Title

Designing microenvironments for optimal outcomes in tissue engineering and regenerative medicine: From biopolymers to culturing conditions

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Abstract

Bone marrow mesenchymal stem cells have been extensively used for tissue engineering and regenerative medicine applications due to their ease of isolation and expansion and their ability to differentiate towards various lineages of mesodermal origin. Despite these properties, their clinical potential is often hampered by the simplicity of the *in vitro* environment and its inability to resemble the complex *in vivo* niche. Herein, different microenvironmental cues (e.g. surface topography, substrate stiffness, mechanical stimulation, oxygen tension and co-culture systems) that have been utilised to enhance the therapeutic efficacy of bone marrow mesenchymal stem cells are discussed.

Keywords

Tissue engineering; Bone marrow mesenchymal stem cells; Microenvironmental cues; Surface topography; Substrate stiffness; Mechanical loading; Low oxygen tension; Co-culture systems

1. Introduction

Regenerative medicine is a rapidly evolving field that aims to functionally restore or replace tissues and organs. Tissue engineering approaches combine different cell types and materials in order to create functional tissue substitutes. The choice of cell source plays a paramount role in tissue engineering applications. Common requirements include a simple harvesting procedure, minimised donor site morbidity, high cell proliferation capacity, high cell expansion capacity without phenotypic drift. Mesenchymal stem cells (MSCs) fulfil these criteria, as they are adult stem cells with the ability to differentiate into mesodermal lineages, such as osteogenic, adipogenic, chondrogenic, neural, muscular, endothelial and tenogenic. MSCs can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cord and peripheral blood. In particular, bone marrow-derived mesenchymal stem cells (BMSCs) have been extensively utilised due to their ease of isolation and their high expansion potential. BMSCs express stem cell specific surface markers, including STRO-1, CD29, CD73, CD90, CD105, CD146, Oct4 and SSEA4. In contrast, BMSCs are negative for the haematopoietic surface markers CD14 and CD34. Importantly, BMSCs express cytokines, such as TNF-α, TGF-β1 and IL-1β and display immunomodulatory properties by supressing the expansion and function of major immune cells, including dendritic cells, T cells, natural killer cells and macrophages. Although BMSCs are considered to be a promising cell type for various clinical applications, their efficacy is often hampered by the simplicity of the *in vitro* culture environment and its inability to recreate the complexity of their specific *in vivo* niche. Commonly utilised *in vitro* cell expansion protocols are based on growing stem cells on plastic surfaces that do not accurately imitate all important tissue-specific microenvironmental features, such as surface topography, substrate stiffness, mechanical stimulation, oxygen tension, localised density and interaction with other cell types (**Figure 1**).
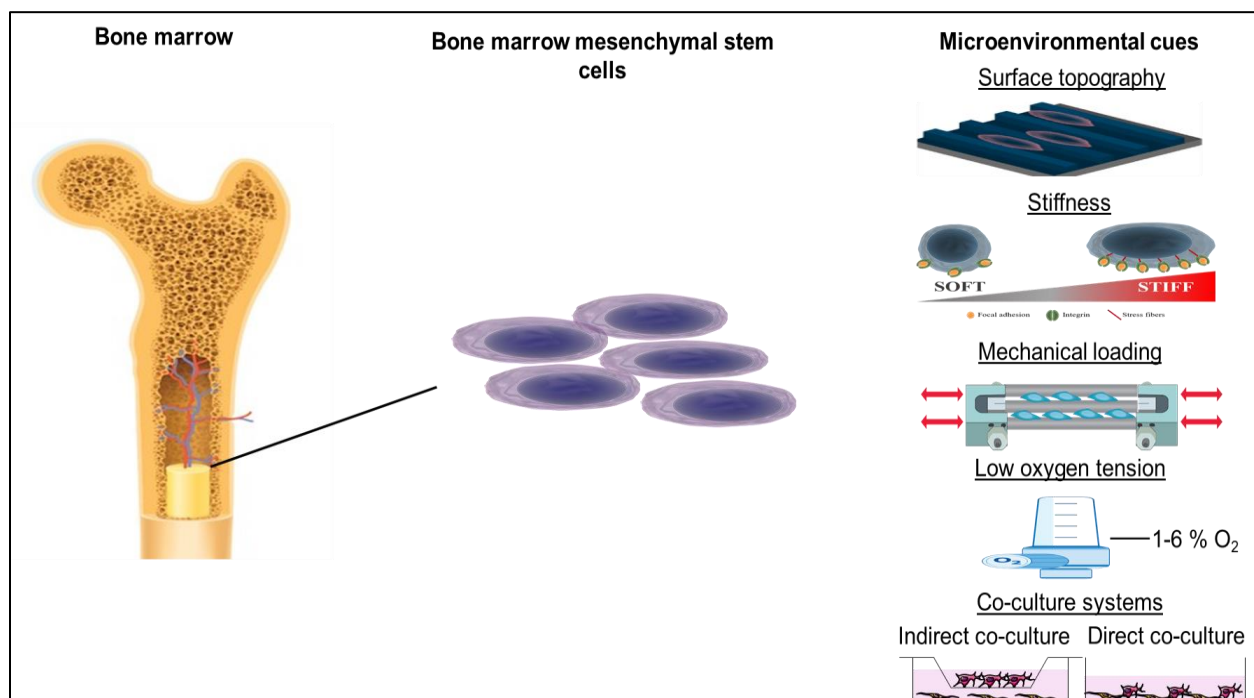
Figure 1: Different *in vitro* microenvironmental cues recapitulating the *in vivo* niche have been employed to modulate bone marrow mesenchymal stem cells behaviour.

