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Engineering *in vitro* microenvironments for cell based therapies and drug discovery

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Traditional *ex vivo* culture setups fail to imitate the native tissue niche, leading to cellular senescence, phenotypic drift, growth arrest and loss of stem cell multipotency. Growing evidence suggests that surface topography, substrate stiffness, mechanical stimulation, oxygen tension and localised density influence cellular functions and longevity, enhance tissue-specific extracellular matrix deposition and direct stem cell differentiation. In this review, we discuss how these cues will facilitate engineering of physiological *in vitro* microenvironments to enable clinical translation of cell based therapies and development of *in vitro* models for drug discovery applications.

Introduction

Recent advances in cell culture systems in the fields of tissue engineering and drug discovery are enabling the development of novel therapeutic solutions for millions of people affected by injuries or diseases, whereas current conventional therapies are inadequate. During the past 20 years, cell based therapies have rapidly expanded in academic, clinical and industrial settings, with current expenditure reaching US$2.5 billion per year and an expected investment of US$14 billion by 2020 [1]. Moreover, the global market of nanomedicine is expected to grow to US$160 billion by 2015 [2], and the drug discovery market is expected to reach US$17 billion by 2015 [3]. However, tissue engineering and drug discovery are still hindered by the inability to develop *in vitro* microenvironments and/or models of physiological and clinical relevance.

Cell based therapies still face the challenge of expanding cell numbers and/or directing stem cell differentiation while maintaining phenotype fidelity and therapeutic potential. Indeed, despite the successful transplantation results that have been achieved by primary somatic cells to date for bladder failure [4], cartilage defects [5], cornea injuries [6], skin burns [7,8] and arterial revascularisation [9–11], their rapid senescence *in vitro* leads to scarce expansion and loss of the native phenotype [12,13]. For instance, significant changes in growth characteristics, cell marker expression and extracellular matrix (ECM) composition are observed in human tenocytes cultured above four passages [14,15], whereas prolonged culture of Schwann cells results in loss of contact inhibition and accelerated mitotic activity, suggesting a preoncogenic transformation [16]. Similar is the situation with stem cell culture. Among embryonic and adult stem cells, mesenchymal stem cells (MSCs) show great potential in the field of regenerative medicine [17–20]. MSC-based therapies have been used for the repair of bone defects [21–23], reconstruction of articular cartilage [24,25], peripheral nerve repair [26–28], remyelination and axonal regrowth in spinal cord injuries [29–31], scarred myocardium repair [32–34] and tendon augmentation [35–37]. However, several studies demonstrate that human MSCs retain their morphology, proliferative capacity and differentiation ability up to three-to-four passages [38–40], whereas skeletal muscle stem cells lose their regenerative capacity within a few days in culture [41,42].

In the field of drug discovery and gene therapy there is an urgent need to generate physiological and pathophysiological cell systems and tissue-like constructs for diagnostics, disease models and drug or gene screening purposes. Indeed, although considerable advances have been made in the development of *ex vivo* culture models [43], drug discovery still utilises primitive *in vitro* models or expensive animal models of questionable clinical relevance [44,45]. To regulate cell behaviour it is imperative to achieve a spatiotemporal control over the mechanical and biochemical cues

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of the *in vitro* extracellular microenvironment. Although the effect of soluble factors *in vitro* is generally taken into account in cell culture systems, the potential of modulating oxygen levels and ECM-related physical stimuli, such as substrate topography, substrate stiffness, mechanical loading and localised density, is often underestimated. Understanding how cells respond to these cues and subsequently improving the accuracy of current *in vitro* microenvironments will offer control over cell fate, avoid changes in cell phenotype and function and, ultimately, will facilitate clinical translation of cell based and pharmacological therapies.

Substrate topography

Topographical cues are under investigation as a means to control cellular growth, regulate cell adhesion, guide cell motility and direct stem cell differentiation [46,47]. The rationale of this approach is based on the fact that the ECM is composed of topographical features, such as grooves, ridges, whorls and pits, ranging from the nano- to the micro-scale [48]. It has been postulated [49,50] that cells sense topographical features and regulate their behaviour accordingly through focal adhesion interactions that elicit a cascade of cellular and molecular events (Fig. 1a).

