Mesenchymal Stem Cells Exploit Extracellular Matrix as Mechanotransducer

Bojun Li*, Cameron Moshfegh*, Zhe Lin, Jörg Albuschies & Viola Vogel

Department of Health Sciences and Technology, ETH Zurich, Ch-8093 Zurich, Switzerland.

While stem cells can sense and respond to physical properties of their environment, the molecular aspects how physical information is translated into biochemical signals remain unknown. Here we show that human mesenchymal stem cells (hMSCs) harvest and assemble plasma fibronectin into their extracellular matrix (ECM) fibrils within 24 hours. hMSCs pro-actively pull on newly assembled fibronectin ECM fibrils, and the fibers are more stretched on rigid than on soft fibronectin-coated polyacrylamide gels. Culturing hMSCs on single stretched fibronectin fibers upregulates hMSC osteogenesis. Osteogenesis was increased when \( \alpha_5\beta_3 \) integrins were blocked on relaxed fibronectin fibers, and decreased when \( \alpha_5\beta_1 \) integrins were blocked or when epidermal growth factor (EGF) receptor signaling was inhibited on stretched fibronectin fibers. This suggests that hMSCs utilize their own contractile forces to translate environmental cues into differential biochemical signals by stretching fibronectin fibrils. Mechanoregulation of fibronectin fibrils may thus serve as check point to regulate hMSC osteogenesis.

Human mesenchymal stem cells (hMSCs) are adult multipotent stem cells residing within the bone marrow and capable of differentiating into cell types of mesodermal origin including osteogenic, chondrogenic, adipogenic and myogenic lineages, as well as into neurons, vascular endothelial cells and cardiomyocytes. Due to this diverse differentiation potential, great efforts are underway to exploit hMSCs for regenerative therapies. One major unsolved challenge is how to guide the specific differentiation of hMSCs in engineered environments and under physiological conditions. Besides biochemical stimuli, various physical properties of synthetic materials have been shown to correlate with hMSC differentiation, including adhesive micro-patterns that dictate cell shape, as well as substrate rigidity and nanotopography. Among these physical properties, rigidity gained significant attention after it had been shown that protein-coated polyacrylamide gels with variable rigidities guide the differentiation of hMSCs, independently of soluble factors. In general, cells do not contact the polymer directly, but they are only in physical contact with the adhesive protein layer that is covalently coupled to the surface of the polymer. A recent study now revealed that keratinocytes and hMSCs do not feel the rigidity of a polymer per se, but respond to the mechanical feedback provided by the protein coating which is differentially anchored to the underlying polymer network architecture of soft and rigid polyacrylamide gels. The mechanics by which the protein layer is coupled to the polymer thus correlate with stem cell fate. These findings revitalized the discussions about how physical parameters of the combined system (i.e. the materials coupled to the protein coating) are recognized by cells and how they might impact their functions. Encapsulation of murine MSCs in 3D hydrogels with different rigidities furthermore suggested that MSCs might respond to their increased ability to cluster integrin ligands in flexible environments. Furthermore, it has never been investigated whether the early ECM that cells assemble in response to the effective mechanical properties of substrate plays an active role in translating physical environmental cues into biochemical signals that ultimately regulate stem cell differentiation, and whether early exposure to ECM fibrils might alter this behavior.

Fibrillar fibronectin (Fn) is the first ECM that is actively assembled by many cell types upon injury, either by harvesting plasma Fn or by producing their own. It plays a crucial role in wound healing, as well as during the healing of tissues in contact with implants and in early embryonic development. Cells can remodel ECM or their surrounding protein environment through the generation of traction forces which leads to the stretching of Fn fibrils. Stretching of Fn fibrils may activate cryptic binding sites or destroy binding motives that are only displayed on unstretched fibers (Fig. 1), including the multivalent binding motifs on fibronectin for bacterial adhesins. Our study builds upon the observation that the conformation of surface-bound Fn can impact...
osteogenic differentiation\textsuperscript{22–24}, and that the tension of early Fn ECM fibrils assembled by fibroblasts is significantly higher when the cells can pull on a protein layer that is covalently linked to rigid compared to soft polyacrylamide surfaces as probed by fluorescence resonance energy transfer (FRET)\textsuperscript{25}.