There is a critical need to optimise *in vitro* culture conditions to fully unlock the therapeutic potential of BMSCs, either in their multipotent state or after guided differentiation towards the required lineage. Herein, we discuss current *in vitro* approaches based on microenvironmental cues, targeting the modulation of BMSC phenotype for improved therapeutic efficacy (summarised in **Table 1**).

Microenvironmental cues	Variable	Outcome
Surface topography	Electrospun aligned fibres	Cell alignment along the direction of fibre orientation
	Imprinted groves, nano-tubes, nano-pillars	Promotes osteogenic differentiation

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Substrate Stiffness	Soft substrate	Differentiation towards neural or adipogenic lineage
	Hard substrate	Differentiation towards osteogenic lineage
Mechanical loading	Compressive loading	Promotes chondrogenic differentiation
	Hydraulic pressure	Increases gene expression of aggrecan, collagen type II and Sox9
	Shear stress	Differentiation into endothelial-like cells
	Tensile Forces	Increased expression of the osteogenic markers
Oxygen tension	Low oxygen tension (1 % – 6 %)	Increased BMSCs proliferation and migration Promote cell-fate commitment and cell differentiation towards mesodermal lineages (e.g. chondrogenic, osteogenic, neural, endothelial, tenogenic)
Co-culture systems	Direct co-culture of endothelial progenitor cells and BMSCs	Increased deposition of calcium with enhanced mineralization
	Indirect co-culture of tendon derived stem cells and tenocytes	Differentiation of BMSCs towards the tenogenic lineage

Table 1: Overview of the influence of the different *in vitro* microenvironmental cues on BMSCs behaviour.

2. Surface topography

Extensive studies have demonstrated that cell behaviour is significantly affected by the composition and surface topography of the culture substrate. Topographical cues are capable of directing stem cells with respect to proliferation, migration, differentiation and quiescence. Electrospinning (ES) is a technique often used to produce scaffolds that mimic ECM organisation. Due to its versatility and controllability ES represents a promising technique to manufacture fibrous three-dimensional scaffolds with controlled fibre diameter and alignment. As a bottom-up approach, it has only minimal processing waste and can be tailored to generate aligned nano- and micro- fibres with tuneable mechanical and structural features. Numerous natural and synthetic polymers have been used to fabricate electrospun scaffolds for tissue engineering applications targeting regeneration of bone, skin, cartilage, tendon and nerve, to mention only a few. Additionally, ES has been investigated for applications in drug delivery systems. It has been demonstrated that anisotropic nano-fibres guide cell alignment along the direction of fibre orientation. Scaffolds composed of aligned fibres enhanced the proliferation and differentiation of MSCs. Studies comparing random and aligned surface topographies, revealed that aligned poly (l-lactic acid) (PLLA) and polycaprolactone (PCL) electrospun scaffolds seeded with BMSCs induced cell orientation along the aligned fibres. Further, aligned scaffolds have promoted tenogenic differentiation of BMSCs through increased expression of tenogenic markers, such as scleraxis, mohawk, tenomodulin, tenascin-C and collagen type I. In more complex tissue engineering approaches, materials made of polyesters and natural polymers exhibiting non-organised topographies have been combined with biological cues. The synergistic effect of electrospun PLLA / gelatin matrices with randomly aligned fibres (diameter range 190-360 nm) and hepatogenic serum was shown to guide hepatic differentiation of BMSCs, demonstrating its potential as a hepatic substitute for restoring damaged liver function.