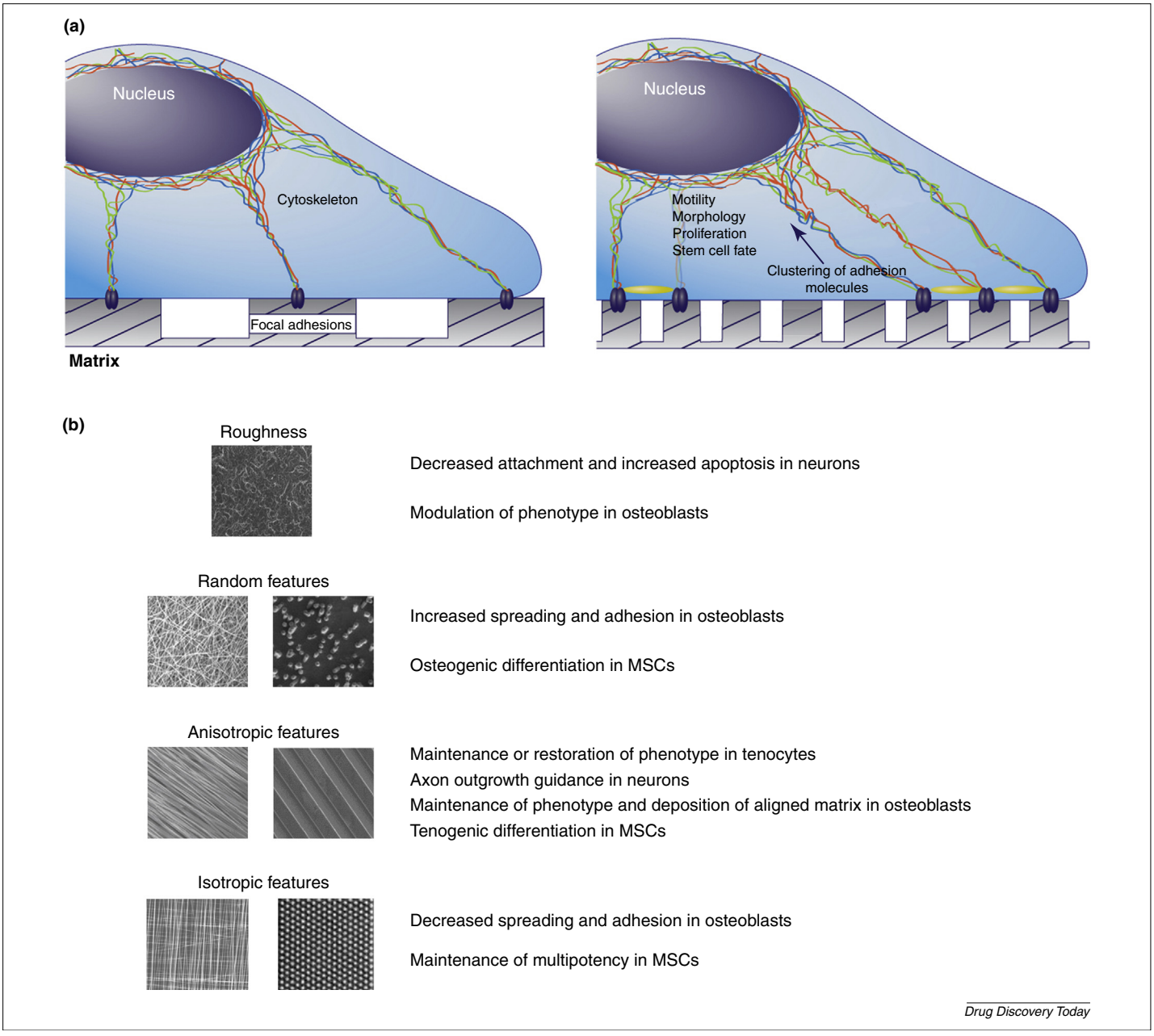

FIGURE 1

(a) Cells sense substrate topography through focal adhesions. Changes in geometry and size of the underlying features of the extracellular matrix (ECM) influence the clustering of integrins and other cell adhesion molecules, thus altering the number and distribution of focal adhesions. Such alterations at focal adhesions could then induce changes in cytoskeletal organisation and structure, which in turn elicit a cascade of cellular and molecular events leading to modifications of cell growth, adhesion, motility and stem cell fate. **(b)** Examples of how topographical cues influence cell behaviour *in vitro*. *Abbreviation*: MSCs, mesenchymal stem cells.

Controlling cell directional growth is showing potential for overcoming the issue of phenotype drift in culture and directing stem cell differentiation (Fig. 1b). Indeed, culturing tenocytes on microgrooved membranes maintains physiological cell morphology, prevents phenotype and functional losses, and can even restore lost phenotype of cells cultured on smooth substrates [51,52]. Anisotropic substrates have also been used for bone tissue engineering application to increase activity and induce aligned matrix deposition in osteoblast cultures [53,54]. Neurons have also been shown to be very sensitive to the substrate topography. Indeed, aligned electrospun nanofibres [55,56] and isoelectric focused collagen films [57] have been employed to guide the outgrowth of dorsal root ganglia neurites successfully. More recently, lithography techniques have been employed to demonstrate that nanotopographies influence neuron adhesion and functionality, with a surprising sensitivity of cells to nanoscale changes [58].

