen), 20% FCS (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 2% 5637 conditioned medium of cell line 5637. The osteosarcoma cell lines SaOS-2 and G292 were purchased from ECACC (Salisbury, UK) and Mg-63 cells from ATCC (Manassas, USA). SaOS-2 cells were cultured McCoy’s 5A (Invitrogen) supplemented with 15% FCS (Invitrogen), 1% penicillin/streptomycin (Invitrogen). G292 and Mg-63 cells were grown in DMEM (4.5 g/l glucose, L-glutamine; Invitrogen) with 10% FCS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Primary human osteoblasts were isolated and cultured as described previously (Hergeth, S.P., Aicher, W.K., Essl, M., Schreiber, T.D., Sasaki, T. and Klein, G. (2008). Characterization and functional analysis of osteoblast-derived fibulins in the human hematopoietic stem cell niche. Exp Hematol. 36, 1022-1034).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the RNeasy® Mini Kit (Qiagen, Hilden) according to the manufacturers’ instructions. RNA was reverse transcribed into cDNA using the SuperScriptTMIII First Strand Synthesis System (Invitrogen). The following PCR was performed with the AmpliTaq DNA Polymerase (Roche Applied Biosystems, Mannheim, Germany). Sequences of the applied primers were as follows: G-CSF receptor forward 5´-CGG TGG TCT CAG AAA GC-3’, G-CSF receptor reverse 5´-AGC TGT GCC CAG GTC TTG-3’, β1-adrenergic receptor forward 5´-GAG AGT GGC TTG CTG ATG TTC-3’, β1-adrenergic receptor reverse 5´-TCT GGC TGG TAG TGT GTC TTG-3’, β2-adrenergic receptor forward 5´-GCT GTG ACT TCT TCA CGA ACC-3’, β2-adrenergic receptor reverse 5´-CCA TGA TGA TGC TCA ACG TCT-3’, β-Actin forward 5´-TCA GAA GGA TTC CTA TGT GGG C-3’ and β-Actin reverse 5´-CCA TCA CGA TGC CAG TGG TA-3’.
**Supplemental Figures**

**Supplemental Figure S1**

**Figure S1.** RT-PCR analysis of G-CSF receptor expression by primary human osteoblasts and CAL-72 cells. No transcript of the G-CSF receptor could be detected in RNA extracted from primary human osteoblasts (pOB) or CAL-72 cells. TF-1 and KG-1a served as a positive control, the RNA-quality was tested by a RT-PCR for β-actin.

**Supplemental Figure S2**

**Figure S2.** RT-PCR analysis of β-adrenergic receptor expression by human osteoblasts. Primary human osteoblasts (pOB) as well as the osteoblast-like cell lines CAL72, Saos2, G292 and Mg63 were investigated by RT-PCR analysis using primers specific for β₁- or β₂-adrenergic receptors. As a control water was included as a template.
Figure S3. Clenbuterol treatment of primary human osteoblasts. (A) The cell height of primary human osteoblasts (measured by confocal microscopy of fluorescently labeled cells at the highest point of the cell) decreases with clenbuterol addition at concentrations as indicated on the x-axis. (B) The ratio of F- to G-actin is lower after treatment of primary osteoblasts with 10 µM clenbuterol. Mean ± s.e.m.; *: p < 0.05, ns: not significant (p > 0.05) in student’s t-test.
Supplemental Figure S4

Figure S4. Schematic representation of the difference between the monolayer and individual cell height. The solid, double headed arrows indicate the individual cell height, while the dotted one on the right represents the monolayer height.

Supplemental Figure S5

Figure S5. Measurement of elastic moduli of cells. (A) Phase contrast image of a triangular cantilever tip armed with a microsphere. (B) Phase contrast image of a cantilever positioned over a cell nucleus before starting the measurement of the cell by indentation. (C) Schematic drawing of the indentation experiment. A laser beam is directed onto the tip of the cantilever and the reflected beam is detected. The cantilever approaches the cell over the nucleus and as it indents the cell, the cantilever bends and the position of the reflected laser beam changes on the detector. (D) From the obtained data force distance curves are plotted (red, solid curve) and by fitting the curves using the Hertz model (blue, dashed line) the elastic modulus E of the cell is determined.