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Why the impact of mechanical stimuli on stem cells remains a challenge

Roman Goetzke[1,2,*], Antonio Sechi[2,*], Laura De Laporte[3,*], Sabine Neuss[4,5,*], Wolfgang Wagner[1, 2,*]

1 Helmholtz Institute for Biomedical Engineering, Stem Cell Biology and Cellular Engineering, RWTH Aachen University Medical School, Aachen, Germany
2 Institute for Biomedical Engineering – Cell Biology, RWTH Aachen University Medical School, Aachen, Germany
3 DWI − Leibniz-Institute for Interactive Materials, 52074 Aachen, Germany
4 Helmholtz Institute for Biomedical Engineering, Biointerface Group, RWTH Aachen University Medical School, Aachen, Germany
5 Institute of Pathology, RWTH Aachen University Medical School, Aachen, Germany
* equal contribution

Correspondence: Wolfgang Wagner, M.D., Ph.D., Helmholtz Institute for Biomedical Engineering, Stem Cell Biology and Cellular Engineering, RWTH Aachen University Medical School, Pauwelsstrasse 20, 52074 Aachen, Germany, phone: +49-241-8088611, E-mail: wwagner@ukaachen.de; and Sabine Neuss, Ph.D., Helmholtz Institute for Biomedical Engineering, Biointerface Group and Institute of Pathology, RWTH Aachen University, Pauwelsstrasse 30, 52074 Aachen, Germany, phone: +49-214-8080622, E-mail: sneuss-stein@ukaachen.de

Abstract

Mechanical stimulation affects growth and differentiation of stem cells. This may be used to guide lineage-specific cell fate decisions and therefore opens fascinating opportunities for stem cell biology and regenerative medicine. Several studies demonstrated functional and molecular effects of mechanical stimulation but on first sight these results often appear to be inconsistent. Comparison of such studies is hampered by a multitude of relevant parameters that act in concert. There are notorious differences between species, cell types, and culture conditions. Furthermore, the utilized culture substrates have complex features, such as surface chemistry, elasticity, and topography. Cell culture substrates can vary from simple, flat materials to complex 3D scaffolds. Last but not least, mechanical forces can be applied with different frequency, amplitude, and strength. It is therefore a prerequisite to take all these parameters into consideration when ascribing their specific functional relevance – and to only modulate one parameter at the time if the relevance of this parameter is addressed. Such research questions can only be investigated by interdisciplinary cooperation. In this review, we focus particularly on mesenchymal stem cells and pluripotent stem cells to discuss relevant parameters that contribute to the kaleidoscope of mechanical stimulation of stem cells.

Introduction

Mesenchymal stem cells (MSC) are widely used in mechanobiology, since they give rise to mesodermal tissues with diverse mechanical properties, such as rigid bone, cartilage, soft adipose tissue, and marrow stroma [1, 2]. In addition, they can be easily isolated from many tissues and comprise a multipotent subpopulation with multi-lineage differentiation potential

and some self-renewal ability. MSC are currently used in many preclinical and clinical studies, where they have shown to support for example regeneration of cartilage, bone, and cardiac muscle tissue [3]. On the other hand, it is not trivial to standardise MSC: they are a highly heterogeneous cell population with multiple subsets [4] and there are donor- and species-specific differences [5, 6], as well as molecular differences depending on the tissue of origin [7]. Furthermore, MSC undergo replicative senescence during culture expansion [8]. The lack of a precise molecular definition of MSC and the heterogeneity of cell preparations may hamper reproducibility of results [9].

In contrast, pluripotent stem cells (PSC), such as induced pluripotent stem cells (iPSC) and embryonic stem cells (ESC), resemble a relatively defined and primitive ground state of cellular differentiation [10]. They can give rise to cells of all three germ-layers – and ultimately all cell types of our body. Furthermore, they have an indefinite capacity to self-renew and lack markers of replicative senescence and aging [11, 12]. However, directed lineage-specific differentiation of PSC into fully matured and functional cell types by defined *in vitro* culture conditions remains a bottleneck for regenerative medicine. There is evidence that differentiation of pluripotent cells needs to be governed by a three-dimensional (3D) environment using functional biomaterials that mimic extracellular cell structures and properties of native tissues [13].

To address effects of mechanical stimulation on stem cells, it is therefore important to choose the best suited starting material and culture conditions. Many mechanobiological studies have been performed with murine or rat-derived cells, albeit these cells do not fully match their human counterparts and there are clearly functional differences between cell types derived from different species [14, 15]. Furthermore, culture conditions, such as medium composition, can have a tremendous impact on cell function [16, 17]. Thus, even for conventional cell culture it is not trivial to fully standardize cell populations – and this becomes much more complex if the relevance of mechanical stimuli is addressed because various chemical, physical, and biological parameters affect cell fate decisions (Figure 1). In this review, we discuss the relevance of different biomaterial features, mechanical stimuli, and the molecular mechanisms involved in mechanoresponses of stem cells, with particular focus on MSC and PSC.

Effects of biomaterial chemistry and protein adsorption from culture media

One of the first systematic, high-throughput approaches to test interactions between stem cells and different biomaterials was described by Anderson and coworkers in 2004: 1,700 synthetic polymers were spotted in nanoliter-scale on a polymer microarray [18]. This pioneering work with human embryonic stem cells was later expanded to other cell types and materials with additional chemical groups [19], extracellular matrix molecules [20], and proteins of cell-conditioned medium [21]. The studies indicated that there are cell type- and species-specific interactions and compatibilities with certain biomaterials [19, 5]. For example, polymer spots of defined chemistry could induce e.g. neuronal fate of ESC or support maintenance of stem cell plasticity [18]. In 2012, the first high-throughput biomaterial arrays were combined with computational models that predicted cell adhesion on a library of biomaterials with different molecular properties using solely computational descriptors [22]. Thereby, the need for experimental measurements such as contact angle or mechanical

properties was minimized. Although such systematic approaches for the analysis of stem cell-biomaterial interactions already resulted in the identification of biomaterials suitable for specific stem cell applications, they did not allow for systematic predictions of stem cell behaviour on tailored biomaterials.

Culture media usually contain serum or serum replacement, which also impacts on the surface chemistry of biomaterials. For instance, MSC culture medium is supplemented with fetal calf serum (FCS) or human platelet lysate (hPL) [23], both containing plasma proteins that can adsorb to biomaterials and thereby alter cell responses [24]. Adhesion and binding of plasma proteins depends on diverse biomaterial characteristics, such as surface roughness, charge, and wettability and is affected by the available protein repertoire in the culture medium [25]. Therefore, it is not trivial to discern cellular responses to biomaterials from indirect effects due to the rapid adsorption of proteins from the culture medium.