Here we asked whether Fn fibrils in early ECM play a mechano-regulatory role by translating microenvironmental inputs into biochemical signals that might regulate hMSC differentiation. Since stretching of Fn fibers changes their rigidity\textsuperscript{19}, as well as their biochemical display of binding sites\textsuperscript{19–21,26}, a single Fn fiber stretch assay was prepared to distinguish the differential effects of fiber rigidity versus Fn’s biochemical display on the differentiation of hMSC\textsuperscript{27}.

Results
hMSCs stretch their own Fn fibrils in early ECM more on rigid than on soft polyacrylamide gels. Alterations of the rigidity of protein-coated polyacrylamide gels have been shown to correlate with hMSC differentiation\textsuperscript{7}, but the mechanism remains unknown. To investigate how hMSCs translate physical properties of their environment into a biochemical signal that regulates differentiation, hMSCs were seeded at a low seeding density (3 \times 10^3/cm\textsuperscript{2}) on a layer of Fn that was covalently linked to polyacrylamide gels of different rigidities. The hMSCs were cultured for 1 day in mixed induction medium (50/50 vol\% adipogenic/osteogenic induction medium), which contained trace amounts of FRET labeled Fn. hMSCs were able to assemble Fn ECM within the first 24 hours on polyacrylamide gels (Fig. 2a–c) (on polyacrylamide) and on glass (Fig. S1).

To be able to probe the Fn fiber strain, Fn was labeled for FRET with donors and acceptors (Fig. 1). Briefly, the native cyanines on FnIII\textsubscript{1} and FnIII\textsubscript{5} of human plasma Fn were site-specifically labeled with Alexa Fluor 546 acceptors (A), whereas amines were randomly labeled with Alexa Fluor 488 donors (D)\textsuperscript{32}. The relative emission intensities of the FRET pair were quantified by calculating the ratio of measured acceptor to donor intensities (I\textsubscript{A}/I\textsubscript{D}). Local stretching of Fn fibrils by cellular traction forces leads to an increase in the average distance between acceptor and donor fluorophores and thus to a reduction in FRET (I\textsubscript{A}/I\textsubscript{D} ratio)\textsuperscript{25}. Fn-FRET I\textsubscript{A}/I\textsubscript{D} ratios were color-coded within the range of 0.05 to 1.0 to yield FRET ratio-images (Fig. 2d–f). The FRET false color scheme represents the relative stretching of Fn fibrils with a color range of red to blue indicating folded to unfolded states of Fn, respectively. As shown in Fig. 2g, the FRET ratio was lowest on rigid substrates, and increased with decreasing rigidity. This change of Fn strain was confirmed by analysis of the average FRET I\textsubscript{A}/I\textsubscript{D} ratios of 10 Fn matrices (Fig. S2).

After culturing the hMSCs in mixed induction medium supplemented with traces of Fn-FRET for 7 days, hMSC osteogenic and adipogenic differentiation were checked by histochemical staining for ALP and OilRedO, respectively. As previously described\textsuperscript{7} and confirmed here, hMSCs can differentiate into adipogenic and osteogenic cell lineages in mixed induction medium after 7 days culture (Fig. S3). Within the reported rigidity sensing regime, from 0.1 kPa to 42 kPa\textsuperscript{8}, we found that osteogenic differentiation of hMSCs as probed by ALP staining was upregulated with increasing substrate rigidity. The hMSC osteogenic differentiation correlated well with the rigidity of Fn-coated polyacrylamide gels (Fig. 2h, i–k) as reported before\textsuperscript{6}. We describe for the first time that the changes in the rigidity of fibronectin-coated polyacrylamide gels correlate with significant shifts in the Fn-FRET ratios within early ECM that hMSCs produce. The same correlations between Fn fibril tensions and polyacrylamide rigidities were also observed on collagen I-coated polyacrylamide gels (Fig. S4).

Consequently, the rigidity of polyacrylamide gels coated with a layer of adhesion proteins not only correlated with stem cell differentiation, but also with the mechanical strain of early Fn fibrils assembled by the still undifferentiated hMSCs within the first few hours. This is in agreement with previous reports showing that fibroblasts pull more strongly on their Fn fibrils of early ECM when cultured on rigid versus soft polyacrylamide gels\textsuperscript{25}.

