

Review Article

Controlling Self-Renewal and Differentiation of Stem Cells via Mechanical Cues

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The control of stem cell response *in vitro*, including self-renewal and lineage commitment, has been proved to be directed by mechanical cues, even in the absence of biochemical stimuli. Through integrin-mediated focal adhesions, cells are able to anchor onto the underlying substrate, sense the surrounding microenvironment, and react to its properties. Substrate-cell and cell-cell interactions activate specific mechanotransduction pathways that regulate stem cell fate. Mechanical factors, including substrate stiffness, surface nanotopography, microgeometry, and extracellular forces can all have significant influence on regulating stem cell activities. In this paper, we review all the most recent literature on the effect of purely mechanical cues on stem cell response, and we introduce the concept of “force isotropy” relevant to cytoskeletal forces and relevant to extracellular loads acting on cells, to provide an interpretation of how the effects of insoluble biophysical signals can be used to direct stem cells fate *in vitro*.

1. Introduction

Cell therapies represent the most challenging and, potentially, the most successful application of stem cells (SCs). SCs are defined as cells with the ability both to self-renew and to differentiate into specialized cells, in response to appropriate signals [1]. Because of their ability to differentiate into different types of functional cells, SCs possess great potential in therapeutics to restore and regenerate native tissues. A typical strategy based on SCs consists in engineering tissues by using cells coupled with suitable biomaterials to mimic the *in vivo* biochemical and biophysical microenvironment [2]; this approach has shown promising results in treating irreparable damage of native tissues caused by diseases or injuries [3]. However, before SC-based therapies are applied in clinics, a fundamental issue needs to be elucidated to gain a precise control over SCs response, in terms of self-renewal and differentiation, specifically, a broader understanding of the interplay between SCs, the surrounding microenvironment components (growth factors, cell-cell contacts, and cell-extracellular matrix interactions), and external forces [4], which is currently lacking.

Recent studies have shown that mechanical cues, including the stiffness of the substrate, the nanotopography of the adhesion surface, and extracellular forces, are able to direct stem cell fate *in vitro*, even in the absence of biochemical factors [5–7]. The goal of this paper is to offer a biomechanical perspective of the biological response of SCs to these cues; in particular, we describe the existing literature on the effects of purely mechanical cues on SC response *in vitro*, and we provide an interpretation of how these known factors can be used to control SC fate. To this purpose, we introduce the concept of “force isotropy” relevant to cytoskeletal forces and relevant to extracellular loads acting on cells.

2. Stem Cell Sources

Stem cells are characterized by two unique properties with respect to specialized cells; *in vivo*, they are able to divide by maintaining their stemness; this process is termed self-renewal. Additionally, all stem cells retain the capacity to progressively differentiate into mature cell types.