As in somatic cell culture, topographical features have been shown to influence stem cell behaviour significantly. Specifically, it has been demonstrated that substrate topography, in synergy with biochemical cues, such as laminin coating, enhances and regulates differentiation of hippocampal neural stem/progenitor cells [59,60]. Similarly, culturing human MSCs on nanoscale pits made by colloidal lithography induced osteogenesis in the absence of osteogenic media, whereas reducing the level offset in pit placement induced a switch from osteogenic stimulation to maintenance of MSC phenotype and multipotency [61,62]. Of significant importance was a high-throughput study that revealed previously unknown, surface topographies able to induce MSC proliferation or osteogenic differentiation [63]. MSCs cultured within microchannels formed using soft-lithography produced oriented cartilage structures with improved mechanical properties [64]. In an attempt to reproduce the complexity of native ECM and to commit MSCs to tenogenic lineage, bioimprints were produced using tendon sections as the template. This replica, when coated with collagen type I, supported tenogenesis of MSCs without requiring exogenous growth factors [65].

The manipulation of surface topography is a powerful tool not only for tissue engineering applications but also for the development of cell based patterned arrays for drug screening [66]. Indeed, controlling cell–substrate interaction through topography results in the modulation of other events such as ion channel function, differentiation and gene expression [67]. For instance, a recent study suggested the use of topographical substrates to develop neural cell based assays for drug discovery targeting ion channel function; compared with flat polystyrene surfaces, microbead arrayed substrates were capable of cell spreading and enhanced voltage-gated calcium channel responsiveness of neural progenitor cells [67]. Collectively, these studies suggest that there are crucial size and geometry features for each cell type specific response. In the near future, we anticipate that a deeper understanding of cell–substrate interactions and improvement of nano- and micro-fabrication technologies will guide the design and production of 'intelligent' nanotopographic surfaces that will direct cell behaviour. However, it is imperative for imprinting technologies to become financially viable, because the current associated expenditure prohibits scaling up and subsequent commercialisation of the newly developed knowledge.

Substrate stiffness

Cells are customarily cultured on rigid polystyrene surfaces that are many orders of magnitude stiffer than most tissues, leading to abnormal cell behaviour. This abnormal change in matrix stiffness influences terminally differentiated cell signalling, motility, morphology and proliferation, as well as stem cell fate [50,68,69]. Cell response to rigidity has been correlated to the stiffness or elasticity of the tissue from which they are derived [70,71]. At focal adhesions (Fig. 2a) cells exert forces to the ECM through actin–myosin contractions and respond to the resistance of the matrix [68,72] through, still poorly understood, chemical signalling mechanisms [70,73].

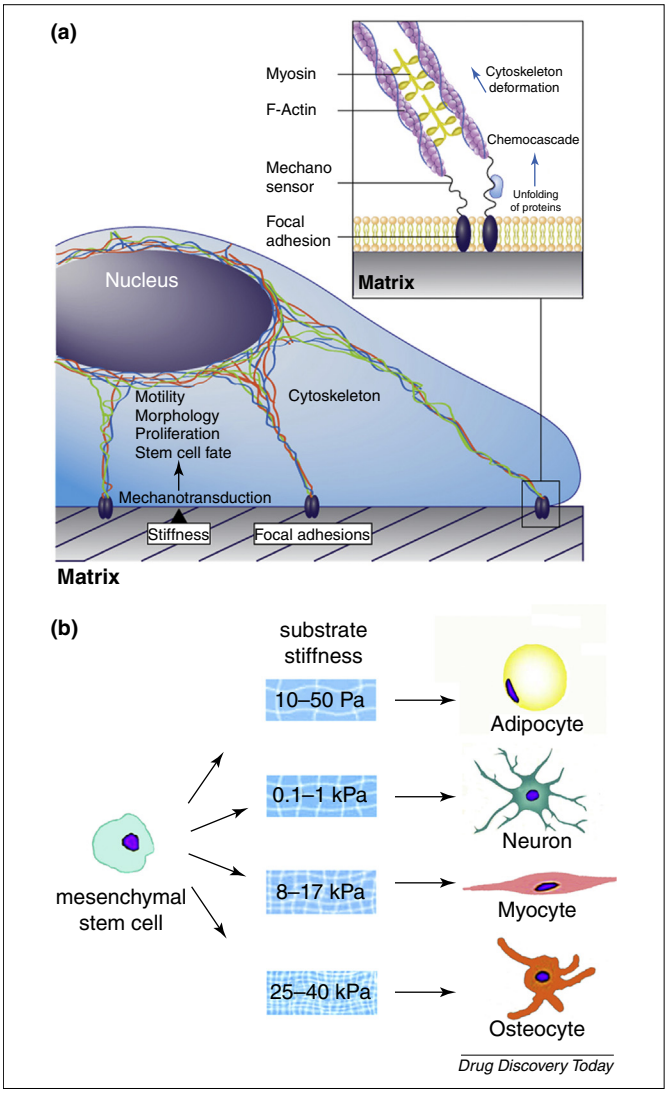

FIGURE 2

(a) By pulling on the matrix at focal adhesions cells transmit forces to the substrate and respond to its resistance, which depends on the stiffness, or elasticity, of the matrix or environment. Substrate stiffness influences cell motility, morphology, proliferation and stem cell fate. The force generated by cells to deform the matrix is transmitted through the cytoskeleton to an interior target such as the nucleus. The resulting tension induces conformational changes or unfolding of focal adhesions or other proteins, leading to activation of signalling pathways (inset). **(b)** Mesenchymal stem cells (MSCs) become adipocyte-like, neuron-like, myocyte-like and osteoblast-like when cultured on substrates having elasticity typical of fat, brain, muscle and cross-linked collagen of osteoid, respectively.