In contrast, the same correlations between Fn fibril strains and altered polymer rigidity cannot be seen for hMSCs cultured on PDMS surfaces where a layer of either Fn or collagen I was covalently bound to the substrate. The mechanical strain of early ECM Fn fibrils assembled on Fn (Fig. S5) or collagen I (Fig. S6) covalently coated PDMS substrates with varying rigidities remained constant. In contrast to polyacrylamide, PDMS does not have a porous nanoscale architecture which leads to a much more homogenous anchorage of the protein layer\textsuperscript{14}. Remarkably and in agreement with our previous report, the altered rigidity of PDMS did not influence hMSC differentiation\textsuperscript{11}. Taken together, it is not substrate rigidity of the polymer per se that correlates with the upregulation of osteogenic differentiation. Instead and most importantly, variations of substrate rigidity affected the differentiation of hMSCs only in those cases where the mechanical strain of early Fn fibrils was altered too.
Figure 2 | The bulk rigidity of polyacrylamide gels correlates with the osteogenic differentiation of hMSCs, as well as with the Fn strain of early ECM. 

(a–c) Merged images of hMSC-assembled Fn fibrils in early ECM (fluorescence image) with brightfield images of hMSCs cultured for 24 hours on rigid (a, ~42 kPa), medium (b, ~7 kPa) or soft (c, ~0.1 kPa) Fn-functionalized polyacrylamide gels at a seeding density of $3 \times 10^3$ cells/cm$^2$ in mixed induction medium supplemented with Fn-FRET. (d–f) Ratiometric Fn-FRET I$_D$/I$_A$ images. Merged images of hMSC-assembled 24 hour Fn ECM (FRET false colors) with brightfield images of hMSCs cultured on rigid (d), medium (e) or soft (f) Fn-functionalized polyacrylamide substrates at a seeding density of $3 \times 10^3$ cells/cm$^2$ for 24 hours in mixed induction medium supplemented with Fn-FRET. The FRET false color scheme represents the relative stretching of Fn fibrils with a color range of red to blue indicating folded to unfolded states of Fn, respectively. (g) Histograms of Fn-FRET I$_D$/I$_A$ ratios of hMSC-assembled Fn ECM on rigid (black curve), medium (purple curve) or soft (pink curve) Fn-functionalized polyacrylamide substrates after 24 hours. (h) Percentage of OilRedO and ALP positive hMSCs. Results are shown as the mean ± s.d. (n = 3). (i–k) Brightfield images of hMSCs cultured on rigid (i), medium (j) or soft (k) Fn-functionalized polyacrylamide gels at a seeding density of $3 \times 10^3$ cells/cm$^2$ for 7 days in mixed induction medium supplemented with trace amounts of Fn-FRET, with histochemical staining for ALP (blue, blue arrows) and OilRedO (red, red arrows). Scale bars: 50 μm.
differentiation can be regulated by stretching Fn fibers, hMSCs were cultured on single Fn fibers that were physically coupled to stretchable silicone sheets\(^{27}\). The stretching of Fn fibers increases their rigidity\(^{19}\) and might simultaneously switch the exposure of molecular binding sites\(^{21,26}\). However, the rigidity of these deposited Fn fibers was more than 0.8 MPa\(^{26}\), and the stiffness of our silicone sheets exceeded 1 MPa (data not shown) which is much higher than the range of cell-sensed rigidities that affect hMSC differentiation\(^{8}\). Therefore, physiologically relevant rigidity alterations could be excluded in this system. By stretching the silicone sheets, Fn fibers of relaxed (20% fiber strain\(^{19}\)) (Fig. 3a) and stretched (300% fiber strain\(^{19}\)) (Fig. 3b) conformations were prepared, followed by Fn crosslinking with 4% formaldehyde to further reduce potential rigidity differences\(^{35}\). Undifferentiated hMSCs adopted a highly elongated spindle shape (Fig. 3a and 3b) and were not able to change the Fn conformation of deposited fibers by tensile forces (Fig. 3a, 3b and Fig. S7).