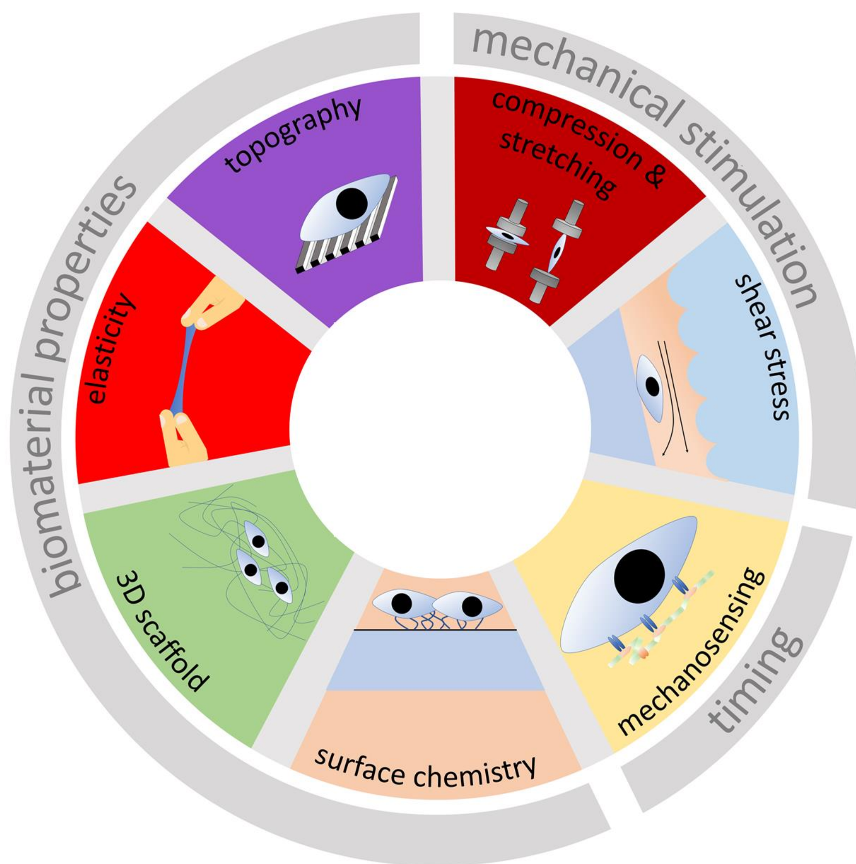

Figure 1. Hallmarks of mechanobiology.
Stem cells sense and respond to various different biomaterial properties (including topography, elasticity, 3D environment, and surface chemistry) and mechanical stimulation (e.g. compression, stretching, or shear stress). In addition, timing and cell-intrinsic mechanisms for mechanosensing are relevant for the cellular response.

Effects of matrix architecture

Most cells of the human body grow in a complex 3D microenvironment consisting of extracellular matrix (ECM), which directs cell behaviour [26]. Thus, it is not surprising that *in vitro* cell responses vary extensively on flat surfaces *versus* 3D structures [27]. Artificial 3D cell niches have been generated with natural and/or synthetic biomaterials that allow integrins in the cell membrane to cluster and form focal adhesions with a spatial distribution to mimic a more physiologic microenvironment. A multitude of 3D scaffold architectures exist, including hydrogels and electrospun fibres, woven, or non-woven fibres [28]. These different scaffolds vary, e.g., in biochemical, mechanical, and structural properties.

Hydrogels are often used as scaffold in tissue engineering due to their unique properties [29, 30]. They are formed by crosslinking synthetic and/or biological components and contain more than 90% water [31]. Depending on their backbone chemistry, type and density of crosslinks, the mechanical properties of hydrogels can be fine-tuned. Furthermore, hydrogels can be functionalised with proteins or peptides to promote cell adhesion or differentiation. Cell spreading and migration within or on hydrogels is highly dependent on their stiffness [32], viscoelasticity [33], strain-stiffening [34], fibre architecture [35], degradation rate [36], and porosity [37].

Pore size is an essential aspect of scaffolds: nanometer pores limit cell migration and could hinder diffusion of nutrients and waste transport depending on their size, and in the case of large pores (325 µm and larger), cell attachment can be limited due to the decrease in specific surface area [38]. Bone marrow-derived MSC, for example, reside in microporous trabecular bone with a porosity ranging from 50-90% and pore sizes in the order of 1 mm [39]. Approaches to mimic these structures, e.g., by using gelatin-based foams with 100-150 µm pores, resulted in MSC with elongated morphology and pronounced cell protrusions grabbing the surrounding matrix, whereas cells in smaller pores were spherical [40]. The increase in cytoskeleton tension inside the 100-150 µm pores, together with the upregulation of α_2- and α_5-integrins, accelerated osteogenic differentiation and maturation.

Understanding how stem cells respond to the mechanics of fibrous microenvironments remains a challenge. Fibrous scaffolds can be easily produced at low costs via electrospinning and this method can be directed to generate scaffolds of defined geometry and thickness [41, 42]. Such fibres can also be generated with tailored surface topography, which impacts on growth and differentiation of MSC [43, 44]. In addition, an electrospun material system has been developed to control the mechanical and adhesive features of fibrillar structures [35]. These synthetic fibre mats elucidated some mechanisms that regulate stem cell sensing of ECM stiffness in fibrous networks [35]. In contrast to flat hydrogel surfaces, these fibres form a 3D mesh to better mimic the fibrous ECM structure and can be tuned in stiffness, density, and alignment. Such a fibre alignment is also feasible in hydrogels e.g. via aligned spinning, electrical and mechanical stretching, or magnetic orientation [45, 46]. Furthermore, it is possible to directly fabricate polymeric scaffolds with an ECM-like nanofibrous topography and defined architectures using extrusion-based 3D printing. Such 3D printed scaffolds with both defined architectures and nanoscale ECM-mimicking morphologies have potential applications in cartilage and bone regeneration [47]. When a polycaprolactone (PCL) fibre mesh is embedded in a poly(ethylene glycol) (PEG)-fibrinogen

hydrogel and mixed with connective tissue growth factor, human iPSC-derived MSC demonstrated stronger fibroblastic commitment compared to osteogenesis and adipogenesis [48]. Three-dimensional fibrous scaffolds were created by coiled flexible fibres resulting in more 3D cell spreading and fibre contraction due to cell forces. This led to milder myofibroblastic activities of human MSC differentiated into fibroblastic phenotypes compared to stiffer 2D fibre meshes [49]. Notably, stiffer fibres led to suppression of spreading and proliferation of MSC [35], while more flexible fibres led to a significant higher proliferation rate due to the ability of the cells to better reorganize the surrounding material and cluster cell ligands.

Effects of substrate elasticity

Many substrates allow for tuning of their elastic modulus and thereby alter stem cell behaviour. For instance, substrate elasticity in the range of 12 kPa was suggested to induce self-renewal of skeletal muscle stem cells in comparison to rigid plastic (~10^6 kPa) [50]. Pioneering studies to investigate the effect of hydrogel stiffness on stem cell fate were performed in 2D cultures on elastic hydrogels [51]. Here, MSC were sensitive to the elasticity of the hydrogels, with soft brain-mimicking matrices triggering neurogenesis, stiffer muscle-like gels inducing myogenesis, and rigid constructs mimicking collagenous bone leading to osteogenesis [52]. We found that differentiation of MSC on polydimethylsiloxane (PDMS) gels favoured differentiation into adipogenic or osteogenic lineages when cultured on soft and rigid PDMS, respectively [44]. However, this tendency was no more evident if cells were transferred to rigid tissue culture plastic (TCP) before adipogenic and osteogenic differentiation was initiated, albeit they were initially continuously culture expanded on different elasticity (Figure 2A,B). Furthermore, global gene expression and DNA methylation profiles of MSC continuously cultured on soft PDMS *versus* stiff TCP hardly revealed any differences [44]. These results suggest that matrix elasticity influences cell behaviour as long as the cells are on the elastic substrate but does not have a major impact on epigenetic cell fate decisions, which are maintained when the cells are again cultured on stiff plastic.

