



Review

Environmental physical cues determine the lineage specification of mesenchymal stem cells

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ABSTRACT

Background: Physical cues of cellular environment affect cell fate and differentiation. For example, an environment with high stiffness drives mesenchymal stem cells (MSCs) to undergo osteogenic differentiation, while low stiffness leads to lipogenic differentiation. Such effects could be independent of chemical/biochemical inducers.

Scope of review: Stiffness and/or topography of cellular environment can control MSC differentiation and fate determination. In addition, physical factors such as tension, which resulted from profound cytoskeleton reorganization during MSC differentiation, affect the gene expression essential for the differentiation. Although physical cues control MSC lineage specification probably by reorganizing and tuning cytoskeleton, the full mechanism is largely unclear. It also remains elusive how physical signals are sensed by cells and transformed into biochemical and biological signals. More importantly, it becomes pivotal to define explicitly the physical cue(s) essential for cell differentiation and fate decision. With a focus on MSC, we present herein current understanding of the interplay between i) physical cue and factors and ii) MSC differentiation and fate determination.

Major conclusions: Biophysical cues can initiate or strengthen the biochemical signaling for MSC fate determination and differentiation. Physical properties of cellular environment direct the structural adaptation and functional coupling of the cells to their environment.

General significance: These observations not only open a simple avenue to engineer cell fate *in vitro*, but also start to reveal the physical elements that regulate and determine cell fate.

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1. Physical cues are important for the lineage specification of MSCs

MSCs were found to undergo osteogenic differentiation *in vitro* with supplements such as dexamethasone and β -glycerophosphate to the culture medium [1]. Later, MSCs were found to commit lipogenic, chondrogenic, and osteogenic differentiation *in vitro* induced by chemicals [2]. Dexamethasone, isobutylmethylxanthine, insulin, and indomethacin induce adipogenic differentiation; transforming growth factor β 3 prompts chondrogenic differentiation; while dexamethasone, β -glycerol phosphate, and ascorbate drive osteogenic differentiation [3–11]. Hence, chemical inducers play a major role in MSC lineage specification. It was unknown whether mechanical/physical cues could induce stem cell differentiation, though the extracellular matrix (ECM) properties were found to regulate cell shape, cell survival, cell

differentiation, and cytoskeletal mechanics [12–14]. Also, the chemically induced MSC differentiation involves the changes in cellular physical status such as stiffness and adhesiveness, and inhibition of these physical status changes impedes or reverses MSC differentiation [15].

ECM-controlled cell spreading can determine human MSC differentiation and fate through RhoA and Rho-associated protein kinase (Rock) signaling [16]. Osteogenic differentiation of MSCs requires extensive cell spreading and high RhoA activity; while adipogenic differentiation of MSCs needs limited cell spreading and low RhoA signaling [17,18]. MSC differentiation and fate can also be determined by the plasticity/stiffness and geometric cue of ECM microenvironment [19–21]. The MSCs spread on the ECMs with osteoid-like rigidity become bone, with intermediate stiffness commit to muscular lineage, and with brain-like softness undergo neuronal differentiation. MSCs form robust stress fibers and focal adhesions in response to rigid ECM microenvironment and fewer stress fibers and focal adhesions to soft microenvironment [19]. The rigidity of 3-dimensional (3D) ECM microenvironment can also regulate MSC lineage specification through altering integrin-ECM binding and ECM ligand distribution in microenvironment [22]. Thus, it is likely that microenvironment-induced reorganization of cellular/cytoskeletal force controls the differentiation and fate determination of MSCs.

Abbreviations: 3D, 3-dimensional; BMP, bone morphogenetic protein; ECM, extracellular matrix; FN, fibronectin; LN, laminin; MLC, myosin light chain; MSC, mesenchymal stem cell; PAAM, polyarylamide; PDMS, polydimethylsiloxane; PEG, polyethylene glycol; Rock, Rho-associated protein kinase

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2. Geometrical cue, mechanical cue, and biochemical cue: applications of hydrogel and elastomeric micropost

Cell-compatible hydrogels are natural, semi-synthetic, or synthesized polymeric materials that are engineered to resemble the extracellular environment of the body's tissues [23]. Changeable chemical composition and pliable physical properties of hydrogel make it an ideal *in vitro* model to simplify the study of complex biological conditions and events like MSC lineage specification. Modulation of the crosslinker quantity can selectively vary the physical properties of hydrogel such as stiffness and porosity without affecting the chemical composition of the gel.