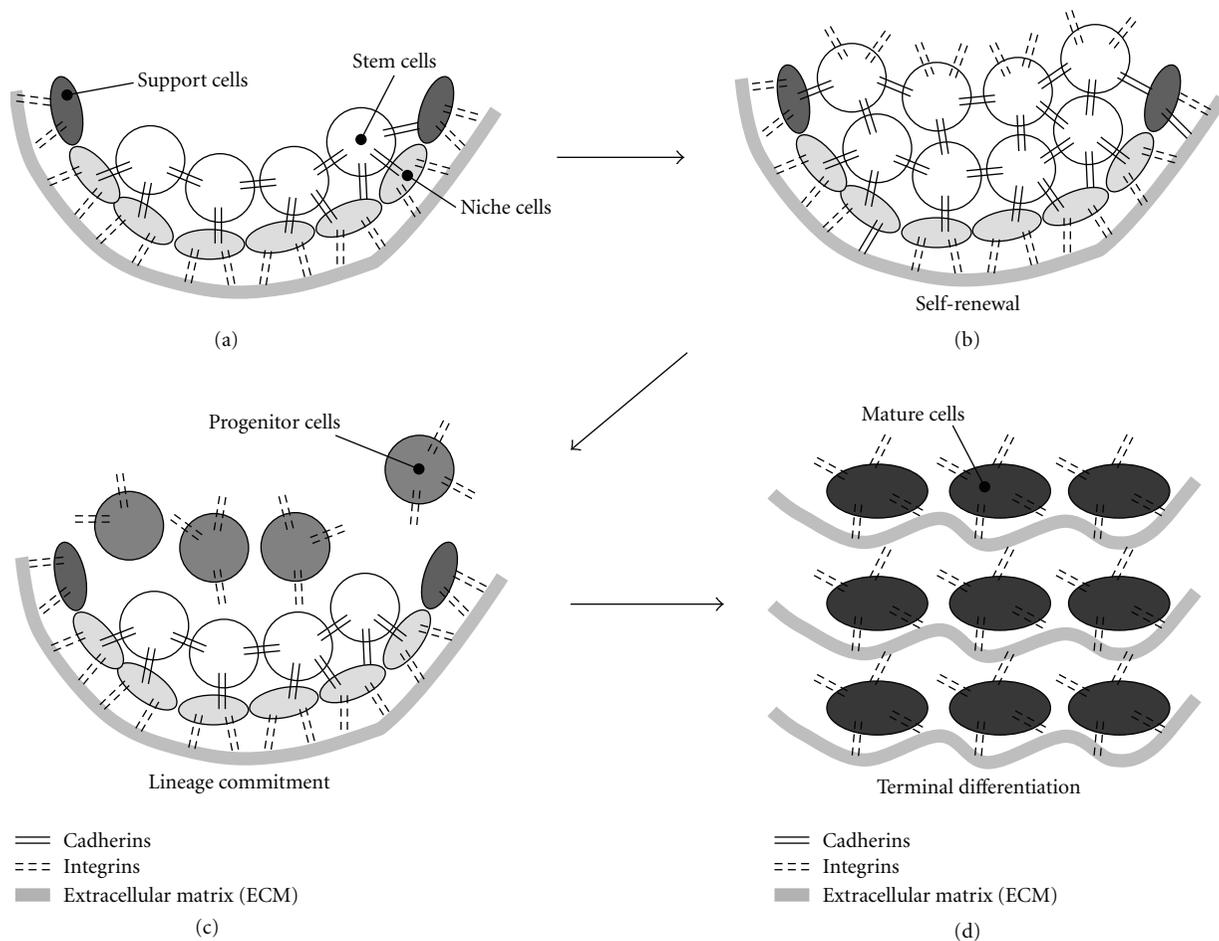


FIGURE 1: The adult mammalian stem cell niche is defined as a microenvironment that facilitates (a) the survival and (b) the self-renewing capacity of the stem cells, as well as (c) the production of actively dividing precursors leading to the generation of a differentiated progeny. The stem cells retain their function as long as they remain anchored to the supporting cells and their divisions occur in such a way that one daughter cell keeps its contact with the supporting cell while the other one loses this contact, migrates from the niche, and (d) proceeds to generate terminally differentiated cells.

Stem cells are often classified into three categories, based on the source from which they are obtained.

- (1) Embryonic stem cells (ESCs) are obtained from embryonic sources and were first isolated through the *in vitro* fertilization of preimplantation blastocysts [14]. They are characterized by their high telomerase activity and pluripotent differentiation potential.
- (2) Adult stem cells (ASCs), such as mesenchymal stem cells (MSCs), are multipotent progenitor cells that can be isolated from a variety of adult tissues including bone marrow, bone, skin, blood vessels, and muscle [15]. They have been shown to differentiate into various cell types, characteristic of the tissues in which they reside [2]. ASCs in somatic tissues represent cellular stores capable of maintaining tissue homeostasis, regenerating tissue, and maintaining organ function [16].
- (3) Induced pluripotent stem cells (iPS) are obtained by genetic reprogramming of somatic differentiated cells

into a dedifferentiated state resembling embryonic stem cells [17–19]. These cells represent a promising method of obtaining autologous pluripotent stem cells sourced from adult tissues.

3. The Stem Cell Niche

The stem cell niche concept was firstly introduced in 1978 [20] and represents the natural microenvironment that surrounds stem cells; it can be defined as an anatomical and functional entity that plays a crucial role in maintaining tissue homeostasis and tissue repair and regeneration in case of injuries [16]. It is believed that the stem cell niche provides a complex array of physical signals, including cell-cell contacts and cell-matrix adhesions, and biochemical signals, such as growth factors, to stem cells in a temporal and spatial manner; the integration of both local and systemic cues in the niche guides these cells to proliferation and fate specification [21, 22].

The native stem cell niche (Figure 1(a)) consists of SCs adhered to a group of supporting cells, soluble environment factors at specific sites embedded in an extracellular matrix (ECM) serving as a mechanical support and also as a reservoir for cellular signalling molecules [23]. The ECM is composed of tissue-type-dependent fibrous structural proteins (collagens, fibronectin, laminin, elastin and vitronectin) and glycosaminoglycan (GAG) network. Collagen and elastin, networks provide tissue with mechanical resistance to shear and tensile stress. GAGs, thanks to the presence of hydrophilic groups in their backbone, provide the swollen of the matrix, and consequently, the compressive strength to the tissue. Cells interact with ECM components that contain various binding domains; these interactions occur through receptors known as integrins [24–27]. Furthermore, cells interact with neighboring cells through receptors referred to as cadherins. The regulation of SCs activities by its surrounding microenvironment is related to these receptors that act as physical anchors able to activate cell transduction pathways [28]. As long as SCs remain constrained to supporting cells, self-renewal occurs and stemness is maintained (Figure 1(b)); when cell division takes place, one daughter cell keeps in contact with the supporting cells while the other one adheres to the ECM, migrates from the niche (Figure 1(c)), and generates a lineage of committed cells (Figure 1(d)). A further important issue in the natural stem cell niche is represented by oxygen concentration, which is a critical component able to regulate SC commitment [29].

4. Biochemical Control of Stem Cell Response

Self-renewal and lineage commitment of SCs have shown to be influenced by several cues, which can be subdivided into two classes: biochemical cues and biophysical cues. Biochemical cues are provided by reciprocal interactions between the cell, soluble bioactive agents, and the ECM.

Soluble factors include growth factors, morphogenetic factors, cytokines, enzymes, and small cell-permeable molecules, such as transforming growth factors (TGF), bone morphogenetic protein (BMP), vitamin C, sodium pyruvate, retinoic acids (RAs), and other small molecules [30, 31]. These factors, when added to the cell culture, or secreted by stem cells or niche cells, diffuse and bind to cell membrane receptors activating cellular signal pathways able to alter SCs gene expression [1, 32].