Matrix elasticity was also suggested to have major impact on stemness and differentiation of PSC. There are several approaches to better maintain pluripotency of PSC in 2D on optimized soft hydrogels [53, 54]. Furthermore, three-dimensional culture systems with changeable properties were used to not only prolong self-renewal but also to trigger cell fate decisions in PSC. For instance, comparison of human iPSC grown inside degradable and non-degradable PEG hydrogels of various stiffness (300-1200 Pa) revealed enhanced proliferation and pluripotency marker expression in the soft and degradable gels [55], suggesting that spatial confinement and degradability of the 3D matrix play a crucial role for pluripotency. However, contradicting results have been demonstrated with a hydrogel system combining alginate and collagen, which is switchable between the cross-linked (~21 kPa) and non-cross-linked state (~5 kPa). Here, human ESC cultured within stiff, cross-linked gels remained their self-renewal capacity, whereas switching to the non-cross-linked state induced differentiation of ESC into mesodermal and endodermal lineages despite the use of pluripotency-supporting medium [56]. Accordingly, soft matrices have further shown to be supportive for maturation of iPSC-derived cell types including cardiomyocytes [57], neurons [58, 59] and hepatocytes [60]. More recently, we analysed if substrate elasticity affects differentiation of iPSC towards MSC. To this end, we generated iPSC-derived MSC on a very

soft hydrogel derived from human platelet lysate (hPL-gel) and on rigid TCP [61, 62]. Against our expectations, there were no differences in morphology, immunophenotype, *in vitro* differentiation potential, gene expression, and DNA methylation profiles (Figure 2C). However, we found differences in expression of Yes-associated protein (YAP) and its paralog transcriptional coactivator with PDZ-binding motif (TAZ): these mechanotransducers were predominantly localized in the nucleus on stiff TCP, whereas on soft hPL-gels YAP and TAZ were more pronounced in the cytoplasm [61]. Hence, although the cells sensed the underlying soft substrate, elasticity might be less relevant for differentiation of iPSC towards MSC than anticipated.

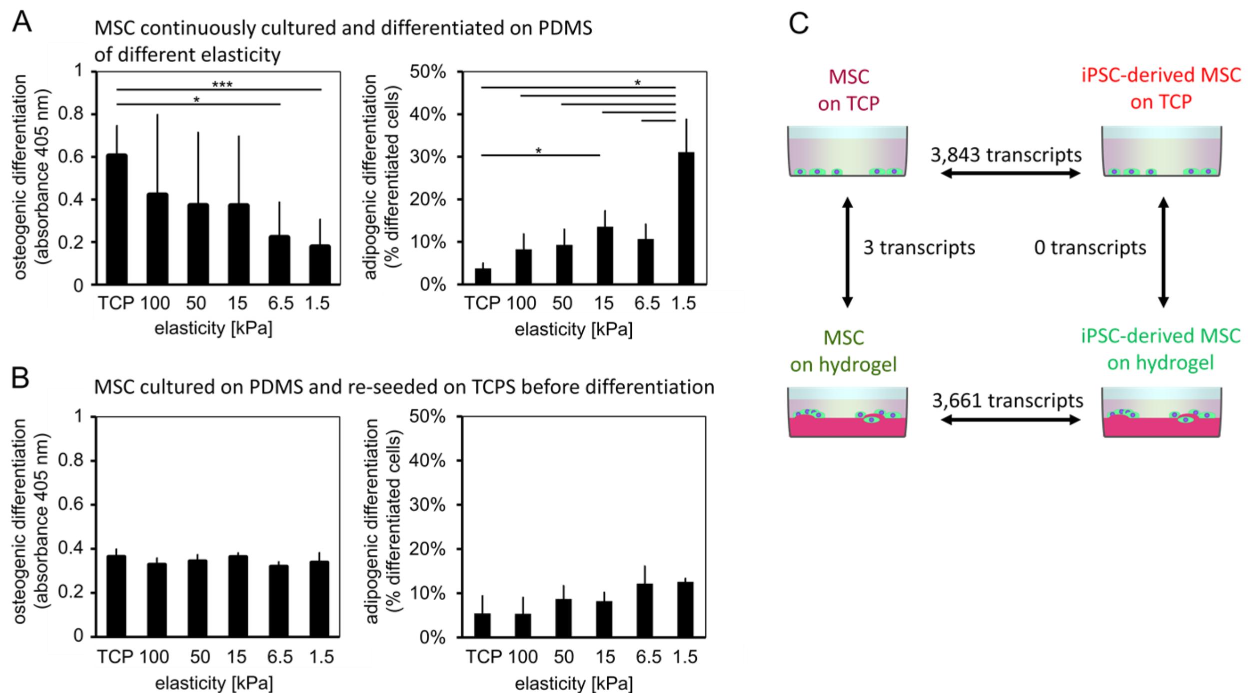

Figure 2. Effects of matrix elasticity may be transient while the cells are cultured on the substrate. A) MSC were isolated from bone marrow and continuously culture expanded on either tissue culture plastic (TCP) or polydimethylsiloxane (PDMS) with different elastic moduli. Osteogenic and adipogenic differentiation was then induced on the substrates and analysed by semiquantitative analysis of calcium phosphate precipitates and the percentage of cells with lipid-droplets, respectively. Osteogenic differentiation was enhanced on stiff substrates, whereas adipogenic differentiation was more pronounced on softer materials (*$p < 0.05$). **B)** However, if MSC were continuously cultured on the substrates as indicated and then re-seeded on TCP before induction of *in vitro* differentiation, the above-mentioned propensity of lineage-specific differentiation was not observed. Additional information on these results is provided in Schellenberg et al. 2014 [44]. **C)** MSC and iPSC-derived MSC were generated in parallel on TCP and a very soft hydrogel of human platelet lysate. Unexpectedly, the different culture conditions hardly evoked significant differences in global gene expression analysis. The number of significant transcripts is indicated (adjusted p-value < 0.05). Further details are provided in Goetzke et al. 2017 [61].

Stem cells undoubtedly feel the mechanical properties of hydrogels, such as viscoelasticity, which can be controlled by several molecular features. Better control of these features leads to better understanding of how stem cells respond to mechanical cues. However, many natural hydrogels, such as Matrigel^{TM}, have an ill-defined chemistry. Thus, it is impossible to decouple their biochemical, physical, and mechanical properties. Therefore, synthetic hydrogels are applied with specific molecular chemistries to control their viscoelasticity, structure, and degradation rate. To enable high-throughput screenings of multiple parameters simultaneously, a robotic liquid dispensing technique was developed. The effect of elasticity, protein modification, and cell-cell interaction on stem cell fate in a PEG-based hydrogel microarray was investigated [63]. A follow-up study using this technology elucidated which properties of the 3D cultures mostly affected stem cell behaviour [64]. Even though soluble factors were still dominating, the mechanical and physical characteristics of the PEG-based hydrogels played a significant role, followed by a smaller effect of the proteins coupled to the hydrogels and the initial cell seeding density. Accordingly, self-renewal and proliferation of mouse embryonic stem cells was optimal in the case of hydrogels with intermediate and low stiffness [64].