For example, collagen-coated polyacrylamide (PAAm) gel induces the differentiation of MSCs and epidermal stem cells, and the stiffness or elastic modulus of PAAm gel regulates the fate commitment of these stem cells [21]. But the PAAm gels with different stiffnesses differ not only in gel porosity or topography but also in collagen-anchorage density. At constant stiffness, the concentration and distance of collagens that are either cross-linked to PAAm gel or embedded in polyethylene glycol (PEG) gel affect epidermal stem cell differentiation, suggesting that the stem cells exert mechanical force on surrounding ECM and gauge the mechanical feedback of the ECM for cell-fate decision [21]. This result, together with the observation that polydimethylsiloxane (PDMS) gels of different stiffnesses does not affect the differentiation and fate commitment of MSCs and epidermal stem cells [21], also excludes that stiffness is essential for stem cell differentiation. However, a recent study showed that varying porosity without altering stiffness of PAAm gel does not significantly change protein tethering, substrate deformations, or the osteogenic and adipogenic differentiation of human adipose- and marrow-derived MSCs [24]. Even with varied protein tethering, MSC lineage specification, surface-protein unfolding, or underlying substrate deformations remain affected. MSC differentiation is also unaffected even without protein tethering. Hence, based on this study, environmental stiffness can regulate MSC differentiation in protein tethering- and environment porosity-independent manner.

Despite of various advantages of hydrogels, hydrogel manipulation could alter surface chemistry, backbone flexibility, and binding property of gel-immobilized ligands. Micromolded elastomeric micropost array is an alternative approach to understand how cells sense changes in microenvironmental rigidity, which is controlled by hexagonally spaced PDMS microposts with different heights [25]. Elastomeric micropost array can decouple microenvironmental rigidity from adhesive and surface properties and correlate subcellular traction forces with focal adhesions. Bone lineage commitment is reflected by higher traction force and more focal adhesions of MSCs during differentiation, while fat formation is manifested by lower traction force and fewer or less developed focal adhesions [26].

The geometric cue that triggers cell spreading is more important for MSC differentiation than the size of cell spreading area [15]. MSCs grown in a confined region that 1) is elongated and spindle-shaped or with sharp edges and 2) favors focal adhesion formation and cytoskeleton organization will commit to osteogenic lineage, while MSCs grown in a relatively rounded region will become adipocytic. Moreover, sharp geometrically patterned edges generate high stress concentration and high density of focal adhesions [15,27].

Other mechanical factors also play roles in MSC differentiation. Under microgravity, stem cells tend to differentiate into adipocytes with the activation of lipogenic factors such as PPAR γ 2, while osteogenic differentiation is reduced [28]. Shear stress can also induce osteogenic differentiation [29]. Strain inhibits adipogenesis [30] but stimulates osteogenesis [31]. Cyclic compression can cause MSCs undergo chondrogenesis [32].

Together, 1) a microenvironment with geometry, stiffness, and ECM ligand that favor the development of stress fibers and/or focal adhesions preferentially induces the osteogenic differentiation of MSC and 2) a

microenvironment that inhibits focal adhesion or stress fiber favors adipogenic differentiation (Fig. 1). Although cytoskeleton reorganization and cytoskeleton-adhesion receptor connection are important in MSC differentiation and lineage specification [14,16,19,21], how the changes in stress fiber and focal adhesion alter MSC fate remain unclear. It is likely that soft ECM microenvironments cannot stabilize integrin-ECM binding, leading to integrin internalization [33]. Subsequently, focal adhesions could not form easily, stress could not be concentrated, and cells would not sense and transduce sufficient mechanical signals. Stiff ECM microenvironments make integrin-ECM binding relatively stable and stronger and allow more robust formation of focal adhesions and better tethering of stress fibers to the ECM-bound integrins at the plasma membrane [33,34] (Fig. 2).