Regarding the biochemical cues provided by the reciprocal interaction between the cell and the ECM, a strategy that has been developed to modulate cell attachment, proliferation, and differentiation consists in the use natural biopolymers to build scaffold materials [33–38]. Also synthetic biomaterials have been exploited. A property that has been shown to regulate SCs activity is chemical functionalisation of the substrate surface, for example, by anchoring monomers representing the ECM binding sites, including RGD and IKVAV [39–48], or other functional groups, for example, $-\text{CH}_3$, $-\text{NH}_2$, $-\text{SH}$, $-\text{OH}$, and $-\text{COOH}$ [49].

Even though the effect of biochemical cues, including soluble factors and ECM ligands [50–52], has been widely investigated *in vitro*, the regulation of self-renewal and lineage commitment of SCs by these key factors is still poorly understood and difficult to mimic; actually, in living systems, ECM components play a crucial role in the controlled delivery of molecular signalling molecules. Furthermore, the spatial and temporal organization of ECM adhesive ligands *in vivo* is finely tuned and difficult to achieve through engineering methods.

Besides applying strategies to control SC fate using biochemical factors, there is increasing evidence that mechanical factors are potent enough to control their fate *in vitro*. For instance, it has been demonstrated that the matrix elasticity can influence the lineage commitment of MSCs into neurons, osteoblasts, and myoblasts [53]. Other than the substrate stiffness, these mechanical factors include the surface topography of the biomaterial scaffold, its three-dimensional (3D) geometry, and the external forces applied to cells [4, 54–56], as will be discussed in the next section.

5. Mechanical Regulation of Stem Cell Fate

Cells have the ability to actively sense their microenvironment and to react to the properties of their surroundings [57]. Anchorage-dependent cells are able to anchor onto the underlying substrate through focal adhesions formed by clusters of proteins, including integrins, that are transmembrane cell adhesion proteins [45]. The cell cytoskeleton, mechanically linked to the focal adhesions, is a network of filamentous proteins that extends throughout the cell cytoplasm in eukaryotic cells, and consists of actin, microtubules, and intermediate filaments [58]. The mechanical connections between the matrix and the cytoskeleton allow cells to exert traction forces that are transmitted to the cell nucleus through intracellular pathways; the resulting force triggers signalling transduction into biochemical signals that affect SC response, for example, the synthesis of specific transcription factors in the nucleus [59].

Various mechanotransduction pathways have been proposed, including the Ras/MAPK, the PI3K/Akt, RhoA/ROCK, Wnt/ β -catenin, and the TGF- β pathways, which are generally integrin-based, and mechano-sensitive ion channels. An extensive review of known mechanotransduction pathways can be found in [60].

6. The Concept of “Force Isotropy”

At the single cell level, cell adhesion to a material with specific engineering properties, such as surface nanotopography, stiffness, and microgeometry, can induce cytoskeletal tensional states due to the complex system of traction forces exerted by cells on the surrounding microenvironment; as mentioned above, these forces trigger signalling transduction cascades that provide intracellular events that regulate cell behaviour [61]. The cytoskeletal tensional state at the generic time point depends on the balance between the intracellular actomyosin contractility and the reaction forces exerted