The above-mentioned hydrogels have elastic properties, which, however, do not fully represent the native ECM. In reality, cells are surrounded by a visco-elastic matrix with stress relaxing properties. As cells gauge resistance to the traction forces they exert on their surrounding, they receive a different feedback from a material when it has the ability to dissipate the energy and reorganize its structure. Cells that normally do not spread well on soft elastic hydrogels now have the ability to attach to soft viscous hydrogels with a large spreading area, which may be explained by their ability to cluster cell ligands [65]. Nuclear translocation of the Yes-associated protein (YAP) is also enhanced with faster relation rates. When MSC were encapsulated into visco-elastic gels, they took an osteogenic route in stiff hydrogels, while softer hydrogels induced adipogenesis, which was consistent with fully elastic hydrogels [66]. However, the relaxation rate of the visco-elastic hydrogels significantly affected stem cell differentiation. While slow relaxation of the soft gels promoted adipogenesis (low nuclear YAP levels), rapid relaxation of stiff gels significantly enhanced osteogenesis and bone forming activity (high nuclear YAP levels), even in gels where the MSC portrayed similar cell morphologies [67]. It was found that integrin-ligand clustering and myosin contractility influenced this particular behaviour.

The above-mentioned results challenge lineage-specific differentiation of stem cells by defined elasticity. In particular for regenerative medicine, it is also crucial to prevent abnormal differentiation that is usually only observed under pathological conditions. For example, increased expression of α-smooth muscle actin (SMA) in human MSC on stiffer substrates may reflect myofibroblastic activity and thus fibrosis [68]. Furthermore, α-SMA is highly expressed in carcinoma-associated fibroblasts (CAF). Increased stiffness of the ECM in the tumour microenvironment may therefore induce differentiation of MSC to CAF, triggering for example enhanced proliferation and survival of mammary cancer cells [69]. Thus, culture conditions can also evoke phenotypes that are relevant for disease development.

Modulation of the elastic modulus of biomaterials may also impact on the topography. Particularly, polyacrylamide hydrogels (PA) have marked differences in porosity, spanning a

range of micro- to nanometer pore sizes for different stiffness [70]. Such topographic features impact on growth and differentiation of MSC and iPSC [71]. With regard to MSC, however, it has been suggested that modulating substrate porosity of PA without altering stiffness does not affect their osteogenic and adipogenic differentiation potential [72].

Effects of matrix topography

Engineering topographical cues has recently attracted growing interest in controlling stem cell fate. A simple, yet elegant way to guide cell behaviour is to control cell shape and size. In general, small and roundish geometric cues limit the flattening of human MSC and thereby favour adipogenic differentiation [73, 74]. Conversely, larger substrate geometries promote cell spreading and hence differentiation towards osteoblasts. Notably, guidance provided by such geometric cues was independent from adipogenic and osteogenic soluble factors [73], indicating that substrate topography is sufficient for controlling stem cell behaviour. Moreover, the area *per se* is not the crucial parameter of the topographical cue, but rather its aspect ratio (width *versus* length) as indicated by the observation that geometries with higher aspect ratios (but with same areas) promote predominantly osteogenesis of human MSC [73]. MSC further behave differently on convex and concave structures. Convex curvatures promote osteogenic differentiation, which may be attributed to more pronounced pulling forces on the nucleus, associated with an increased level of Lamin A [75].

New screening platforms of matrix topography, such as the BioSurface Structure Array (BSSA) and the Nano-TopoChip, provide opportunities to identify and study effects of specific features for guided renewal and differentiation of stem cells [76, 77]. These chips combine a large library of topographies, so called Topo-Units, with lateral dimensions ranging from 200 - 1000 nm, that enable single cell screening on substructures down to nanometre scale [78]. In the case of the Nano-TopoChip, synergistic effects of topography and material chemistry on stem cell response have been studied. To better mimic the cellular microenvironment of stem cells, more complex substrates, including wires/tubes, have been fabricated. For instance, in the absence of soluble osteogenic factors short and very thin silicon wires (~160 nm diameter and ~8 μm length) better supported osteogenic gene expression (*RUNX2* and *COL1A1*) in human MSC as compared to longer nanowires (~190 nm diameter and ~26 μm length) [79]. Remarkably, the osteogenic potential of MSC cultured on shorter wires correlated with expression levels of F-actin, vinculin, and α2-integrin [79]. Furthermore, in studies using vertically spotted titanium oxide nanotubes, it was shown that osteogenic differentiation of human MSC was promoted when the diameter of the nanotubes was 100 nm, whereas osteogenic markers were hardly upregulated if their diameter was 50 nm or smaller [80]. However, in the case of rat MSC, osteogenic differentiation was increased with titanium oxide nanotubes of 15 nm in diameter, whereas on larger nanotubes (≥ 50 nm) cells adhered poorly and underwent apoptosis [81].

Similarly, stem cells also respond to nano- and micro-scale groove/ridge surface topographies. This becomes particularly evident on PDMS substrates with wrinkle gradients (amplitudes from 49 to 2561 nm; wavelengths from 464 to 7121 nm) [82]. Human MSC seeded on these wrinkle gradients clearly showed directed migration, cellular alignment, F-actin alignment, and the amount and length of filopodia were significantly enhanced with increasing wrinkle size. Furthermore, cells on micro-topographic surfaces revealed an

increase in focal adhesions with uniform alignment, compared to nano-scale wrinkles. We could demonstrate that arrays with ridge sizes of 650 nm enhanced both adipogenic and osteogenic differentiation of MSC cultured in their appropriate differentiation medium, indicating that, in addition to biochemical cues, mechanical stimuli for both differentiation programs are crucial [71]. When cultured on polyimide arrays with 15 µm wide ridges, MSC preferentially differentiated towards the adipogenic lineage, whereas reducing the ridge size to 2 µm promoted osteogenic differentiation.

More recently, we demonstrated that sub-micron groove/ridge topographies can also be exploited to regulate the behaviour of individual human iPSC and even of iPSC colonies [83]. iPSC colonies acquired an elongated morphology parallel to the groove/ridge arrays. Thus, behaviour of iPSC can not only be regulated by controlling the shape of single cells but also the shape of whole stem cell colonies (Figure 3). Within colonies, single iPSC develop apical stress fibres oriented along the major axis of the arrays, which also determined the orientation of the cell division machinery. Notably, BMP4-induced differentiation of iPSC colonies was promoted by sub-micron groove/ridge substrates, which can be attributed to the larger edge region of elongated colonies [83]. Similarly, growing human ESC colonies on small circular patterns of 200 µm promoted their endodermal differentiation, whereas ESC colonies on larger patterns (400-800 µm) retained their self-renewal capability as indicated by high expression levels of Oct4 [84]. Hence, it is clear that special care must be taken when designing novel biomaterials through the right balance of shape, area, and aspect ratio of their topographic features.