3. Cytoskeletal tension and MSC lineage specification: application of cytoskeleton regulators

Cytochalasin D disrupts actin cytoskeleton by inhibiting actin polymerization and was used in several studies for the differentiation of different stem cells [35,36]. Disruption of actin cytoskeleton leads to adipogenic differentiation of MSCs and embryonic stem cells, accompanied by a decrease of Young's modulus of the cells during the course of differentiation. Young's modulus, also known as elastic modulus, is used to describe the stiffness or elasticity of materials. Actin cytoskeleton disruption in bone marrow stromal cells, which represent a population of multipotent MSCs, results in neuronal differentiation [37]. Therefore, with disrupted actin cytoskeleton, MSCs tend to differentiate into soft tissues such as fat and nerve, supporting that actin cytoskeleton organization can control MSC lineage specification.

Blebbistatin inhibits non-muscle myosin II [38] and promotes adipogenic differentiation of MSCs [39]. Non-muscle myosin II is required for actin fiber bundling and contractile force generation. Without myosin II, cytoskeletal tensile force and cellular contractility are diminished [40]. The contractility is important for cells to sense the stiffness of microenvironment, re-program the gene expression profile of MSC, and exert forces to deform ECM [19]. Rock inhibitor Y27632 enhances adipogenic and reduces osteogenic differentiation of MSCs [41], by inhibiting stress fiber and focal adhesion formations. Besides cytoskeleton-regulatory reagents, virus-mediated cellular delivery of RhoA, which promotes stress fiber and focal adhesion formations [42], and miRNA-mediated alteration of focal adhesion kinase level also regulate MSC differentiation [16, 43–45]. Again, the studies from cellular aspect support the notion that focal adhesions and stress fibers regulate MSC differentiation and fate determination.

Although disassembly of microtubules with nocodazole promotes bone morphogenesis and osteoblast differentiation [46], the mechanism is unclear. According to the tensegrity model [47], tension is accumulated in actin fibers while microtubules balance the tension so that the cell will not collapse. It is likely that, during osteogenic differentiation, the compression within microtubules is also increased to balance the elevated inward cellular tension from actin fibers.

4. Cell shape and MSC lineage specification

MSC differentiation can be controlled by the shape of MSCs [15, 48–50] (Fig. 3) and results in the cells with different shapes to form functionally different tissues [16]. For example, osteogenic differentiation leads to a flattened or spread cell shape while adipogenic differentiation a rounded or non-spread cell shape. The MSCs being adapted to a cell shape that increases actomyosin contractility promote osteogenesis [15], consistent with the finding that myosin inhibition reduces osteogenesis. Cell shape manipulation also alters Rac1 activity [49], which promotes the formation of actin meshwork and antagonizes actomyosin contractility; while Rac1 inhibition promotes osteoblastic differentiation [51]. In addition to osteogenic and adipogenic differentiation, cell

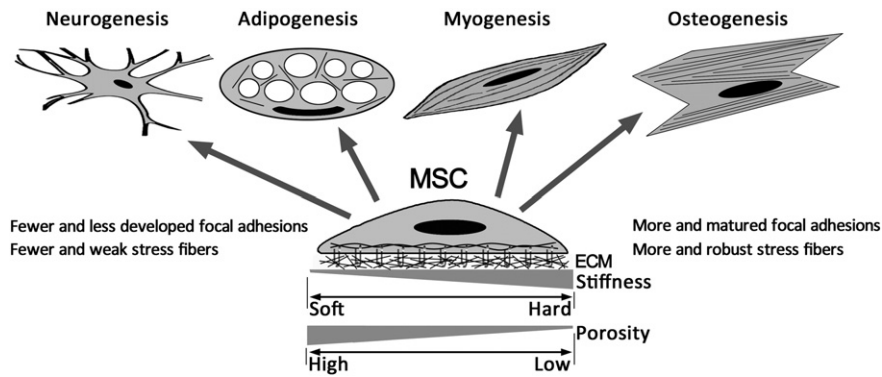


Fig. 1. Environmental physical cues regulate MSC fate and differentiation. MSC differentiation and lineage commitment can be controlled by physical cues such as the stiffness and topography of ECM environments, through the changes in actin cytoskeleton and cell adhesion structures.

shape also modulates myocardial [52], neuronal [53], and myogenic [52, 54,55] differentiation. Cell shape also contributes to the maintenance of differentiated cells. Chondrocytes, for example, gradually lose their cartilage phenotype when flattened [56].