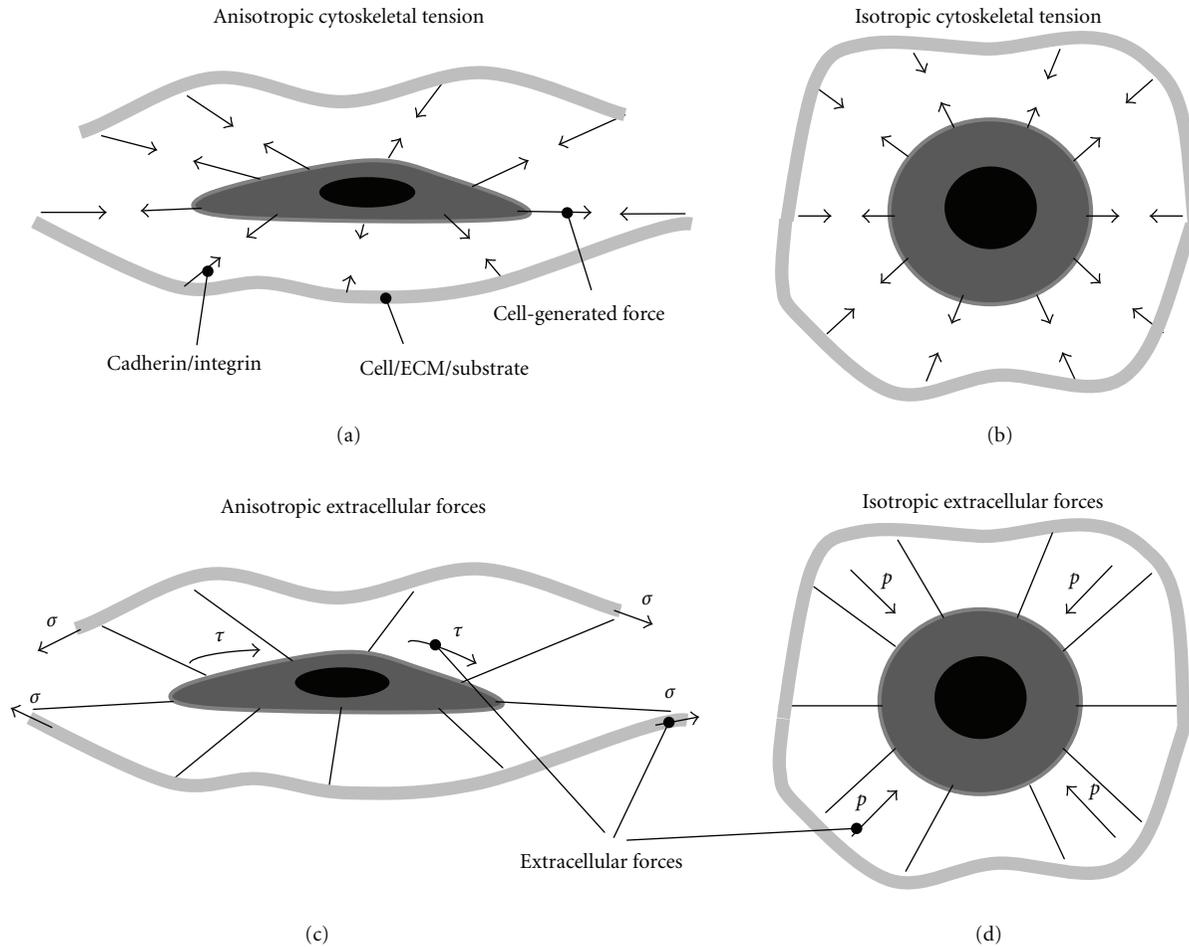


FIGURE 2: The concept of force isotropy applied to cells: (a) anisotropic and (b) isotropic cytoskeletal tension caused by cell contraction either in the niche, or during cell migration through the extracellular matrix; (c) anisotropic and (d) isotropic extracellular forces; σ is cyclic matrix stress, τ is cyclic fluid-induced shear, and p is cyclic pressurization.

by the underlying substrate, determined by its engineering properties. If cell adhesion-mediated traction forces, exerted by the cell on the matrix or adhesion substrate, have different magnitudes at varying spatial orientations, as shown by arrows in Figure 2(a), then the cell nucleus tends to elongate; this condition is what we define as “anisotropic cytoskeletal tension”. On the contrary, isotropic cytoskeletal tension is characterized by adhesion-mediated traction forces of similar magnitude at varying orientation (Figure 2(b)); in this condition, the cell nucleus tends to maintain a roundish morphology.

In addition to the cytoskeletal tension related to cell adhesion, also extracellular forces are able to influence SC commitment [56]; extracellular forces, including pressurization of interstitial fluids, flow-induced shear, and matrix strain, are dynamically superimposed to the cell cytoskeletal tension in native tissue. The concept of force isotropy may well be extended to extracellular forces. When adhering cells are subjected to anisotropic loads, including cyclic tension or shear stress (Figure 2(c)), or to isotropic loads, such as cyclic hydrostatic pressure (Figure 2(d)), they will respond to these forces, which are superimposed to cytoskeletal

tension, by dynamically varying the spatial distribution of focal adhesions, thus the nucleus shape. Increasing levels of anisotropy, both in terms of cytoskeletal tension and in terms of extracellular forces, can be obtained *in vitro* by applying engineering strategies, as will be discussed in the next section.

7. Engineering Strategies to Mimic Force Isotropy States

The ability of various types of cells to respond to mechanical differences in the extracellular environment has been thoroughly reviewed by Discher et al. [4, 54]. The different engineering strategies developed to study how the cytoskeletal tension and extracellular forces affect SCs response are summarized below.

7.1. Substrate Stiffness. A biophysical factor that has been proved to strongly affect adult SC behaviour is the substrates stiffness [62]. By varying the mechanical properties of the matrix (Figures 3(a) and 3(d)), for example from soft to relatively rigid by controlling the level of polymer