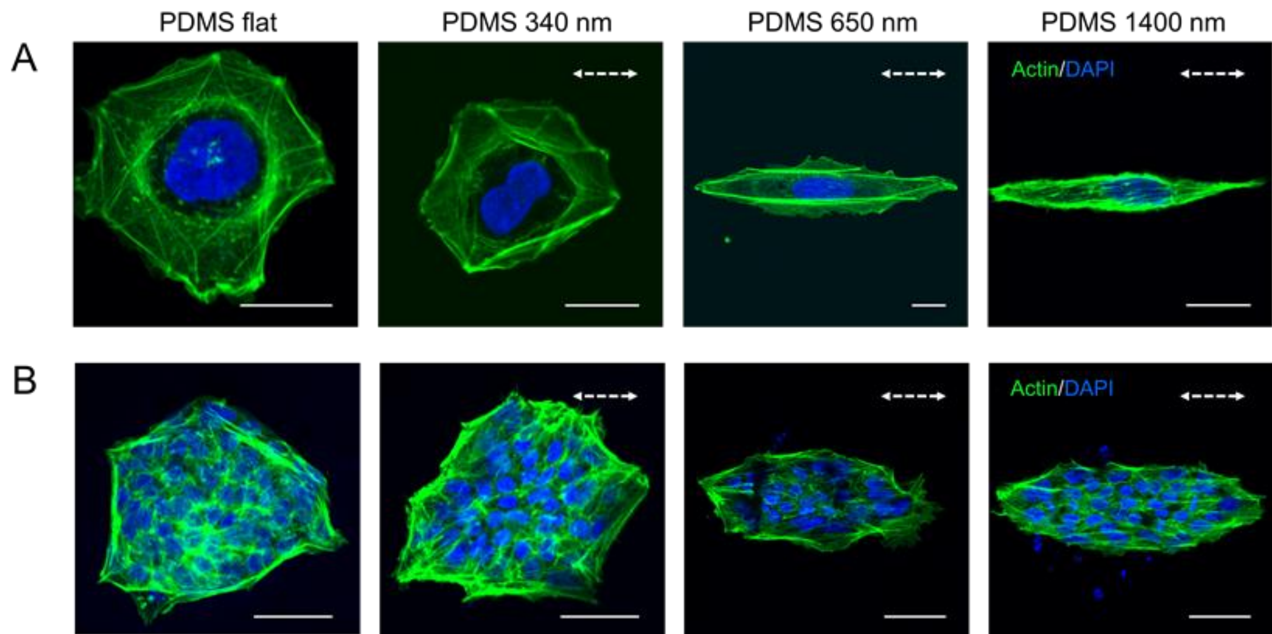

Figure 3. Grooves affect morphology of single iPSC and of iPSC colonies.
Confocal microscopy images of single iPSC **(A)** and iPSC colonies **(B)** seeded on PDMS substrates structured with grooves of different periodicities (nm periodicity is indicated). Cells were stained for actin (green) and nuclei (blue). The dotted white arrow always indicates the direction of the sub-micron grooves. Scale bars = 20 µm for (A) and 50 µm for (B). Further details are provided in Abagnale et al. 2017 [83].

Effects of ligand spacing

Ligand spacing is of central relevance when analysing effects of biomaterial topography. Seminal studies used hexagonal arrays of gold nanoparticles functionalised with a peptide containing the cell adhesion sequence arginine-glycine-aspartic acid (RGD) demonstrating that spatial confinement of $\alpha v\beta 3$ integrins is crucial for cell adhesion and spreading on biomaterials [85, 86]. Specifically, the murine osteoblast precursor cell line MC3T3 poorly adhered to surfaces with ligand spacing equal or bigger than 73 nm [85]. Furthermore, larger ligand spacing (108 nm) induced faster focal adhesion turnover and enhanced lamellipodia protrusion velocity [86]. In line with these observations, human hematopoietic stem cells better adhered to the biomaterial surface when the ligand spacing was less than 20 nm and not at all for spacing of 58 nm [87]. As cell spreading and stretching is linked with differentiation processes, ligand spacing may also affect cell fate: alginate was chemically modified with RGD peptides and osteogenic differentiation of MC3T3 cells was more prominent if RGD islands were spaced by 36 nm as compared to 78 nm [88]. In a more comprehensive study, Frith and colleagues demonstrated that osteogenesis of human MSC is reduced on surfaces with increased lateral RGD ligand spacing while adipogenic differentiation is increased [89]. It needs to be taken into account that ligand spacing may also be modified by protein adsorption from the culture media or secreted ECM products by the cells themselves.

Impact of compression and stretching on stem cell fate

In 1892, Julius Wolff already hypothesised that bones adapt to received loads and that increasing loads result in bone remodelling and finally stronger bones – which is today known as Wolff´s law [90]. During the past 50 years, it has become clear that compression forces *in vivo* – in particular compression forces applied at high frequencies – have an impact on cell fate decisions, thus influencing bone homeostasis, growth, and osteogenesis [91, 92, 93]. In contrast, the lack of forces, as in the case of the weightless state during space travels, results in bone loss, amongst other severe physiological problems [94, 95]. In this regard, *in vivo* studies with unloaded hind limbs of rats demonstrated inhibited expression of osteogenic-related *RUNX2*, while adipogenic-related *PPAR-γ* expression was increased [96, 97].

Stretching of substrates with cultured cells has a strong impact on stem cell behaviour. Flexible matrices can usually be stretched uniaxially, biaxially, or equiaxially with defined frequencies and tensional strength. In terms of stem cell differentiation, mainly pathways of the myogenic fate are affected by stretching strategies. It has been shown that myogenic differentiation of MSC can be induced by cyclic stretching without any addition of soluble factors [98]. In addition, stretching stimulated tenocytic differentiation of MSC, identifying RhoA/Rock together with cytoskeletal organization and Focal adhesion kinase (FAK) as relevant signalling factors [99]. Other relevant signals involved in stretch-induced differentiation processes are microRNAs, e.g., miRNA-21 [100], and NFκB activation, which increase resistance of MSC to apoptosis and enhance their angiogenic capacity after mechanical stretching [101].

Besides MSC, pluripotent stem cells have demonstrated to be affected by stretching, inducing maturation towards cardiomyocytes with cyclic uniaxial stretching [102]. Human embryonic stem cell-derived cardiomyocytes (hESC-CM) were cultured on absorbable gelatin sponges which were either cultured under static conditions or stretching culture conditions in which they were uniaxially cycled between stretched (displacement of 12% with a frequency of 1.25 Hz) and relaxed state. Cyclic stretching led to increased expression of genes and proteins associated with mature cardiomyocytes in hESC-CM and higher beating frequency compared to the static control [102]. Similarly, static and dynamic stretching of hESC-CM embedded in collagen strips (5% displacement at 1 Hz frequency) mediated higher expression of integrin ß1 and vinculin, apart from better cell alignment, longer sarcomere formation and augmented gene expression levels of maturation markers [103]. These studies show that controlled stretching can lead to more mature hESC-CM preparations, however, mechanical stimulation alone does not lead to fully functional cardiac tissue as in ventricular muscle [103].