The ratio of alkaline phosphatase (ALP) positive cells was much higher on stretched fibers than on relaxed fibers. These results are independent of whether the hMSCs were cultured on single Fn fibers either in mixed (50/50 vol% adipogenic/osteogenic induction medium) or pure osteogenic induction medium for 7 days (Fig. 3e and 3f). The experiments were repeated several times. In mixed induction medium, a total of 196 hMSCs on the stretched crosslinked fibers and 120 hMSCs on relaxed crosslinked fibers were analyzed. On stretched crosslinked fibers about 21% of the cells showed osteogenic differentiation, while on relaxed crosslinked fibers only about 4% stained ALP positive. In pure osteogenic induction medium, a total of 210 hMSCs on the stretched crosslinked fibers and 187 hMSCs on relaxed crosslinked fibers were analyzed. About 41% of the cells showed osteogenic differentiation on stretched crosslinked fibers, and about 22% showed osteogenic differentiation on relaxed crosslinked fibers. The same trend was also observed on fibers that had not been crosslinked, or on Fn fibers with 20% (relaxed) and 300% (stretched) strain on the same silicone sheet (Fn fibers were deposited perpendicular to each other onto the same silicone sheet; stretching of the sheet results in stretching and relaxation of fibers at the same time). This confirms that stretch-induced changes in the rigidity of the silicone sheet did not drive the increased hMSC osteogenic differentiation on stretched Fn fibers. Also small changes of cell shape on single Fn fibers did not positively correlate with the differentiation path (Fig. 3g and 3h). These results show that stretched Fn fibers enhance hMSC osteogenesis. Importantly, these findings are not dependent on changes of cell shape (Fig. 3g and 3h) or changes in Fn fiber rigidity. Together with the previous observation that cells pull more strongly on Fn fibers of early ECM fibrils on protein-coated rigid than soft polyacrylamide gels (Fig. 3), this suggests that the mechanical strain of early Fn ECM might be involved in regulating hMSC differentiation.

Adipogenesis on single Fn fibers was not observed, even in adipogenic culture medium. When hMSCs were cultured on single Fn fibers (Fig. 3c and 3d), no OilRedO positive hMSCs were observed after 7 days, neither in mixed (Fig. 3e) nor in pure adipogenic induction medium (Fig. 3f). Previously it has been shown that cell shape regulated adipogenesis of hMSCs and that a spread cell shape inhibited adipogenesis\(^{2}\). hMSCs were forced to assume an elongated cell shape on Fn fibers. hMSCs either did not differentiate into adipocytes while in contact with Fn fibers, or they detached from the fibers.

Blocking αvβ3 integrin binding upregulates osteogenesis on relaxed fibers, while blocking αvβ1 slightly downregulates osteogenesis on stretched Fn fibers. Previous studies on Fn adsorbed to surfaces presenting different chemistries\(^{\ast}\) suggested that differential activation of the two most prominent Fn-binding integrins, αvβ1 and αvβ3, may be involved in the regulation of hMSC osteogenesis by Fn conformation. To test for the differential roles of αvβ1 versus αvβ3 integrins in recognizing whether Fn fibers are stretched or relaxed, hMSCs were cultured on single Fn fibers in pure osteogenic induction medium (Fig. 4). In the presence of both function-blocking antibodies against αvβ1 (clone JB5S) and αvβ3 (clone 23C6), hMSCs were not able to attach to single Fn fibers. This confirmed that the antibodies were active. The continued presence of the αvβ3 antibody alone allowed cell attachment and increased the ratio of ALP positive.