Cell shape and intracellular tension are correlated. On the one hand, intracellular tension increases when cell spreading area is increased [25, 57]. The highest force tends to be localized to protrusions or corners when either non-stem cells [57] or MSCs [15] are cultured on a confined region. On the other hand, cytoskeletal tension regulates cell shape and

focal adhesion formation [58]. Since cell shape could directly reflect the physical environment of cells, the geometry of MSC niche is likely to play roles in MSC differentiation by, in parts, modulating cytoskeletal tension.

5. Physical cue-initiated signaling to specify MSC lineage

Cells on firm substrate tend to develop mature focal adhesions with extensive cell spreading, while cells on flexible substrate develop a

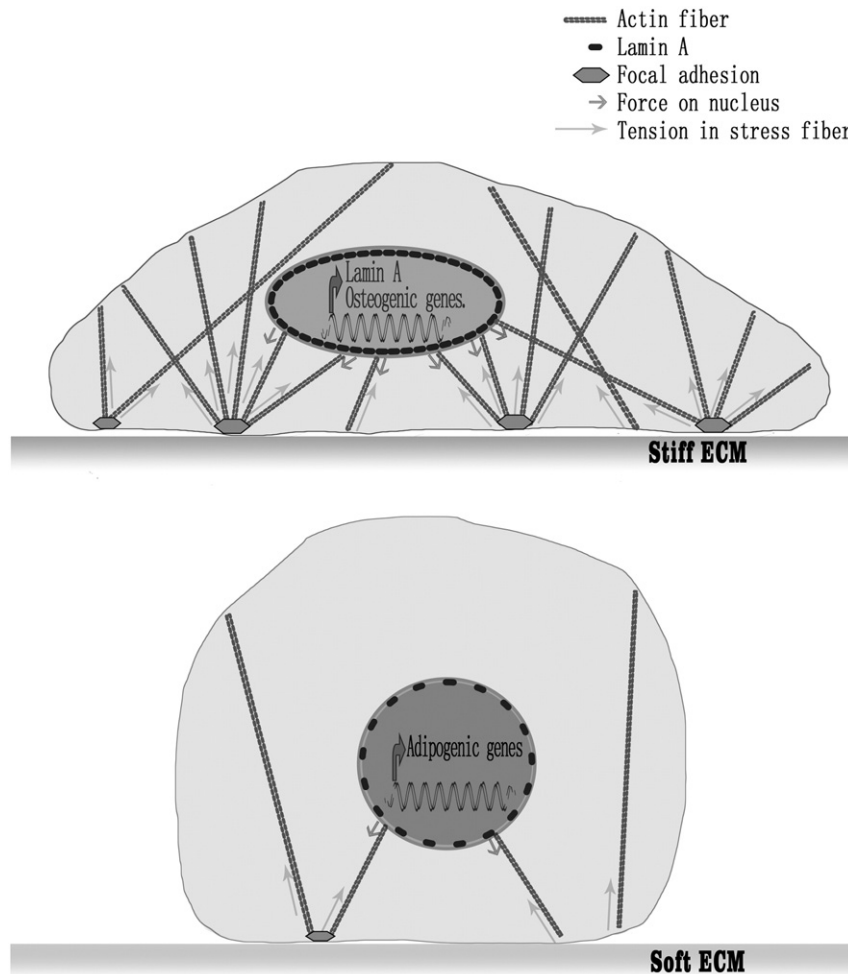


Fig. 2. Environmental physical cues affect gene expression by altering cytoskeletal and nuclearskeletal tension. ECM with high stiffness increases intracellular tension, leading to the deformation of nucleus and upregulation of lamin A and osteogenic gene expression. ECM with low stiffness decreases intracellular tension, leading to the upregulation of adipogenic gene expression.