The first cells reported to sense and respond to substrate stiffness were fibroblasts and epithelial cells [74]. It was observed that cells increased motility or lamellipodial activity on flexible substrates, whereas on stiff matrices cells were more rigid and well spread. More recently, it was demonstrated that fibroblasts preferentially move towards stiffer regions of matrix compliance, a process termed 'durotaxis' [75]. Studies with stem cells demonstrate that matrix elasticity is effective, although insufficient to induce terminal differentiation of MSCs, which showed early osteogenic, myogenic and neuronal phenotypes after culture on stiff, intermediate and soft gels, respectively [72] (Fig. 2b). The ability of MSCs to sense matrix elasticity probably involves non-muscle myosin II, as suggested by the lineage specification blockage of MSCs on all substrates after treatment with blebbistatin [72]. Further studies utilising microarrays with different rigidity impacted the differentiation of human MSCs: osteogenic lineage was favoured on rigid arrays, whereas adipogenic differentiation was enhanced on soft substrates [76,77]. In the neural field, soft hydrogels conjugated with full-length laminin directed differentiation of neural stem cells (NSCs) towards neurons, whereas stiff hydrogels resulted in equal proportions of neurons and astrocytes. Rho GTPase activity increased with stiffness and modulated lineage specification towards astrocyte differentiation [78]. Of note, it has also been shown that the use of substrates mimicking the elasticity of *in vivo* stem cell niches enhances *in vitro* self-renewal of a variety of stem cells including muscle stem cells [42], embryonic stem cells (ESCs) [79,80] and haematopoietic stem cells [81].

Although these fundamental findings have been mainly obtained through 2D cultures, an increasing body of evidence suggests the importance of investigating the cell matrix interactions in a 3D microenvironment, because the different spatiotemporal distribution of cell adhesion sites significantly influences cell behaviour [82]. Indeed, it has been shown that the different viscoelastic properties of biomaterials influence proliferation and matrix remodelling of fibroblasts in 3D hydrogels [82]. It has also been demonstrated that the dedifferentiation process observed in chondrocytes in monolayer cultures can be reversed upon transfer into a 3D environment [83]. Understanding how 3D matrices influence cell behaviour, including the subsequent proteolytic degradation of the biomaterial matrix, has a potential application in bioprinting technology, where living cells together with hydrogel-based scaffolds are precisely deposited in a certain 3D pattern to fabricate *de novo* organs [84,85]. To move the organ printing one step closer to reality, new hydrogels should be tailored to enhance a specific spatiotemporal response in each cell type, for example, cellular migration, differentiation or gradual matrix degradation.

In the field of drug discovery there is increasing evidence that changes in matrix stiffness influence cancer cell behaviour and sensitivity to therapeutic drugs. Cancer progression in soft tissues is typically associated with an increase in ECM rigidity [86,87]. Accordingly, hepatocellular carcinoma cell lines cultured on gels of variable stiffness showed increased proliferation and chemotherapeutic resistance on rigid matrices [87]. By contrast, the addition of an antibody directed to an integrin-associated protein had little effect on the viability of lung cancer cells on soft gels, owing to reduced adhesion, whereas it was effective against cells spread on rigid substrates [88]. With respect to gene delivery applications, ECM stiffness has been found to mediate cell ability to uptake exogenous molecules. In particular, it has been shown that high substrate rigidity leads to a higher efficiency of nonviral gene delivery and expression in cells, probably through the regulation of cell proliferation [89,90].

Overall, these observations clearly indicate the importance of controlling matrix stiffness *in vitro* to optimise cell culture conditions and modulate the fate of terminally differentiated cells and stem cells. Despite the significant strides achieved, further multifactorial studies employing new biomaterials, advances in nano- and micro-fabrication technologies, microfluidics systems and biochemical or biological signalling will be essential to address the mechanisms of cell stiffness sensing systematically. Appropriate design of substrate stiffness will also enable development of more-realistic and -efficient drug delivery systems.