**Figure 3** Osteogenic differentiation of hMSCs is upregulated on stretched single Fn fibers. (a and b) Brightfield images of hMSCs merged with Fn-FRET images of relaxed (a) or stretched (b) single Fn fibers after cell attachment and culture for 2 days (Scale bars: 10 μm). (c and d) Brightfield images of hMSCs cultured on crosslinked, relaxed ((c), 20% fiber strain) or stretched ((d), 300% fiber strain) single Fn fibers in mixed induction medium for 7 days, with histochemical staining for ALP (blue). Scale bars: 100 μm. (e and f) Percentage of OilRedO and ALP positive hMSCs when cultured on single Fn fibers in mixed (e) or single (f) induction medium for 7 days. No OilRedO positive hMSCs could be detected on stretched or relaxed Fn fibers. Data shown represent mean ± s.d. (n = 5). Two asterisks: p < 0.01 versus 20% Fn fiber strain (p = 0.0003 for (e), p = 0.0006 for (f)). (g and h) Analysis of cell length (g) and aspect ratio (h) of ALP positive (blue) and ALP negative (yellow) hMSCs on relaxed and stretched single Fn fibers. Data shown represent mean ± s.d. (n = 8).
hMSCs (from 22% to 30%, p < 0.01) on relaxed Fn fibers after 7 days, but did not do so on the stretched Fn fibers (Fig. 4a). Blocking α5β1 binding slightly decreased the ratio of ALP positive hMSCs (from 41% to 31%, p < 0.05) on stretched Fn fibers, but had no effect on hMSCs on relaxed Fn fibers (Fig. 4a). This shows that both integrins are involved in mediating cell attachment and differentially regulate the differentiation of hMSCs on Fn fibers. Importantly, the two integrins show different dependencies to Fn stretching. This suggests that the conformational change of Fn caused by the stretching fibers might regulate integrin binding which could finally impact hMSC osteogenesis on fibers. However, the increase in osteogenesis on stretched Fn fibers could not be fully reverted, neither by blocking of α5β1 nor αvβ3. This suggests that additional mechanisms might exist that regulate the mechanosensitive osteogenesis of hMSCs in contact with mechanically strained Fn fibers. One possible mechanism may be the change of ligand density caused by the stretching of fibers. There is a possibility that stretching of Fn fibers decreases the ligand density on the surface of manually pulled fibers. It was reported that ligand density could impact cell activities. However, it has not been reported that a decrease in the density of some binding sites of Fn could promote osteogenesis. Also, a change in ligand density alone is unlikely to cause different integrin signaling. Therefore, on manually pulled Fn fibers, different integrin signaling caused by stretching of fibers may combine with other mechanisms to regulate hMSC osteogenesis.

**Stretch-induced upregulation of osteogenesis on single Fn fibers is decreased upon inhibition of the EGF receptor (ErbB).** Integrins have been reported to cooperate synergistically with growth factor receptors to regulate cell activities. For example, integrin α5β1 mediates Fn-dependent epithelial cell proliferation through epidermal growth factor (EGF) receptor activation, and potent synergistic signaling between α5β1 integrin and the growth factor receptors when the integrin and growth factor binding sites are presented at the right distance from each other. The ErbB family of transmembrane receptors belongs to the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. Since it was recently shown that ErbB3 and ErbB4 (also called HER3 and HER4) are involved in the regulation of the rigidity response of fibroblasts that were spreading on PDMS substrates coated with adsorbed (not crosslinked) Fns, we tested whether ErbBs are also involved in the mechanosensing of Fn fiber strain. hMSCs were cultured on single Fn fibers for 7 days in pure osteogenic induction medium containing the ErbB inhibitor GW572016 (Lapatinib). GW572016 treatment only decreased the ratio of ALP positive hMSCs on stretched Fn fibers (from 41% to 27%, p < 0.01), but had no effect on the differentiation on relaxed Fn fibers (Fig. 4b). These results imply that ErbBs by themselves or downstream signaling are indeed involved in distinguishing between stretched and relaxed Fn fibers.

**Discussion**

Based on many previous studies performed on flat polymeric surfaces or in hydrogels, it was demonstrated that physical properties of biomaterials can correlate with hMSC osteogenesis, but the underlying mechanism remains unknown. To shed light into the question of how cells sense physical properties of synthetic materials, we investigated whether the early ECM that cells assemble on materials plays an essential role in translating physical properties into biochemical signals that can regulate cell function. We showed here that hMSCs assembled early ECM by harvesting plasma Fn from the medium within the first 24 hours, and stretched their own Fn ECM fibrils. Importantly, hMSCs could regulate the strain of the earlyFn ECM by stretching the Fn ECM fibrils more on protein-coated rigid than on soft polyacrylamide gels. Manually stretched Fn fibers upregulated hMSC osteogenesis independently of cell shape and fiber rigidity. In contrast to polyacrylamide hydrogels whose nanoscale surface architecture is altered as function of its bulk rigidity, we would like to propose that smooth PDMS substrates with varying rigidities did not impact hMSC differentiation because the rigidity variations here did not affect the Fn strains in early ECM (Fig. S5 and S6). This suggests that hMSCs exploit the strain of Fn ECM fibrils, which they self-adjust by their own traction forces, as a mechano-regulated checkpoint regulating hMSC osteogenic differentiation in response to the effective mechanical properties of protein-coated substrates.