Surface patterning orientation has also been shown to affect neural differentiation of BMSCs. Random and aligned PCL fibres have been micropatterned with fibrin at different angles. A 45° angle promoted neurogenesis of BMSCs in basal media with a significant increase in neurogenic markers, such as microtubule-associated protein 2, nestin, neurogenic differentiation factor 1 and class III β-tubulin. To further assess cell-material interactions in depth, the influence of topographical cues and material chemistry was assessed by an extensive gene characterisation utilising RNA sequencing (RNA-Seq). BMSCs seeded on electrospun PCL fibres with a fibre diameter of 603 ± 197 nm showed that surface topography significantly altered gene expression more than the chemistry of the scaffold.

Although electrospinning has shown promising results, it offers limited control over topographical features. Lithography technologies have emerged from the demand for constructs with precise dimensional features such as grooves, pillars, pits and wells down to the nano-scale. One of the main advantages of imprint lithography is the accurate fabrication of topographies on a wide range of materials without altering their bulk properties. Soft lithography is the most commonly used, utilising elastomeric polymers to obtain patterns based on methods such as embossing, moulding and printing. The effect of topography on cell differentiation has been extensively studied using polymeric patterned substrates seeded with stem cells which have been differentiated towards chondrogenic, adipogenic, tenogenic and neural lineages. Advanced fabrication technologies have started elucidating the biological mechanisms that trigger a cell's morphological response to substrate topography. The conversion of filopodia into lamellipodia has shown to play an important role for cells responding to a material's topography. It has been shown that a microsphere array pattern promoted cell adhesion and proliferation of BMSCs due to accelerated lamellipodia formation and cell spreading with recognition and conversion of filopodia into lamellipodia. Further studies on BMSCs showed that micro-grooved topography affected primary cilia structure and function via the WNT signalling pathway. Further, substrate topography was shown to maintain stem cell multipotent phenotype, highlighting the potential

use for expansion prior to differentiation or transplantation. PCL substrates with a nanoscale pits have been shown to maintain BMSC phenotype and stem cell markers expression, such as STRO-1 and activated leukocyte cell adhesion molecule, CD166, up to eight weeks in basal conditions *in vitro*.

The differentiation ability of BMSCs is largely affected by cell adhesion, cell shape and size, elongation and position of focal adhesion points and cell-cell interactions, all of which indicate that surface topography will play a key role in control of stem cell lineage commitment. For instance, titanium substrates with different geometries, including groves, nano-tubes and nano-pillars have been shown to promote osteogenesis in BMSCs. Dots on PCL substrates with a diameter of 120 nm led to osteogenic differentiation of BMSCs, even in the absence of osteogenic media, with an increased activation of the extracellular signal-regulated kinases (ERK) - mitogen-activated protein (MAP) kinase cascade, which is crucial for osteogenic conversion. Polylactic-co-glycolic acid (PLGA) 85/15 films with varying width and spacing values of micropillars (between 0.8 and 6.4 μm values) were shown to induce severe deformation of cell nuclei. In the presence of lineage-specific differentiation media, osteogenesis was enhanced while adipogenesis was supressed. Polyesters, such as PCL, PLA and polyglycolide (PGA) were used to produce substrates with nano-pillar and nano-hole topographies, showing increased expression of chondrogenic markers, such as collagen II, aggrecan and proteoglycan 4 (PRG4) and enhanced formation of hyaline cartilage of BMSCs, whereas nano-grating resulted in insignificant chondrogenesis. Further, BMSCs seeded on thermoplastic polyurethane (TPU) with grid-like square cavities exhibited upregulated gene expression of anti-inflammatory markers involved in wound healing, indicating the potential of these topographical cues to enhance tissue regeneration.

Given the endless variations that can be introduced in the geometry of imprinted patterns, recent efforts are being directed towards developing high-throughput approaches for simultaneous assessment of multiple surfaces. A multi-patterned "biochip", containing cues with sizes from 10 to 1000 nm, was explored in order to evaluate cellular-migration selectivity. BMSCs actively migrated towards the

patterns of preference, such as nano-grooves, while avoiding repelling topographies, such as nano-squared surfaces, as squares do not feature a topographical continuity necessary for cell migration. A similar approach has been developed to investigate the optimal topographies for osteogenic differentiation of BMSCs. Thousands of topographies were imprinted on a titanium-coated surface denominated TopoChip. Surfaces with highest alkaline phosphatase (ALP) expression have features of 10 to 30 μm with a moderate spacing of 5 to 10 μm. The cells were confined between the structures and were relatively narrow compared to cells on flat surfaces resembling a network of tubes. Additionally, these surfaces showed an upregulation of the osteogenic markers osteocalcin, osteopontin and bone sialoprotein. However, the TopoChip does not allow for a detailed investigation of the underlying mechanisms between stem cells and topographic cues. The TopoWellPlate, combining the previous described TopoChip and a 96 well plate, allows for the analysis of multiple genes and secreted proteins. Topographical cues are an important tool for control of *in vitro* BMSC culture. The use of optimal surface topographies for stem cell expansion is of particular interest when considering clinical translation of cell-based therapies. However, most approaches are focussed on surface patterning which does not allow for cell infiltration and does not provide the necessary three-dimensional architecture to accurately mimic the cells' native microenvironment. Discrepancies between *in vitro* and *in vivo* work have already been identified with regards to cell and tissue orientation. Further, the materials typically used frequently lack suitable mechanical properties and fail to emulate tissue stiffness in order to match the needs of BMSCs to commit to certain lineages.