Oxygen tension

Physiological oxygen levels range from 5% to 13% in blood and 2% to 9% in most tissues. Current cell culture studies are generally performed at 21% O_2, which should be considered as a hyperoxic environment, and hypoxic environments (<2% O_2) have a strong impact on cellular biology [91]. Indeed, *in vitro* and *in vivo* data convincingly demonstrate that molecular oxygen levels regulate cell behaviour and play a significant part in developmental processes, such as angiogenesis, haematopoiesis and morphogenesis [91]. Therefore, a considerable research effort has been directed towards optimisation of oxygen supplementation for *in vitro* engineering (Fig. 3) of various tissues [92], including cartilage [93,94], tendon [95], bone [96,97], intervertebral disc [98], nucleus pulposus [99] and heart [100,101].

Oxygen homeostasis is regulated by hypoxia-inducible transcription factor 1 (HIF-1) which facilitates oxygen delivery and adaptation to oxygen deprivation [102]. Hypoxia upregulates the activity of specific prolyl hydroxylases involved in the stabilisation of HIF, which in turn transcriptionally activates a variety of genes linked to biological processes, such as angiogenesis and glucose metabolism [103,104]. In fibroblast cultures hypoxia has been shown to increase mRNA levels of procollagen α1(I) [105] and transforming growth factor beta 1 (TGF-β1) [106], a fundamental regulator of ECM formation [107,108], enhancing ECM production. In addition, low oxygen concentrations have been employed in cardiovascular tissue engineering to enhance ECM formation and maturation by human myofibroblasts [101].

Recent studies also demonstrate that oxygen tension is of paramount importance in maintaining stem cell niche and stem cell commitment towards a specific lineage [109]. In MSC cultures it has been shown that low oxygen tension (5% O_2) retains their undifferentiated and multipotent status [110,111]. Moreover, different oxygen concentrations have been employed to stabilise a chondrogenic phenotype or to promote hypertrophy of cartilaginous grafts, suggesting a possible application for cartilage repair therapies or endochondral bone repair strategies, respectively [112]. Human ESCs maintain pluripotency at oxygen tensions between 3% and 5%, whereas they spontaneously differentiate when cultured at 21% O_2 [113]. This is not surprising considering the relatively oxygen-poor environment in which the mammalian embryo develops. Also, NSCs, which physiologically reside in a