Compression and stretching forces can be modulated in mechanoreactors with precise amplitude and frequency. Osteogenic differentiation of MSC seems to be particularly favoured by cyclic compression of MSC seeded on a polycaprolactone-tricalcium phosphate (PCL-TCP) scaffold with a frequency of 1 Hz for four hours a day, similar to physiological conditions [104]. The very same conditions, however, can result in chondrogenic differentiation of MSC, when changing the scaffold material to Polyvinyl-acetat-poly(ε-caprolactone) (PVA-PCL) and adding TGF-β_3. In addition to the type of substrate, the magnitude of dynamic compressive strain also has a strong impact on the fate of MSC. Compressive strains up to 10% supported osteogenic differentiation, while 15% and higher

strains rather directed cells towards a chondrogenic lineage [105]. In contrast, other groups demonstrated that compression with a lower magnitude of strain (5%) at a lower frequency (0.1 Hz) also resulted in chondrogenic differentiation of MSC [106]. While MSC embedded in a PEG-based hydrogel chose a chondrogenic lineage due to a 10% strain at 1 Hz with or without the presence of transforming growth factor 1, chondrogenic differentiation of ESC-derived (human embryoid body-derived) cells was only enhanced by mechanical stimulation in the presence of the directing growth factor [107]. In addition, besides mechanical stimulation, the biofunctional domains inside the gel modulate mechanotransduction [108, 109]. Thus, although critical parameters can be precisely controlled in bioreactors, the results presented in the literature are difficult to compare due to differences in patient-specific cells, tissue of origin of the cells, biomaterial substrates, and the parameters of mechanical stimulation.

Impact of shear stress on stem cell fate

Besides being subjected to stretch and compression, cells are also notoriously influenced by shear stress that arises by flow of culture medium inside bioreactors/perfusion chambers. For example, particular profiles of shear stress led to activation of different mitogen-activated protein kinase (MAPK) signalling pathways inside MSC [110] and osteogenic differentiation of MSC [111]. In a systematic study, defined shear stress parameters including magnitude, frequency, and duration were varied, demonstrating that a 2 Pa shear magnitude at 2 Hz frequency reliably guided MSC towards an osteogenic fate [112]. Furthermore, it has been suggested that iPSC derived endothelial cells cultured in perfusion reactors under low shear rates ($\leq$5 dyne/cm^2) or high shear rates ($\geq$10 dyne/cm^2) show venous or arterial characteristics, respectively [113, 114].

To translate mechanical stimuli, including shear stress, osmotic force, or fluid flow, from the outside into a biochemical signal inside the cell, primary, non-motile cilia have been identified as mechanosensory organelles that reach from the cell surface into the local environment [115, 116]. Therefore, inconsistencies in cell differentiation upon application of shear stress might be due to the fact that not all stem cells equally express cilia. For instance, it has been shown that only 1% of ovine bone marrow cells possess primary cilia [117]. On the other hand, human MSC, which were applied to oscillatory fluid flow, showed increased osteogenic marker expression and higher proliferation rates mediated by mechanotransduction through primary cilia [115]. In accordance, deletion of kinesin family member 3A (Kif3a), a cilia-specific gene in bone marrow-derived MSC, transplanted into mice, led to disruption of cilia and thus decreased amounts of bone formation upon loading [118]. Therefore, primary cilia may play a significant role in stem cell differentiation induced by shear forces, especially for osteogenic differentiation of primary MSC.

Impact of timing and duration of mechanical stimuli on stem cell fate

One relevant factor for cell-fate decisions is time. It could be demonstrated that stem cells, cultured either within or on top of hydrogels not only sense the changes in stiffness but can also memorize previously exhibited properties, depending on the corresponding time scales [119, 120]. Thus, cellular differentiation is highly dependent on the duration of exposure to mechanical signals. Therefore, so called 4D scaffolds are developed to study the effect of material changes in form and function after the scaffold has been manufactured. Using this

technique, it has been shown that MSC cultured in soft hydrogels increased their spreading area and traction forces when the material was stiffened. The longer MSC were cultured in soft gels before stiffening, the more cells became primed toward adipogenic lineage [121]. Similarly, studies using photo-tuneable hydrogels revealed that MSC grown on a stiff hydrogel for 1 day before gel softening became adipogenic, while culturing them on a stiff gel for 10 days led to irreversible osteogenesis and nuclear YAP translocation. The authors further claim that extensive culture on stiff substrates leads to long-term storage and activation of YAP in the nucleus even after transfer to de-activating, soft hydrogels [120]. Alternatively, shape memory polymers (SMPs) are applied to enable a one-time mechanical stimulus on adherent cells [122]. Scaffolds fabricated from an aromatic shape memory polyurethane were first stretched to 50% strain at 65°C and their temporary shape was fixed at 4°C. Subsequently, temperature increase to 37°C initiated shape recovery. MSC cultured on these scaffolds showed elongated shape along the direction of the scaffold fibre upon stretching, even one day after shape recovery [122]. Even though these studies indicate that stem cells possess mechanical memory, changes in the mechanical properties of biomaterials are often irreversible, prohibiting determination of the time window, in which stem cells are the most mechano-sensitive. Recently, a hydrogel was developed with the ability to alter its stiffness in a reversible manner over a time span of 9 hours, using complementary DNA strands [123]. The dynamic hydrogel system elucidated that the temporal window to mechanically induce neurogenesis in neuronal stem cells by gel softening is between the first 12 to 36 hours of receiving chemical differentiation cues. This sensitivity was associated with reduced levels of both nuclear and cytoplasmic transcriptional coactivator Yes-associated protein (YAP).

Mechanisms of mechanotransduction

Cells interact with the matrix via α/β-integrin heterodimers. For human MSC, particularly α2- and α5-integrins seem to be involved in osteogenic differentiation [124, 125]. Interaction of human MSC with stiff substrates led to upregulation of α2-integrins, whereas their functional inhibition reduced the expression of osteogenic markers [125]. On a matrix of intermediate stiffness (~ E = 10 kPa), β3-integrin accumulates at focal adhesions [126], whereas soft substrates promote the activation and internalisation of β1-integrins [127]. These observations suggest that integrin receptors play a key role in matrix sensing. However, the relevance of their composition and further downstream signalling cascades can hardly be generalised as individual studies used different mechanical stimuli, cell types, and specific molecular readouts.

Analysis of relevant intracellular signalling cascades is particularly challenging because the different pathways are usually tightly interconnected and vary with cell cycle and growth conditions (Figure 4). Several studies demonstrated that signalling via the Ras homolog gene family member A (RhoA) is essential for acto-myosin contractility (i.e., actin cytoskeleton tension) and is thus crucial for both promoting osteogenic differentiation and inhibiting adipogenic differentiation of human MSC [128, 74, 129]. Notably, RhoA signalling leads to the inhibition of pluripotency-related signalling cascades and reduced expression of the pluripotency markers NANOG, SOX2, and OCT4 in human iPSC [130]. Apart from RhoA signalling, extracellular signal–regulated kinases 1 and 2 (ERK1/2) and mTOR Complex 2 (mTORC2) signalling cascades have been identified as pathways involved in

mechanosensing. For instance, both pathways have been demonstrated to contribute to osteogenic differentiation of human MSC [131, 132].