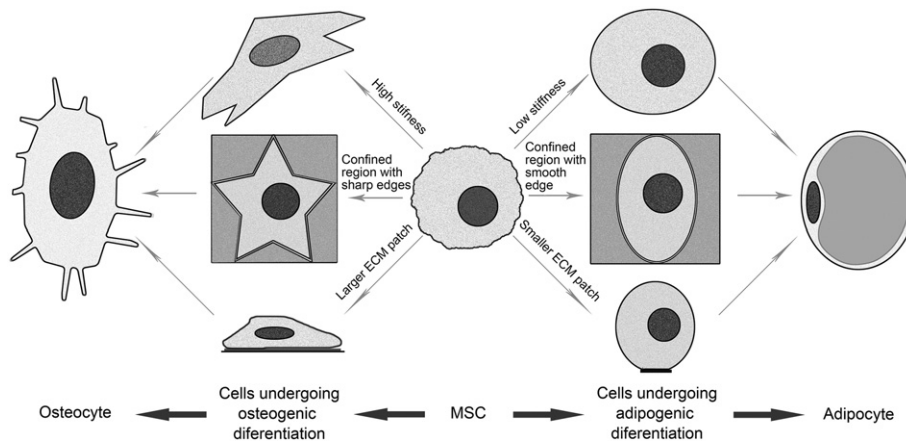


Fig. 3. Physical parameters of ECM regulate MSC fate and differentiation. MSCs positioned on ECM substratum with high stiffness, a confined region with sharp edges, or large size may undergo osteogenic differentiation; while MSCs positioned on ECM substratum with low stiffness, a confined region with smooth edges, or small size may undergo adipogenic differentiation.

relatively dynamic focal complex with limited cell spreading [59]. Focal adhesion formation requires stable binding of clustered integrins to ECM, while blocking integrins with antibodies or integrin binding to soft ECM makes the formation of stable and strong integrin-ECM bonds difficult [33,60]. In addition, stiff ECM induces more phosphorylation/activation of non-muscular myosin light chain (MLC) and subsequently increases intracellular stress or cytoskeleton tension [61], by forming more focal adhesions and stress fibers [47]. We predict that the cell adhesion strengthening process can also modulate MSC lineage specification by building up cytoskeleton tension. Tissue stiffness stabilizes nucleus-skeleton protein lamin A, which in turn upregulates the expression of stress fiber-relevant genes through serum response factor (SRF) and hippo signaling factor YAP1 [62]. Lamin A silencing enhances MSC differentiation on soft matrix to fat, while increases in lamin A levels enhance MSC differentiation on stiff matrix to bone. The retinoic acid (RA) pathway transduces the signal of matrix stiffness to nucleus to regulate lamin A transcription [62] (Fig. 2).

Signaling associated with osteogenic differentiation of MSCs includes RhoA/Rock/MLC, FAK, Ras/MAPK-ERK, NF- κ B, and bone morphogenetic protein (BMP) II [17,44,63,64]. The activities of lipogenic enzymes such as glycerophosphate dehydrogenase and fatty acid synthetase are increased in the adipogenic differentiation of 3T3-F442A cells [65]. In nucleus, lamin A physically stabilizes the nuclear lamina and chromatin against stress and impedes nuclear remodeling under stress [62]. Other mechanical cues such as cyclic compression also trigger osteogenic differentiation [19,66], reinforce cellular tensional structures [67], and thereby likely shares similar signaling mechanisms. However, why calcium deposition is linked to cytoskeleton reorganization remains unclear.

Adipogenic differentiation seems correlated with increased integrin activation and internalization and increased caveolae-mediated endocytosis of BMP receptor [33]. In a soft ECM environment, integrins are more easily internalized so that the formation of stable integrin-ECM bonds is inhibited. Consequently, it is difficult to assemble focal adhesions, form stress fibers, and then mount intracellular tension. In addition, BMP internalization makes the MSCs less likely to differentiate into bone cells. Cell shape, controlled by micro-patterned geometry, seems uncorrelated with adipogenic differentiation [68], and focal adhesions are inessential for adipogenic differentiation. In soft ECM, insulin receptor expression and signaling are enhanced [69], which strengthens the effects of insulin-induced lipogenesis. PPAR γ is required for adipogenesis [70]. Mechanical loading downregulates PPAR γ [71], and PPAR γ agonist reduces focal adhesion formation [72]. Thus, soft ECM likely activates PPAR γ , given that mechanical loading and PPAR γ activity are inversely correlated.