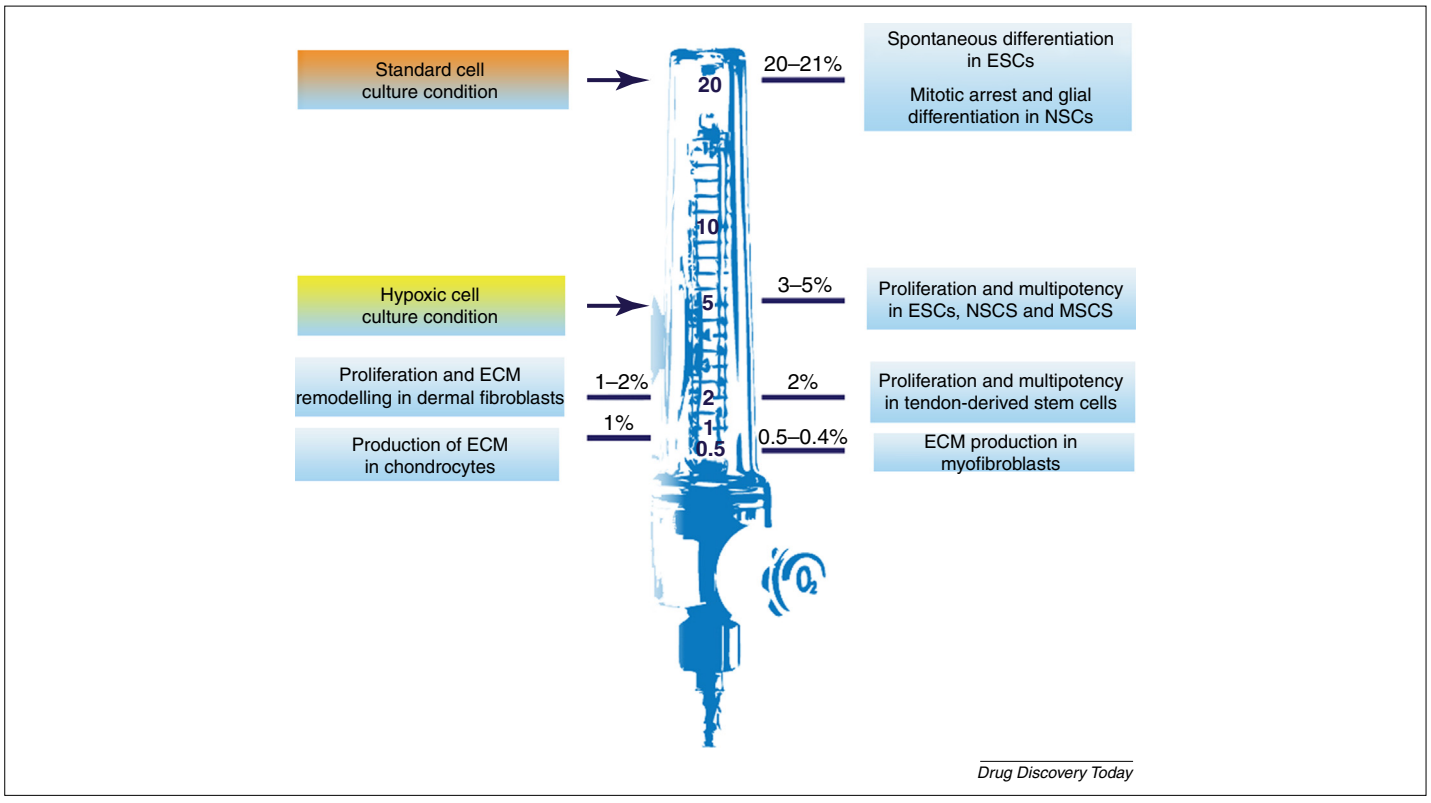

FIGURE 3

Routinely, cells are cultured under hyperoxic conditions (21% O_2), although physiological conditions in several adult and developing tissues are hypoxic. Considerable effort is ongoing to adjust oxygen levels of cultured cells with the desired tissue engineering application. Examples of how hypoxia influences stem cell fate and matrix production are shown. *Abbreviations*: ECM, extracellular matrix; MSCs, mesenchymal stem cells; NSCs, neural stem cells; ESCs, embryonic stem cells.

relatively hypoxic niche, can respond to changes in redox status. Indeed, several studies have demonstrated that mild hypoxic conditions (5% O_2) enhance their proliferation and multipotency [114], whereas cultures at 20% O_2 lead to mitotic arrest and glial differentiation [115]. Of note, in this latter study, when human neural precursors were expanded at 5% O_2 and then differentiated at 20% O_2, oligodendrocyte maturation was greatly enhanced in comparison with cells expanded in 20% oxygen [115], indicating that dynamic control of oxygen tension could be crucial for committing NSCs to a oligodendrocyte lineage in neuroregenerative therapies where remyelination of a damaged axon is required. Recently, low oxygen tension (2%) has been successfully applied for expansion of human tendon-derived stem cells *in vitro*, suggesting that hypoxia could help achieve a sufficient number of these stem cells for clinical application [95].

Because oxygen tension facilitates stem cell expansion, while maintaining phenotype fidelity, it could also be a useful tool to generate stem cells of sufficient quantity and quality for drug screening applications [116]. Moreover, several studies have shown that HIF-1 can be an important target for treating diseases such as cancer, heart failure, stroke and fibrosis [117–119]. Because HIF-1 is overexpressed in hypoxic tissues, including solid tumours, several anticancer agents have been developed to inhibit HIF-1 activity and prevent cancer cell survival [118]. By contrast, enhanced expression of HIF-1 has been used to protect cultured cardiomyocytes against simulated ischemia–reperfusion injury [120,121]. These observations thus suggest that low oxygen levels can be used for drug screening purposes to produce physiologically relevant *in vitro* models of hypoxia-related pathologies.

All in all, the ability to control cell behaviour and stem cell fate by modulating oxygen levels *in vitro* has a tremendous potential for the development of clinically relevant tissue-engineered grafts and functional cell systems for drug discovery. Further studies to determine the *in vivo* physiological and pathological tissue oxygen levels should be carried out and, subsequently, controlled bioreactor systems should be developed to translate these findings into a robust and reproducible culture process.

Mechanical stimulation

Mechanical forces, in the form of strain, compression or shear, have been shown to influence various cellular functions, including signalling pathways, gene expression, cell proliferation and differentiation and secretion of ECM proteins [122,123]. Cells react to mechanical stimuli through a multitude of cell membrane receptors, including integrins at focal adhesions [124,125], microcilia [126] and mechanosensitive ion channels [127]. Stimuli [128,129] can then be transferred mechanically through the cell via the actin microfilament network or by stimulating activity in chemocascades, such as mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) 1/2, which in turn results in a nuclear response (Fig. 4a).

Mechanical loads have been found to be beneficial for the physiological function of various cell types *in vitro* (Fig. 4b). Shear stress and compression have been shown to increase greatly the