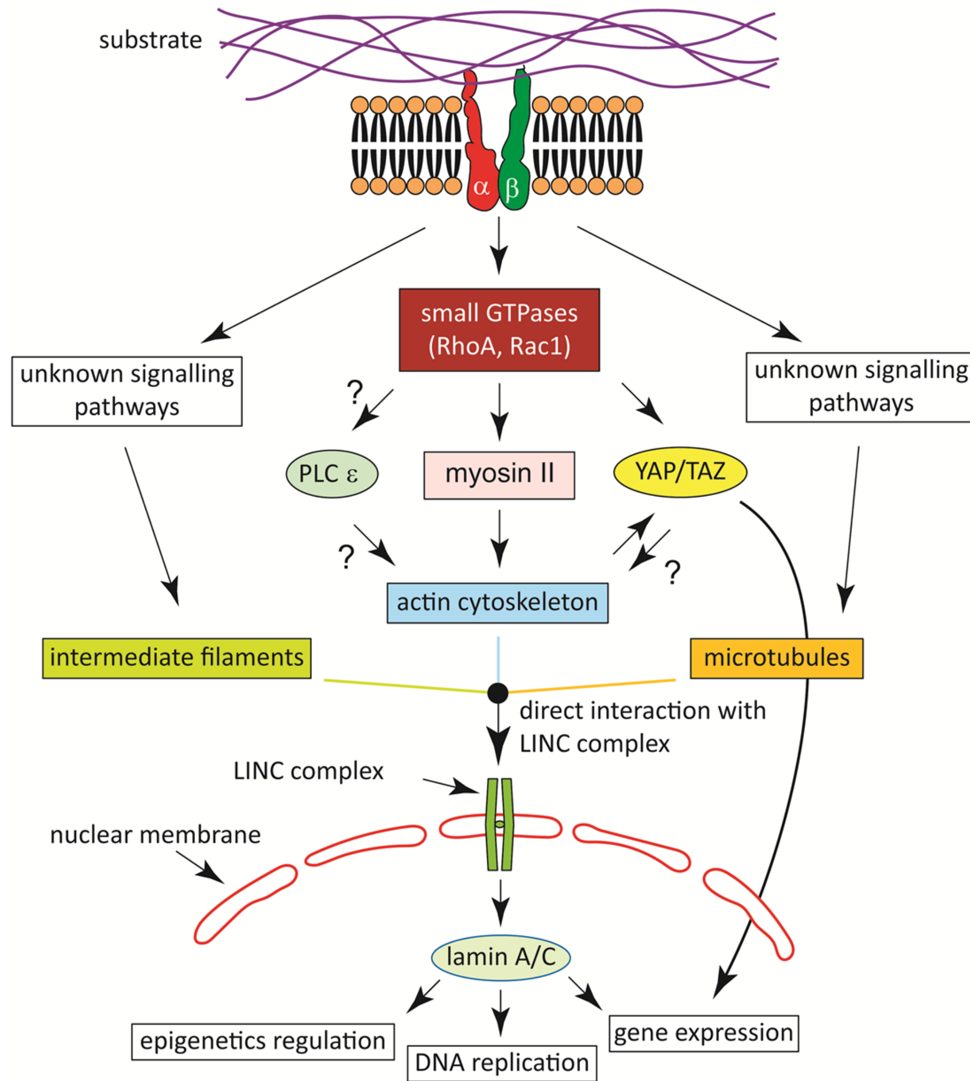

Figure 4. Exemplary depiction of mechanosensing pathways.
Interaction of cells with the extracellular matrix is mediated through α/β-integrin heterodimers. It has been shown that integrins are involved in activating RhoA signaling (amongst other potential pathways), which in turn leads to acto-myosin contractility. The linker of nucleoskeleton and cytoskeleton (LINC) complex interacts with the actin cytoskeleton leading to nuclear lamin A-mediated DNA replication, alterations in gene expression, or epigenetic changes in the nucleus.

Concerning the cell's interpretation of the mechanical stimulus, it was shown that zyxin, a protein associated with focal adhesions, localises to actin stress fibres and the nucleus at high mechanical forces (i.e., actin cytoskeleton tension) but remains confined to focal adhesions at low mechanical forces [133]. Similarly, the tumour suppressor proteins YAP and TAZ, both downstream targets of RhoA signalling, translocate to the nucleus in cells cultured on rigid matrices [134], a condition where cells are under great tensional force. Furthermore, nuclear accumulation of the cytoskeleton-associated protein paxillin has also been associated with mechanosensing [135]. In addition, it has been recently demonstrated that YAP/TAZ activity is regulated by β1-integrins through the RAC1-PAK1 signalling pathway and the interaction with merln/NF2 [136]. Finally, lamins, which are intermediate filaments that contribute to nuclear stiffness and stability [137], have also been implicated in the regulation of mechanoresponses. For example, lamin A levels were highly increased in human MSC cultured on a rigid matrix in osteogenic differentiation conditions, whereas lamin A was suppressed during adipogenic differentiation of MSC on soft substrates. This is in accordance with higher lamin A levels observed in stiff tissues (cartilage, bone) compared to soft ones (marrow, brain) [138]. Although many molecular key players associated with sensing of mechanical stimuli were identified, it is important to investigate and understand in more detail the function of the molecular switches with regard to specific mechanical stimuli and their effect on cell fate.

Conclusion

Several studies have shed new light on the fascinating molecular mechanisms of mechanosensing and the subsequent cell responses occurring at the single-cell level. These studies unequivocally demonstrate that mechanical stimuli direct stem cell function and differentiation. A better insight into these processes might help to design more sophisticated lineage-specific differentiation strategies, which is still a bottleneck in stem cell research. Current protocols for *in vitro* differentiation of stem cells are particularly based on specific culture media and growth factors – but so far these differentiated cell types remain molecularly and functionally distinct from their natural counterparts [139, 61]. A better understanding of mechanobiological interactions of cells with their environment could help to either preserve stem cell properties *in vitro* or to efficiently direct stem cell differentiation.

In general, mechanobiological research is hampered by a potpourri of parameters that impact the molecular and functional response of MSC and iPSC – and this becomes evident by a rapidly growing number of well controlled studies (Table 1). There are differences between species, cell types, culture conditions, biomaterial scaffolds, and mechanical stimuli that impede cross-comparison of studies. In addition, biomaterial-related parameters, such as surface chemistry, (visco-) elasticity, (nano-) topography, and 3D architecture are tightly interwoven and can hardly be addressed as separate parameters. Furthermore, it has to be taken into account that mechanical stimulation involves multiple force parameters such as amplitude, frequency, duration, etc. Timing is of particular relevance as cells may be more or less mechanosensitive if stimulated for different periods during differentiation. To further elucidate these time-relevant processes and better tailor and decouple individual parameters, new biomaterial concepts have to be engineered. Experimental settings have to be carefully controlled to change only one of many relevant parameters at the time. However, it always has to be taken into account that such custom-designed substrates might be difficult to

engineer and standardise. Finally, the functional and molecular readouts should be carefully chosen to enable quantitative and standardized comparison of different studies. These demanding tasks can only be achieved by interdisciplinary cooperation between biology, chemistry, medicine, physics, mechanics, material science and engineering.