We further investigated possible molecular mechanisms by which the stretching of Fn fibers regulates hMSC differentiation. Differential integrin α5β1 and αvβ3 binding is shown here to be
mechano-regulated by adjustments of Fn fiber strain (Fig. 4a). Previous studies suggest differential integrin signaling as the major mechanism by which altered Fn conformations might regulate outside-in signaling and, potentially, osteogenesis. As expected, integrin signaling is differentially affected by stretching Fn fibrils. The continued presence of the αvβ3 function blocking antibody increased the ratio of ALP positive hMSCs on relaxed Fn fibers after 7 days, but did not do so on the stretched Fn fibers (Fig. 4a). In contrast, inhibition of Fn-α2β1 binding slightly reduced the ratio of ALP positive hMSCs on stretched but not on relaxed single Fn fibers (Fig. 4a). This suggests that relaxed Fn fibers preferentially signaled through integrin αvβ3 over α2β1, resulting in decreased hMSC osteogenesis on relaxed fibers. This finding was counterintuitive since the stretch-induced conformational changes of Fn which regulate integrin binding may involve the elongation of the relative distance of the synergy site on FnIII1 with respect to the RGD-loop on FnIII10 as previously reported. We surprisingly found that blocking either of the two integrins still showed a relatively weak effect compared to the stretch-induced increase of hMSC osteogenesis on Fn fibers (Fig. 4a). This gave a first hint towards the existence of additional outside-in signaling cascades that are differentially regulated by Fn fiber strain.

In addition to integrin signaling, a pronounced effect was seen here upon pharmaceutical inhibition of the EGF growth factor receptor ErbB by GW572016 treatment: a decreased ratio of ALP positive hMSCs was seen on stretched Fn fibers, but had no regulatory effect on the osteogenesis of hMSCs on relaxed Fn fibers (Fig. 4b). These results thus imply that ErbBs, or ErbB-affected downstream signaling events, are also involved in distinguishing between stretched and relaxed single Fn fibers. GW572016 not only inhibits ErbB1 but also other ErbB members, including ErbB2, ErbB3 and ErbB4. Among the ErbB family, ErbB3 and ErbB4 are reported to be involved in the regulation of the response of fibroblasts to PDMS substrate rigidity, but the mechanism is unknown. For hMSC osteogenic differentiation, it has been shown that a sustained activation of ErbB1 by surface-tethered EGF increases hMSC osteogenesis via the MAPK/ERK pathway. ErbB2 has no ligand, but instead heterodimerizes with other ErbBs such as ErbB1, and sustained activation of ErbB1 and ErbB2 enhances hMSC osteogenesis. It is unknown whether ErbB receptors are directly associated with Fn fiber strain or whether ErbB-affected downstream signaling is involved. Further studies are clearly needed to gain more detailed insights into the role of EGF and its receptors (ErbBs) in translating the mechanical strain of Fn fibers into an osteogenic response. Finally, FnIII1,2-14 fragments were found previously to bind promiscuously to many growth factor families, including members of the PDGF, FGF, TGF-β families and heparin binding EGF (HB-EGF). While shown here for EGF receptor-mediated signaling, also other growth factor receptors might thus contribute to sensing the mechanical strain of extracellular matrix fibers.

In summary, we now propose a mechanism by which hMSCs can sense the effective physical properties of synthetic materials coated with a protein layer:

hMSCs can harvest plasma Fn from the medium and assemble it into an early provisional ECM already within the first 24 hours (Fig. 2). They stretch the Fn fibrils within early ECM considerably more on protein-coated rigid than on soft polyacrylamide gels (Fig. 2). The stretched Fn fibrils promote hMSC osteogenesis through differential biochemical signaling which may involve αvβ3 and α2β1 integrins and ErbB signaling pathways (Fig. 4). Thus before the hMSCs start to upregulate the expression of their own Fn, hMSCs can start to exploit Fn harvested from the medium or serum as a mechanotransducer to self-adjust the functional display of ECM fibrils.

Taken together, the data suggest that Fnn serves as pivotal mechano-chemical signal converter, and that its mechanical strain might serve as a check point by which hMSCs can translate physical aspects of their environment into biochemical signals that direct hMSC differentiation. The translation of different physical properties may involve a larger set of transmembrane receptors, including integrins and ErbBs. Learning how hMSCs exploit the tension of ECM fibrils to self-regulate their differentiation path is crucial to initiate new ideas how to exploit MSCs for therapeutic purposes, or for the design of advanced biomimetic scaffolds for tissue engineering applications.