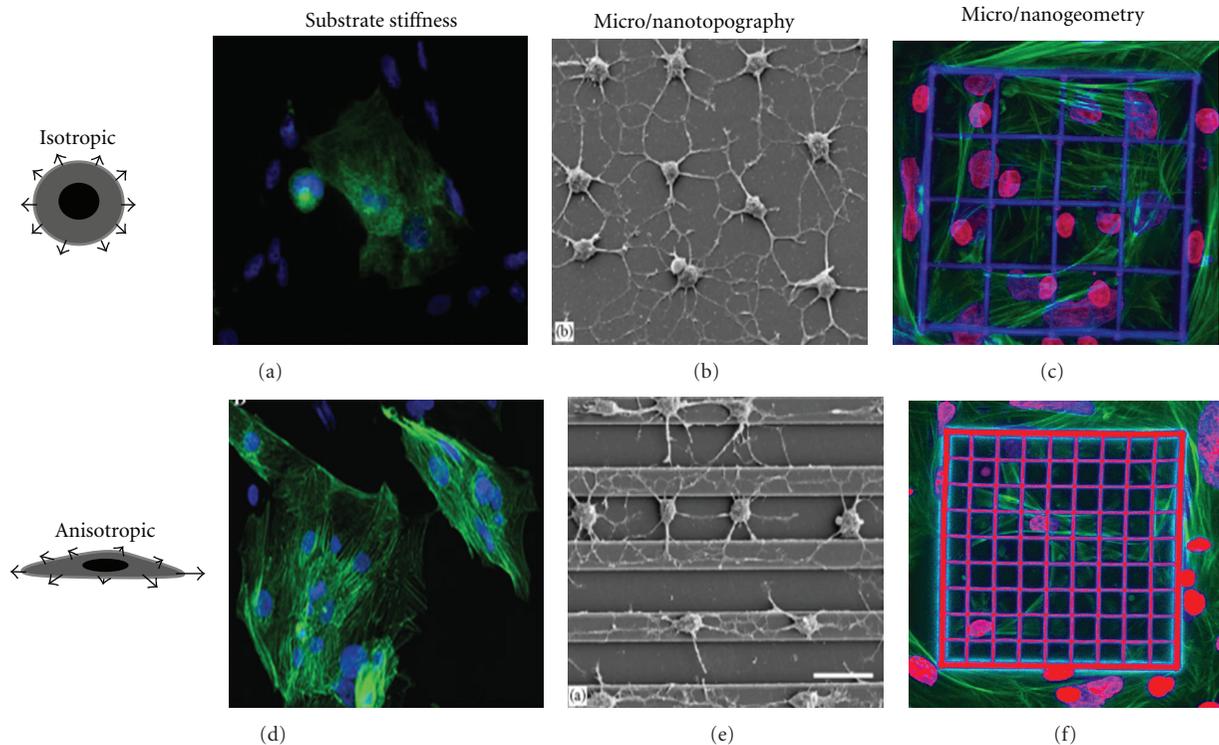


FIGURE 3: Examples of engineering strategies used to study the effects of anisotropic versus isotropic cytoskeletal tension on cultured stem cells. Substrates that mimic (a) soft versus (d) stiff ECM induce differential fates in human embryonic stem cell-derived cardiomyocytes, due to differential patterns of focal adhesions and actin-myosin stress fibres (in green, nuclei in blue). Reprinted and adapted from [8] with permission from John Wiley and Sons. In rat neural progenitor stem cells, (b) nonpatterned substrates induce formation of cell protrusions oriented randomly, while (e) micropatterned surfaces that mimic the native presentation and orientation of ECM proteins to cells induce alignment of elaborated processes in the direction of the grooves. Reprinted and adapted from [9] with permission from Elsevier. In rat mesenchymal stem cells, ultraprecise microscaffolds (c) with pore dimension of 20 microns allow for cell migration and isotropic attachment to the internal 3D lattice, whereas (f) for pores below 10 microns in size, cell migration into the scaffold pores is limited, inducing cell spreading on the top surface of the micro-scaffold (dividing nuclei in pink, cytoskeletal actin in green). Reprinted and adapted from [10] with permission from Elsevier.

crosslinking, it's possible to direct SC fate. Regarding MSCs, when they are cultured on soft substrates that mimic the elasticity of brain tissue (characterised by a stiffness of 0.1–1 kPa, in terms of Young modulus), neuronal precursors were generated; matrices with intermediate stiffness mimicking muscle (8–17 kPa) induced myogenic commitment while comparatively rigid matrices mimicking collagenous bone (25–40 kPa) proved to direct osteogenic lineage [8, 53, 63–70].

Substrate stiffness was found to also strongly influence ESCs behaviour. For example, in [71] it has been found that cell spreading increases as a function of substrate stiffness. In a further study [72], in which a wide range of substrate stiffness was investigated (41–2700 kPa), it was assessed that mouse ESCs cultured for 5 days on softer substrates were able to self-renew even in the absence of leukemia inhibitory factor (LIF).

7.2. Nanotopography. Another key factor that has been demonstrated to direct self-renewal and commitment of SCs is the surface topography (Figures 3(b) and 3(e)) at the

micro- or nano-scales (reviewed in Nikkhah et al. [73]). Various patterns combined with different adult SC types have been investigated: nanostructured surfaces [74, 75] affect self-renewal; nanogratings of 350 nm width compared to flat substrates can induce an upregulation on neuronal markers of MSCs [76] even in the absence of retinoic acid as a biochemical cue; neuronal commitment in human MSCs was also observed in nanofibrous scaffolds, with fibre diameter in the range of 230 ± 31 nm, using neural induction factors [77]. Myogenic commitment of MSCs in the absence of differentiating medium has been observed in aligned/nonaligned nanofibres having different average diameters [78] and in low roughness silk-tropoelastin scaffolds [79]. Grooves and grids, inducing cells alignment and elongation, show a greater effect on osteogenic differentiation compared to flat surfaces in MSCs [80–82]. Also random topographies have been exploited, including controlled disorder nanopits; the disordered ones were able to induce MSCs to differentiate towards osteogenic lineage [83–85]. The height of topographical features may influence MSCs differentiation [86] and adhesion [87]. In this study, the authors demonstrated that SC behaviour is a function