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Table 1. Selection of studies investigating mechanical stimulation of MSC and PSC.

	Species	Cell type	Mechanical stimulus	Parameters tested	Duration	Readout	Ref.
(Visco-) Elasticity	Human	MuSC	Elasticity of PEG hydrogels	2-42 kPa elasticity	2-7 days	Cell survival, stemness, proliferation, engraftment potential	[50]
	Human	MSC	Elasticity of polyacrylamide gels	0.1-40 kPa elasticity	1-3 weeks	Morphology, protein and transcript profiles, cytoskeleton organization	[52]
	Human	MSC	Stiffness of fibre mats	140 MPa - 3.1 GPa stiffness	2 days	Cell proliferation and spreading, actin cytoskeleton, FA analysis	[35]
	Human	MSC	Elasticity of PDMS substrates	1.5 kPa and 50 kPa elasticity	3 weeks	Morphology, adipogenic and osteogenic differentiation, gene expression, and DNA methylation profiles	[44]
	Human	MSC, iMSC	Elasticity of hPL-gels	20-30 Pa elasticity	3-5 weeks	Morphology, chondrogenic, adipogenic, and osteogenic differentiation, gene expression and DNA methylation profiles	[61]
	Human	MSC	Elasticity of PEG hydrogels	1-50 kPa elasticity	11-14 days	Proliferation, adipogenic and osteogenic differentiation	[63]
	Mouse	ESC	Elasticity of PEG hydrogels with different soluble factors	0,2-2 kPa	5 days	Self-renewal capacity, proliferation, pluripotency assessment	[64]
	Human		Geometric cues with different area sizes	1,000-5,000 µm^2	1 week	Adipogenic and osteogenic differentiation markers	[73]
Topography	Human	MSC	Geometric cues with different aspect ratios	Aspect ratios of 1:1, 3:2, and 4:1	1 week	Adipogenic and osteogenic differentiation markers, cytoskeleton	[73]
	Human	MSC	Fibrin islands of different area size	1024-10,000 µm^2 area sizes	1 week	Adipogenic and osteogenic differentiation	[74]
	Human	MSC	Convex and concave curvatures	Spherical structures with 250 µm (principle curvature (κ) = 1/125µm^{-1})-750 µm (κ = 1/375µm^{-1}) diameters	1-10 days	Cell migration speed, cytoskeleton tension, osteogenic differentiation	[75]
	Human	MSC	Nano- and micro-size wrinkles	Amplitudes from 49-2561 nm, wavelengths between 464-7121 nm	2 days	Cell viability, cell elongation, cell alignment, F-actin alignment, filopodia length and alignment, FA analysis	[82]
	Human	MSC	Vertically aligned silicon nanowires	~9-26 nm length of wires, ~160-190 nm diameter of wires	1-3 days	Cell viability, spreading area, osteogenic differentiation, FA analysis	[79]
	Human	MSC	TiO_2 nanotubes of different diameters	Diameters of 30-100 nm	3 weeks	Morphology, extracellular matrix, osteogenic differentiation	[80]
	Rat	MSC	TiO_2 nanotubes of different diameter	Diameters of 15-100 nm	2 weeks	Cell adhesion, proliferation, osteogenic differentiation, FA analysis	[81]
	Human	MSC	Polyimide with nano- and macro-scale grooves and ridges	2-15 µm wide ridges with 650 nm periodicity	1-14 days	Morphology, proliferation, FA, adipogenic and osteogenic differentiation, gene expression profiles	[71]
	Human	iPSC	Polyimide with nano-scale grooves	200 nm high grooves with 650 nm periodicity	1-14 days	iPSC colony and single cell shape, cytoskeleton, spatial distribution of pluripotency factors, YAP/TAZ localization	[83]
	Human	ESC	Micro-patterned Matrigel substrates	Diameters of 200-800 µm	2 days	Colony size, pluripotency marker expression	[84]

Compression/Stretch	Human	ESC-CM	Uniaxial, cyclic stretch	12% stretch, 1.25 Hz	3 days	Troponin T expression, protein expression, proliferation, cell distribution, morphology, calcium cycle duration	[102]
	Human	ESC-CM	Uniaxial, steady or cyclic stretch	10% stretch, 1 Hz	3-14 days	Expression of cardiac troponin T, integrin ß1, and vinculin, cell alignment, elastic modulus, sarcomere length, expression of cardiomyocyte maturation markers, beating frequency and twitch force	[103]
	Human	PDLSC	Uniaxial, cyclic stretch	10% stretch, 1 Hz	6-48 hours	Morphology, osteogenic differentiation, analysis of miR-21 levels	[100]
	Human	MSC	Uniaxial, cyclic compression	0,22-1,10% strain, 1 Hz,	4 weeks, 4 hours daily	Cell proliferation, extracellular matrix, osteogenic differentiation	[104]
	Rabbit	MSC	Compressive deformation of PLCL scaffolds	Gel pressing with 5% strain at 0.1 Hz	10 days	Extracellular matrix, chondrogenic differentiation	[106]
Shear stress	Human	MSC	Oscillatory pressure driven flow	1-5 Pa, 0,5-2 Hz	2-4 hours	Osteogenic gene expression, collagen deposition, calcium expression	[112]
	Human	iPS-EC, HUVEC, HUAEC	Microfluidic system with variable shear stress control	0.07-21 dyne/cm^2	1 hour	Expression of *EFNB2*, *RPL32*, *CX43*, and *CX40*	[113]
	Human	iPS-EC, HUVEC, HAEC	Bioreactor system with variable shear stress control	5-10 dyne/cm^2	24 hours	Expression of genes associated with venous and arterial endothelial cells	[114]
	Human	BM MSC	Oscillatory fluid flow in flow chamber	Shear stress of 1 Pa (28 ml/min) – 2 Pa (56 ml/min)	2 hours	Formation of primary cilia, proliferation, osteogenic gene expression	[115]

This table exemplarily summarises studies that investigated mechanical stimuli applied to different stem cells as mentioned in the text. Please note that this selection is incomplete – and we apologize for not being able to highlight all relevant studies in this review. ESC = embryonic stem cells; iPSC = induced pluripotent stem cells; MSC = mesenchymal stem cells; iMSC = iPSC-derived MSC; MuSC = muscle stem cells; CM = cardiomyocytes; PDLSC = periodontal ligament stem cells; HUVEC = human umbilical vein endothelial cells; HAEC = human aortic endothelial cells; HUAEC = human umbilical aortic endothelial cells; iPS-EC = iPSC-derived endothelial cells; FA = focal adhesions; PEG = polyethylene glycol; PDMS = polydimethylsiloxane.