Methods

Cell culture. hMSCs (Lonza) were cultured as specified, either in growth medium (DMEM, 10% FBS, 0.3 mg/ml glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin), osteogenic, adipogenic or mixed (50/50 vol% adipogenic/osteogenic) induction medium (Lonza). Only early passage hMSCs (up to passage 5) were used. To inhibit specific integrin-Fn interactions, hMSCs (5 × 10³ cells/ml) were allowed to attach for 1 hour on single Fn fibrils or Fn fibrils in growth medium supplemented with GW572016 (1 μM, Lapatinib, Axxon Medchem), and were subsequently cultured for 7 days in osteogenic induction medium supplemented with GW572016.

FRET analysis. All images were acquired using an Olympus (http://www.olympus-global.com/) VF-1000 scanning laser Confocal microscope with a 1.35NA 60× oil immersion objective. Alexa Fluor 488 donor and Alexa Fluor 546 acceptor fluorophores of various concentrations were added into a dimeric Fn-FRET as mechanical strain probe to the cell medium. The native cysteines on FnIII1 and FnIII10 were site-specifically labeled with Alexa Fluor 546 acceptor fluorophores (A), whereas amines were randomly labeled with Alexa Fluor 488 donor fluorophores (D). Local stretching of Fn fibrils by cellular traction forces leads to an increase of the average distance between acceptor and donor fluorophores and thus to a reduction in FRET (I_D/I_A).

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Chemical denaturation curve of Fn-FRET in solution. Fn-FRET I_D/I_A ratios were calibrated to different Fn conformations in solution for monomeric and dimeric Fn-FRET in different concentrations of denaturant. Solutions of dimeric Fn-FRET in 0 M, 1 M, 2 M and 3 M GdnHCl and monomeric Fn-FRET in 1 M GdnHCl were added into 2 mm wide channels on Bovine serum albumin (BSA, Sigma-Aldrich) coated glass coverslips separated by 0.25-mm-thick silicone sheets (Specialty Manufacturing, Saginaw, MI). Fn-FRET solutions (about 2 μl) were imaged using the same scanning laser Confocal microscope (Olympus VF-1000, http://www.olympus-global.com/).
Preparation of 2D polycrylamide substrates. In accordance with a previously described protocol, 35 mm glass-bottom dishes were plasma cleaned, silanized using aminopropyltriethoxysilane and treated with glutaraldehyde. The surfaces were covalently functionalized with Fn or collagen I using aminopropyltriethoxysilane and treated with glutaraldehyde. The surfaces were dried, and Fn fibers were prepared by relaxing the silicone sheet to 0.5 times the initial length (corresponding to 20% fiber strain), while stretched Fn fibers were prepared by stretching the silicone sheet to 1.7 times the initial length (corresponding to 300% fiber strain). After stretching or relaxation, Fn fibers were crosslinked with 4% formaldehyde for 1 hour. The silicone sheets were backfilled with 0.1 mg/ml PLL(20)-g(3.5)-PEG(2) for 1 hour and rinsed with PBS.

Cell staining. ALP was stained using the Sigma kit #85 according to the manufacturer’s protocol. For the staining of lipids, cells were fixed with 10% polyacrylamide/0.26% bisacrylamide for the ~42 kPa rigid substrate (41.8 kPa +/−10.7 kPa), 10% polycrylamide/0.05% bisacrylamide for the ~7 kPa medium-stiff substrate (7.3 kPa +/−0.6 kPa) or 3% polycrylamide/0.05% bisacrylamide for the ~0.1 kPa soft substrate (0.13 kPa +/−0.005 kPa) and covered with 12 mm diameter coverslips. Coverslips were removed and the polycrylamide gels were covalently functionalized with Fn or collagen I using sulfo-succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexafluoro (sulfo-SANPAH), Pierce to allow cell attachment. Briefly polycrylamide gels were placed in a 24-well plate and 500 µl of a 0.2 mg/ml solution of sulfo-SANPAH in milli-Q H2O were added to each well. The PDMS surface was irradiated for 5 minutes using the 365 nm UV LED array. The solution was removed and the procedure was repeated once. After washing with 50 mHEPS in PBS (twice), the substrates were coated with 50 µg/ml collagen I or 20 µg/ml Fn (purified by ourselves) in PBS. The Young’s moduli of the polycrylamide gels were determined by atomic force microscopy (AFM) using a silicon nitride tip with an attached polystyrene bead (Novascan, 4.5 µm bead diameter, 10 nm nitrogen constant) and a modified Hertz model as previously described. The AFM-derived Young’s moduli were in good agreement with recent literature values of comparable polycrylamide gel compositions.