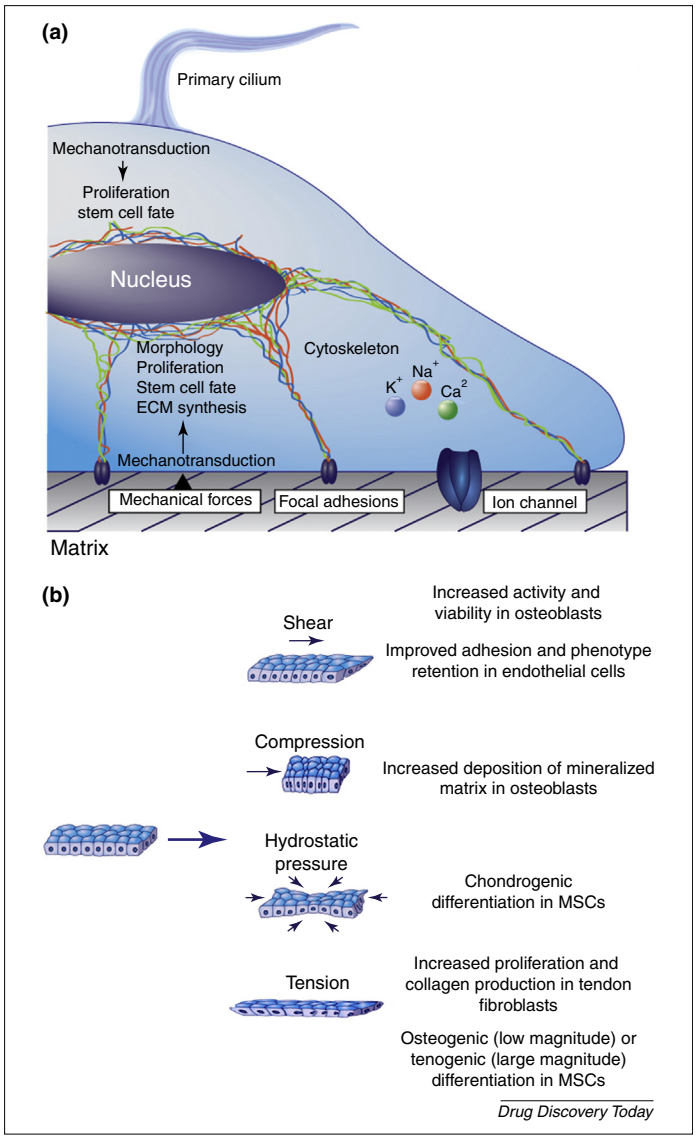

FIGURE 4

(a) Mechanical forces act through integrin receptor proteins, mechanosensitive ion channels or a specialised cell surface projection called primary cilium, which has recently been shown to mediate fluid flow mechanotransduction. Forces are then applied to the nucleus via intracellular mechanisms (e.g. actin filaments or chemocascades), resulting in protein transcription being activated. **(b)** Examples of how mechanical forces have been applied in cell culture studies and tissue engineering strategies to affect stem cell fate, extracellular matrix (ECM) synthesis, and cell phenotype and proliferation. *Abbreviations*: MSCs, mesenchymal stem cells.

activity of osteoblasts on engineered scaffolds [130,131]. Cyclic loading preconditioning of smooth muscle cells has been shown to be crucial for blood vessel tissue engineering [132,133]. Although excessive load induces chondrocyte death, morphological changes and cellular damage, a physiological cyclic load triggers morphological and ultrastructural recovery aspects [134] and significantly increases the amounts of glycosaminoglycans (GAGs) and collagen II after two weeks in culture [135]. In addition, mechanically stimulated tenocytes increase proliferation rate and ECM synthesis, maintain cell phenotype and improve tendon repair biomechanics [136–139], whereas the absence of mechanical load induces alignment perpendicular to the substrate topography orientation of bovine tenocytes [140]. Further work has also showed that cyclic tension regulates matrix remodelling in tendon cells and fibroblast cultures [141,142].

Mechanical loads have also been shown to induce cell differentiation. Human fibroblasts [143] and MSCs [136,144–147] differentiate towards tenogenic lineage after mechanical stimulation. Cyclic compression is the principle mechanism required for MSCs to undergo chondrogenesis [148–150]. It has even been observed that mechanical stimulation is more potent than chemical stimuli in differentiation of human MSCs towards chondrogenic lineage [151]. Moreover, application of mechanical stimuli, such as tension, compression, fluid flow and high-frequency vibration, has been shown to upregulate osteogenesis in MSCs [152–154]. Furthermore, the addition of mechanical stimulation has been shown to improve cell culture models for gene delivery and toxicology assessment. Indeed, endothelial cells that were exposed to a fluid shear stress increased uptake and expression of liposome–DNA–plasmid complexes, compared with cells under static conditions, owing to an enhanced supply of plasmids reaching the cellular surface and increased cellular activity [155]. Recently, a 'lung-on-a-chip' system utilising integrated microfluidics and cyclic mechanical strain imitated breathing movements [156]. In this study, mechanical stress enhanced cellular uptake of nanoparticles in the alveolar epithelium and stimulated their transport into the underlying endothelium. Because the effect of cyclic mechanical strain on lung nanoparticle absorption has never been detected in conventional static cell culture models, this study suggests that a mechanically active microdevice could implement current cell culture systems for toxicology applications [156]. We anticipate that, in the near future, such miniaturised lab-on-a-chip systems will replace or improve the outcome of animal studies by enhancing the predictive power of *in vitro* models. An overwhelming amount of literature clearly demonstrates the positive effects of mechanical stimulation and/or conditioning on cell phenotype maintenance, stem cell differentiation and cellular drug/gene delivery uptake. However, unless the underlying mechanisms are unveiled, as well as bioreactor systems becoming affordable, mechanical stimulation will remain an understudied opportunity for optimising *in vitro* systems.