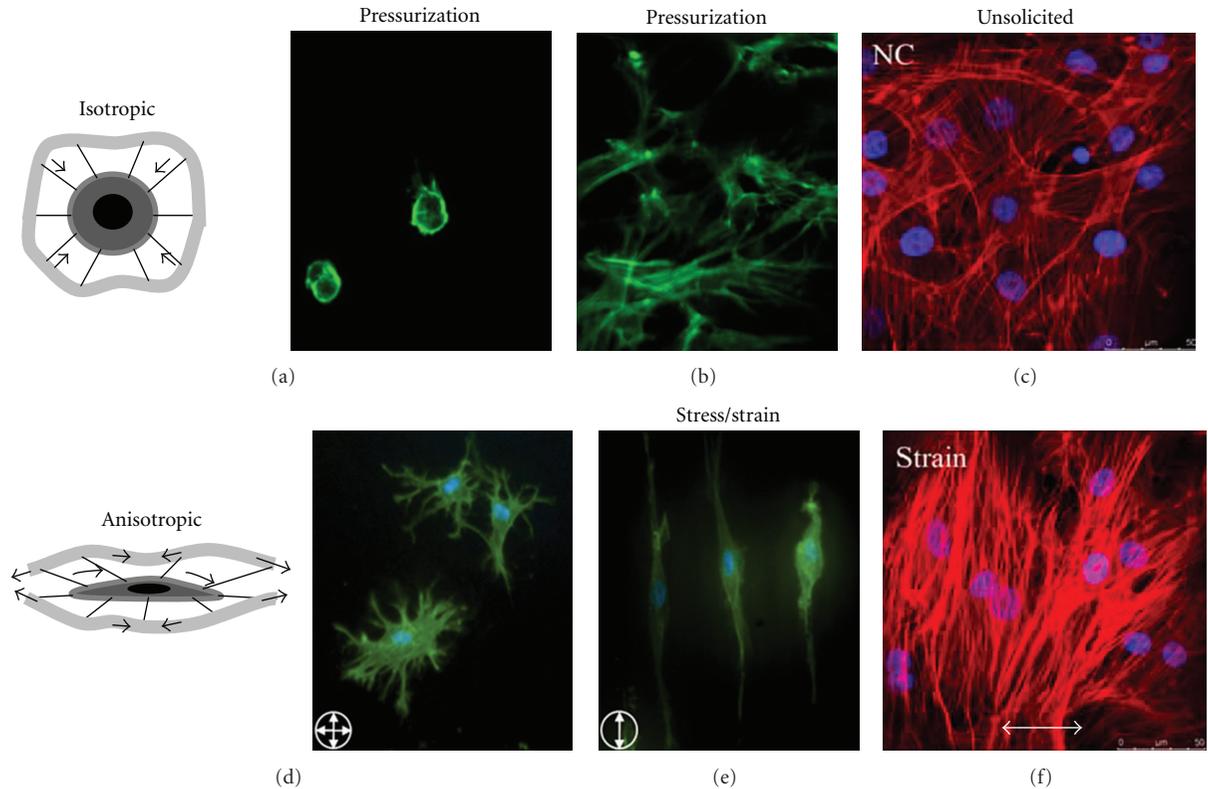


FIGURE 4: Examples of engineering strategies used to study the effects of anisotropic versus isotropic extracellular forces on cultured stem cells. Pig bone marrow-derived stem cells, subjected to cyclic hydrostatic pressure, (a) retain a spherical morphology when encapsulated in softer agarose, whereas (b) spread when encapsulated in stiffer fibrin hydrogels. Reprinted and adapted from [11] with permission from Elsevier. Rat bone marrow-derived stem cells (c) appear randomly distributed in unsolicited culture conditions, whereas (f) anisotropic strain induces cell elongation and upregulation of cardiomyocyte-related markers. Adapted with permission from [12]. In human bone marrow-derived mesenchymal stem cells and meniscal fibrochondrocytes cultured on a randomly organized nanofibrous scaffold, (d) biaxial strain induces a roundish shape of the cell nucleus, with small protrusion in all directions, while (e) uniaxial strain determines an elongated morphology of the cell nucleus. Reprinted and adapted from [13] with permission from Elsevier.

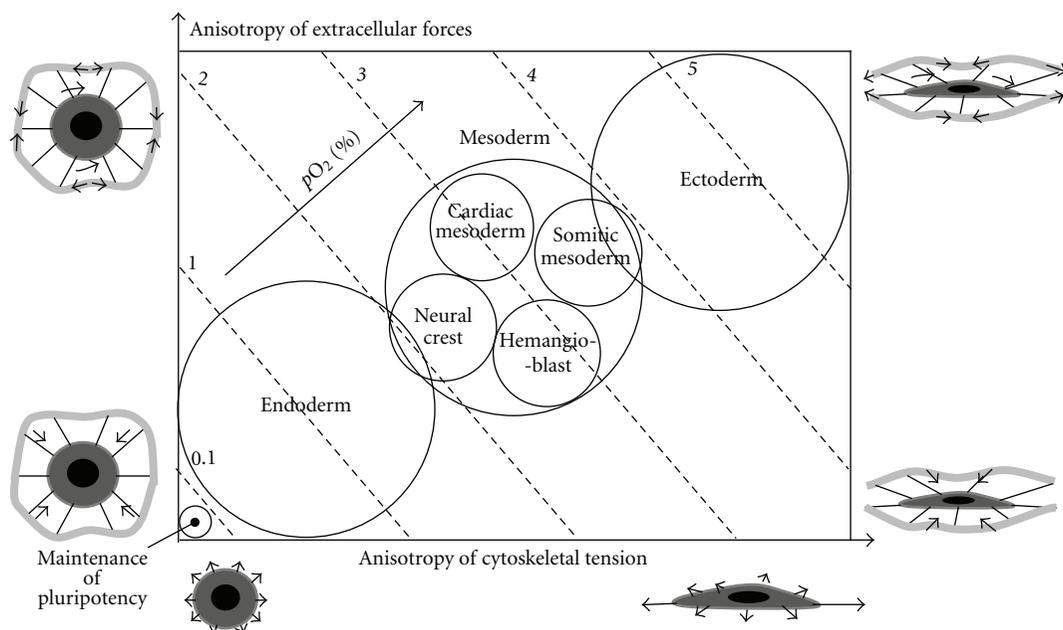


FIGURE 5: Synthesis of *in vitro* experimental evidence of the effects of mechanical cues and oxygen tension on embryonic stem cell fate.