Preparation of 2D PDMS substrates. In accordance with a previously described protocol, 13 mm glass coverslips (thickness no 1, borosilicate glass) were thoroughly cleaned by ultrasonication in milli-Q H2O and ethanol. The two parts of the PDMS kit (Syngard 184, VWR) were mixed in different ratios ranging from 100:1 to 10:1 basiscrosslinker and spread on the glass coverslips. The elastomers were cured at 70 °C overnight. For cell seeding, Fn or collagen I was covalently attached to the PDMS surface using the same sulfo-SANPAH linker as for the Polycrylamide hydrogels.

Statistical analysis. Statistical differences between two groups of data were analyzed with Student’s t-test. Data are presented as means ± s.d.
43. Krammer, A., Craig, D., Thomas, W. E., Schulten, K. & Vogel, V. A structural model for force regulated integrin binding to fibronectin’s RGD-synergy site. *Matrix Biol.* 21, 139–147 (2002).

44. Xia, W., Liu, L.-H., Ho, P. & Spector, N. L. Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016. *Oncogene* 23, 664–653 (2004).

45. Muraoka-Cook, R. S., Feng, S.-M., Strunk, K. E. & Earp, H. S. ErbB4/HER4: role in mammary gland development, differentiation and growth inhibition. *J. Mammary Gland Biol. Neoplasia* 13, 235–246 (2008).

46. Tanama, K., Kawasaki, H. & Wells, A. Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC. *J. Biomed. Biotechnol.* 2010, 795385 (2010).

47. Klapr, L. N. et al. The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc. Natl. Acad. Sci. U. S. A.* 96, 4995–5000 (1999).

48. Yarden, Y. & Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* 2, 127–137 (2001).

49. Platt, M. O., Roman, A. J., Wells, A., Lauffenburger, D. A. & Griffith, L. G. Sustained epidermal growth factor receptor levels and activation by tethered ligand binding enhances osteogenic differentiation of multi-potent marrow stromal cells. *J. Cell. Physiol.* 221, 306–317 (2009).

50. Martino, M. M. & Hubbell, J. a. The 12th-14th type III repeats of fibronectin function as a highly promiscuous growth factor-binding domain. *FASEB J.* 4711–4721 (2010).

51. Kundu, A. K., Khatiwala, C. B. & Putnam, A. J. Extracellular matrix remodeling, integrin expression, and downstream signaling pathways influence the osteogenic differentiation of mesenchymal stem cells on poly(lactide-co-glycolide) substrates. *Tissue Eng. Part A* 15, 273–283 (2009).

52. Baneyx, G., Baugh, L. & Vogel, V. Coexisting conformations of fibronectin in cell culture imaged using fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U. S. A.* 98, 14464–14468 (2001).

53. Engvall, E. & Ruoslahti, E. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer* 20, 1–5 (1977).

54. Mosesson, M. W. & Umfleet, R. A. The cold-insoluble globulin of human plasma. I. Purification, primary characterization, and relationship to fibrinogen and other cold-insoluble fraction components. *J. Biol. Chem.* 245, 5728–5736 (1970).

55. Mosesson, M. W., Chen, A. B. & Huebey, R. M. The cold-insoluble globulin of human plasma: studies of its essential structural features. *Biochem. Biophys. Acta* 386, 509–524 (1975).

56. Mahaffy, R. E., Shih, C. K., MacKintosh, F. C. & Kas, J. Scanning probe-based frequency-dependent microrheology of polymer gels and biological cells. *Phys. Rev. Lett.* 85, 880–883 (2000).

57. Tse, I. R. & Engler, A. J. Preparation of hydrogel substrates with tunable mechanical properties. *Curr. Protoc. Cell. Biol. Chapter* 10, Unit 10.16 (2010).

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**Author contributions**

B.L., C.M. and V.V. designed the research and B.L., C.M. and Z.L. performed it. B.L., C.M., Z.L. and V.V. analyzed the data. J.A. performed AFM indentation measurements and analysis. B.L., C.M. and V.V. wrote the paper.

**Additional information**

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