Integrin signaling, triggered by cell-matrix adhesion, apparently modulates MSC lineage specification, although specific and coordinated roles of individual integrins remain to be determined. Integrin α 2-mediated activation of Rock, FAK, and ERK promotes the osteogenic differentiation of human bone marrow-derived MSCs in stiffer ECM [73]. During adipogenic differentiation of mouse 3T3-L1 preadipocytes, integrin expression is differentially regulated, with a gradual decrease in the level of integrin α 5 and an increase in α 6. Overexpression of integrin α 5 increases proliferation and decreases adipogenic differentiation, while overexpression of integrin α 6 does not affect differentiation [74]. A functional-blocking antibody of α 5 β 1 integrin reduces both formation of bone nodules and expressions of osteogenic genes [75]. Blocking integrin α 5 β 1 increases adipogenesis and decreases osteogenesis of mouse bone marrow-derived MSCs [76].

6. Summary

Extracellular environments in tissues and organs have distinct physical properties, which directly regulate cell behaviors and fates. How biochemical and biophysical factors of microenvironment affect MSC lineage specification remains to be fully elucidated. A relatively better understood example is that tissue stiffness scales with the collagen content extracellularly, the actin cytoskeleton tension intracellularly, and the lamin A level intranuclearly. This directional signaling flow changes the gene expression profile of MSCs and then the cell fate.

In RGD peptide-conjugated 3D alginate gel system, the osteogenic differentiation of MSCs favors an environment with stiffness of $\sim$ 11–30 Kpa [22]. The osteoid without mineralization, which is composed primarily of collagen, has a stiffness of $\sim$ 27 Kpa [19] and provides appropriate mechanical cues for osteoblast differentiation, which in turn leads to osteoid mineralization during bone development or repair. Higher stiffness would inhibit osteogenesis in a 3D environment [22]. Bone has a Young's modulus of $\sim$ 10–20 Gpa [77], which results apparently from calcification after osteogenic differentiation. In contrast, adipose tissue has a Young's modulus of $\sim$ 3 Kpa [78], similar to the stiffness used *in vitro* to induce adipogenic differentiation of MSCs in a 3D environment [22].

In addition to being the crucial machinery that controls cell movement, morphology, division, and organelle transport, cytoskeleton is also a key determinant for stem cell differentiation and fate determination. Although cytoskeleton tension is critical in controlling MSC lineage specification [16,17], why and how cytoskeleton tension regulates MSC lineage specification are not understood. Cytoskeleton tension probably couples microenvironmental mechanical or geometric signals [47] to nucleus-skeleton reorganization. Indeed, lamin A becomes increased in response to extracellular tension, making the nucleus more resistant

to deformation [62]. Stem cell differentiation driven by cytoskeleton and nucleus tension is probably adaptation process that directs cell destiny suitable for the environmental physical properties. Also possibly, when the biochemical signals for differentiation such as hormones and growth factors are at suboptimal concentrations, environmental physical cues serve as a double-check mechanism that promotes the differentiation of stem cells toward specific lineages.

7. Conclusion

Like biochemical signals, the biophysical cues of MSC environment contribute to MSC fate determination and differentiation, which involve multiple signaling pathways. The signaling triggered by biochemical and biophysical cues is probably indistinguishable from each other, especially for downstream signaling events. The biochemical signaling of MSC lineage specification induces profound cytoskeleton reorganization and subsequent changes in cellular biophysical properties such as tension and adhesiveness; while biophysical changes in cytoskeleton and nucleus tension during MSC fate commitment and differentiation also affect and alter biochemical signaling. Thus, biophysical cues and factors i) generate, strengthen, or sustain biochemical signals for MSC lineage specification, to fine-tune the differentiation processes, and ii) play crucial roles in establishing the tissue structures proper for biological functions. However, how the stimulations from environmental physical cues are converted to arrays of cellular signaling remains unclear, how biophysical changes from cytoskeleton reorganization result in expression profile switch in the lineage-specific genes is largely elusive, and how the crosstalk between biochemical and biophysical signals needs further investigation.

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