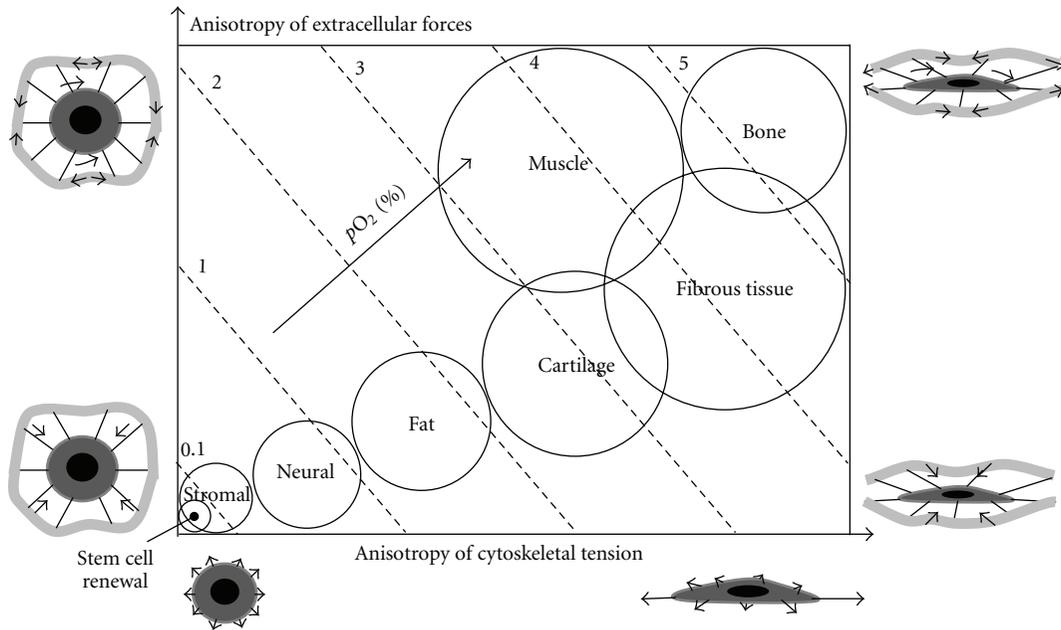


FIGURE 6: Synthesis of *in vitro* experimental evidence of the effects of mechanical cues and oxygen tension on the fate of mesenchymal stem cells derived from the endosteal compartment.

of pillar height and not of the diameter or gaps between nanopits. The pattern shape has been proved to regulate MSCs activities: in [88] round patterns aided adipogenic lineage differentiation while gratings induced elongation and osteogenic transition; also, in [89–92] geometric-specific proliferation and differentiation patterns were observed.

Regarding neuronal progenitor SCs, nanofibres of various size have been investigated and it was observed that cells grown on these fibres were able to differentiate into oligodendrocytes. The proliferation rate increased on smaller fibre diameter [55]. Neural differentiation was observed on grooves chemically modified with laminin [9] (Figures 3(b) and 3(e)).

Nano/microtopography has shown to affect also ESCs behaviour. In [93] mouse ES cell differentiation can be observed when these cells are cultured in the form of embryoid bodies. ESC morphology, including elongation, alignment, and proliferation can be induced by nanoscale grating features [94]. Micro- and nanopatterns of various geometries and sizes to study ESCs response have been exploited [95]; for example, in [96], circular micro domains (200 μm in diameter) were found to direct cardiac differentiation of uniform-sized mouse ESC aggregates. Also nanofibres have been investigated and it has been shown that they induce an upregulation of early development osteogenic markers [97].

7.3. Microgeometry. The three-dimensional (3D) microgeometry of the culture environment strongly contributes to direct cell phenotype [98]. Traditionally, mammalian and SCs have been cultured on 2D substrates. Differences in cell behaviour, including adhesion, migration, proliferation, and gene expression, have been observed between 2D

and 3D cultures. For instance, 2D culture confines cells to a planar environment and restricts the more complex morphologies observed *in vivo*; cells are able to interact through a limited membrane segment with the underlying substrate and neighbouring cells [99, 100]. As a consequence, the mechanotransduction process and the interaction with nutrients, soluble factors, and mechanical cues are altered [101]. These considerations lead an increasing interest in the development of truly 3D scaffolds to mimic the native environment in which cells reside [102]. Different 3D cell culture supports have been developed, including nanofibres, hydrogels, microwells, and other more complex structures (Figures 3(c) and 3(f)). Nanofibrous scaffolds were able to mimic the architecture formed by fibrillar ECM proteins [103, 104]. Another approach to surround cells with a truly 3D environment consists in suspending them in a hydrogel; for example, in [105] osteogenic commitment of rat MSC has been observed in these gels, although in the presence of osteoinductive medium. Very recently, photocurable materials have been used to fabricate 3D scaffolds by two-photon polymerization, a 3D microfabrication technique that allows to fabricate ordered microstructures having a submicron spatial resolution [106, 107]. Cell viability [108], homing, and proliferation were promoted by culturing MSCs on these structures [10, 109].