Localised density

An alternative approach to modulate the *in vitro* microenvironment is based on the principles of macromolecular crowding (MMC), a biophysical phenomenon that directs the intra- and extra-cellular milieu in multicellular organisms and increases, by several orders of magnitude, thermodynamic activities and biological processes [157–160]. MMC uses the principles of volume occupancy, during which large macromolecules occupy volumes larger than their 'real' volume owing to their high hydrodynamic radius, thereby hampering the space for other macromolecules in the system [161,162]. Biological macromolecules such as enzymes and proteins function in a highly dense environment: the extracellular space, whereas highly dilute standard culture media (e.g. 16.78 g/l DMEM:F12, ATCC; 17.22 g/l DMEM high glucose and L-glutamine, Invitrogen) fail to imitate even the most dilute of the body fluids (e.g. 80 g/l blood; 36–50 g/l urine).

The first tissue-engineering-specific application of MMC demonstrated that the addition of neutral or negatively charged molecules in the culture media accelerated collagen type I deposition in *in vitro*

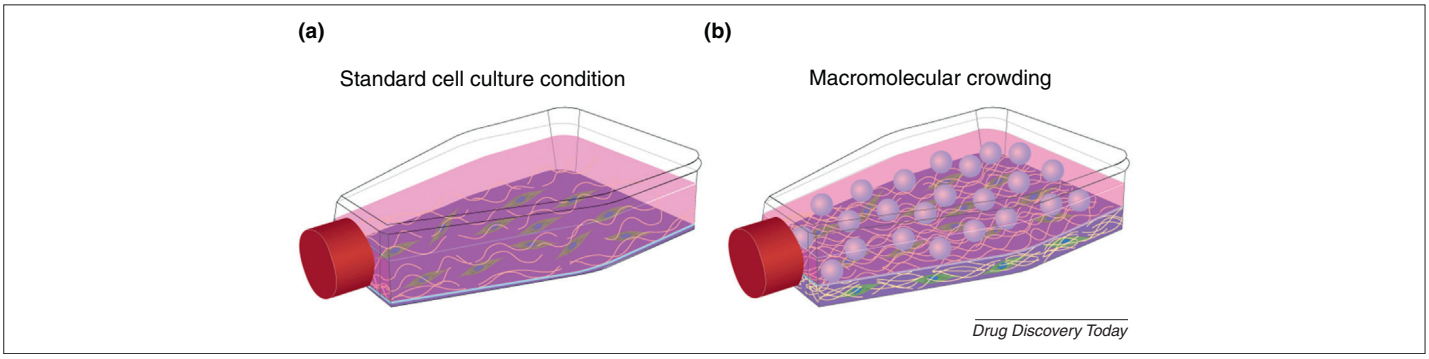

FIGURE 5

(a) In standard cell culture conditions the proteolytic cleavage of procollagen is very slow, resulting in minuscule amounts of insoluble collagen matrix. **(b)** Under macromolecular crowding the conversion of procollagen to collagen is accelerated, resulting in a rich collagenous cell layer.

culture of human fibroblasts (Fig. 5); in fact a >20–30-fold increase was recorded [163,164]. This can be explained by considering that *in vivo* cells are entrapped in the highly crowded extracellular space where the conversion of the *de novo* synthesised procollagen to collagen takes place rapidly, whereas in the dilute culture environment the conversion of procollagen to collagen is very slow [165–167]. Recent studies using human MSCs demonstrated that MMC enhanced collagen I deposition and alignment, which increased alignment of actin cytoskeleton and proliferation but decreased motility [168]. Although the full potential of this technology is under investigation, a promising application of MMC for cell based therapies is combining it with cell sheet technology [9–11,169–171] to accelerate the production of cell sheets rich in ECM [172]. The potential of MMC in drug discovery has also been demonstrated with the development of the 'scar-in-a-jar' model, which provided a fast and quantitative screening system for antifibrotic agents [173].

Concluding remarks

This review emphasises the importance of controlling fundamental, yet underestimated, biophysical and biochemical facets of the *in vitro* microenvironment, namely surface topography, substrate stiffness, mechanical loading, oxygen tension and localised density, to regulate cell behaviour, to control stem cell fate and to develop accurate *in vitro* models for diagnostics and drug discovery purposes. An improved understanding of the mechanisms behind each of these stimuli will enable development of controllable, scalable and more physiologically relevant *in vitro* microenvironments. Multifactorial and interdisciplinary approaches are likely to make an impact in the years to come and lead to clinically relevant *in vitro* tissue equivalents, providing suitable analysis systems are developed.

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