7.4. External Forces. Mechanical forces play an important role in SC adhesion, spreading, proliferation, migration, and differentiation [56]. External forces applied to the cell are able to influence the intracellular cytoskeletal arrangement thus regulating nuclear shape and cell activities. It is difficult to precisely determine the biomechanical and biochemical

mechanisms *in vivo* related to the cytoskeletal conformational changes. Biomimetic mechanical stimulation, including compression, stretching, and fluid-induced shear stress, have, therefore, been used to investigate these mechanisms *in vitro* [110].

Cyclic compression is able to alter MSC phenotype. MSCs subjected to dynamic compression or hydrostatic pressure (Figures 4(a)–4(c)) induced an increase of chondrocyte lineage markers and enhanced ECM deposition [111, 112].

Mechanical strain from applied loading, either unidirectional or biaxial (Figures 4(d)–4(f)), can direct specific stem cell lineages. For example, cyclic stretch has been demonstrated to commit MSCs to a myogenic phenotype [113] and mouse ESCs to a vascular smooth muscle cell phenotype [114]. Mechanical strain can also increase proliferation and inhibit differentiation in mouse and human ESCs [115], as well as promote cell alignment with respect to the direction of the strain [116].

Fluid-induced shear has a significant impact on the fate of SCs, as well. In two-dimensional (2D) systems, it induces osteogenic differentiation by activating multiple intracellular signalling pathways [117].

8. Mechanical Control of Stem Cell Response

In this section we provide a tentative interpretation of how mechanical cues can be used to control SC fate by applying the concept of force isotropy to synthesise the existing literature of this field.

8.1. Embryonic Stem Cells. As shown in various studies, a typical approach to mimic *in vitro* the native microenvironment of ESC consists in confining them, by the use of microwells or microarrays having different dimension and shape, in the aim of allowing them to form 3D microaggregates; using this method, it has been observed that higher cell densities induce cell self-renewal and maintenance of pluripotency [118, 119].

Cells cultured in aggregates are characterized by a maximized cell-to-cell contact, which induces a highly isotropic cytoskeletal tension, resulting in a roundish nuclear morphology, and by low levels of oxygen concentration (Figure 5). Based on the existing literature, at lower levels of anisotropy of cytoskeletal tension, ESCs commit toward endoderm and mesoderm, specifically neuronal [120], cardiomyogenic [27, 96], and haematopoietic [121]. A highly anisotropic cytoskeletal tension directs ectodermal lineage [118].

8.2. Adult Stem Cells. The most studied SC type in mechanobiology are MSCs, due to their very high sensitivity to mechanical stimuli. By making a synthesis of the existing literature [67, 69, 88, 89, 91, 92], it can be noted that a highly isotropic cytoskeletal tension, combined with a low oxygen concentration and with very low extracellular loads, synergistically promote MSCs self-renewal and maintenance of pluripotency (Figure 6). For example, in a study by Winer et al. [67], human MSCs cultured on hydrogels having a

stiffness comparable to bone marrow (0.25 kPa), have shown to self-renew and maintain pluripotency, if compared to cells cultured on more rigid substrates.

Coherently, the existing literature shows that the increase in anisotropy of cytoskeletal forces reduces pluripotency and enhances MSCs lineage commitment. For example, neural precursor cells can be obtained from MSCs by using nano/micropatterned surfaces, including nanofibres and gratings, and/or substrate having a very low stiffness, comparable to that of the brain ECM [53, 55, 76, 77]. All these engineering strategies result in substrates which induce an isotropic cytoskeletal tension, resulting in a very roundish morphology of the cell nucleus.

SC commitment can be induced by applying biophysical cues in a synergistic manner; actually, the superimposition of extracellular loads to cells, having their own cytoskeletal tension state due to adhesive forces, can alter and regulate their fate. MSCs can express various phenotypes including fat, cartilage, muscle, fibrous tissue, and bone. Fibrous tissues, such as tendon, could be obtained by anisotropic traction forces exerted by cells and through the superimposition of anisotropic extracellular loading, such as mechanical stretching [122–124]. Myogenic and cardiomyogenic precursor cells have been obtained through a combination of different types of directional (anisotropic) extracellular loading, such as shear stress and cyclic strain, in 2D systems [12]. Chondrogenic lineage can be achieved by a combination of intrinsic factors (e.g., a moderate substrate stiffness) and isotropic extracellular loads, including cyclic pressurisation and shear stress, as shown in [125]. Regarding adipogenic lineage commitment, it is inhibited by anisotropic extracellular forces like mechanical strain [126], and favoured by isotropic-inducing factors such as a very low substrate stiffness, or a low cell density [92].

9. Conclusion

To gain a precise control over SC response and make successful translation of SC-based therapies to the clinics, the contribution of mechanical factors on SC response must be elucidated and quantified. In this paper, we have analysed the existing knowledge in this field, and we have introduced the concept of “force isotropy,” as a first step towards the interpretation of the existing knowledge. Further research, in which biomechanical cues will be applied in a synergistic manner on truly 3D microenvironments, will likely provide a broader understanding of SC response to these cues.

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