3. Substrate stiffness

Adherent cells are known to respond to the elastic properties of their tissue-specific ECM, by adapting their cytoskeleton, initiating and coordinating signalling cascades. During embryonic development, matrix elasticity has been shown to be integral for driving cell differentiation. In adulthood, matrix

elasticity regulates cell-cycle progression and cell proliferation, making it an important factor in tissue homeostasis. Perturbations in ECM stiffness have been related to fibrosis, muscular dystrophies and malignant cancer progression. For decades, biological effects of matrix stiffness on *in vitro* cell cultures have been neglected by researchers with the generalised use of tissue culture plastic (TCP). Recent advances in material fabrication and characterisation technologies have allowed for the production of cell culture substrates with controlled surface rigidities, providing new insights into the role of this biophysical parameter on cell adhesion, migration, proliferation, morphology and gene expression.

Cells have been reported to sense their surrounding microenvironment via trans-membrane and cytoplasmic proteins that cluster together to form focal adhesion complexes (FAs). FAs act as bridges between the ECM and the cellular cytoskeleton, transmitting forces in an outside-in and inside-out fashion and serving as a key component in an incompletely understood force-sensitive signalling pathway, a process known as mechano-transduction. FAs and the actin cytoskeleton are dynamic structures; their size and degree of organisation directly correlates to the physical properties of the ECM. Stiff substrates provoke a spread-like cellular morphology with numerous FAs complexes and robust actin stress fibres. Conversely, soft substrates are known to induce a circular and constrained cellular morphology with immature FAs and disorganised actin filaments. Cytoskeletal tension is modulated by the activity of myosins, motor-proteins that slide actin filaments past one another. Active stretching of actin filaments results in a traction force from the cell towards the ECM which is proportional to the matrix rigidity. A phenomenon commonly known as durotaxis describes the differential cell migration guided by a rigidity gradient, typically from softer to stiffer substrates.

The elasticity of the ECM in humans ranges from a few Pascal (Pa) in soft tissues, such as brain, to GPa in hard tissues, such as bone. Several *in vitro* studies support the hypothesis that stem cells are able to recognise substrate stiffness and differentiate towards specific lineages. For instance, BMSCs cultured on soft (0.1 to 1 kPa), midrange compliant (8 to 17 kPa) and stiff (25 to 40 kPa) collagen type

I-coated polyacrylamide (PAAm) gels showed upregulated expression of neurogenic, myogenic and osteogenic markers, respectively. Similarly, soft fibronectin-coated PAAm gels (1 kPa) promoted adipogenic differentiation of BMSCs, while BMSCs seeded onto stiffer gels (40 to 68 kPa) underwent osteogenic differentiation. Other studies suggested that lineage commitment of stem cells is not dictated by matrix elasticity alone, but rather by a combination of matrix elasticity and other tissue-specific ECM-associated molecules. For example, BMSCs cultured on stiff PAAm substrates (80 kPa) showed significantly increased osteogenic differentiation potential when grown on collagen I coated gels. On gels coated with collagen IV and laminin I, two proteins marginally present in bone tissue, BMSCs did not exhibit osteogenic differentiation. Similarly, midrange compliant PAAm gels (15 kPa) alone failed to terminally differentiate BMSCs into smooth muscle cells. Instead, a combination of a specific substrate stiffness and transforming growth factor-beta (TGF-β) was required. In fact, TGF-β is widely known to promote BMSC differentiation towards two different types of phenotypes, depending on substrate compliance. In the presence of TGF-β, BMSCs cultured on stiff TCP adopted a smooth muscle cell (SMC)-like phenotype, whilst BMSCs cultured on hydrogels were directed towards a chondrogenic phenotype. It is noteworthy that some of the FA-proteins involved in mechano-transduction pathways, such as focal-adhesion kinases (FAK) and Src family kinases, are also key regulators in growth-factor-mediated signalling cascades. Collectively, matrix stiffness as physical cues work in concert with other biological and biochemical signals, influencing stem cell lineage commitment.

Despite encouraging results, restricted availability of stiffness-tuneable biodegradable materials, lack of standardised procedures for the measurement of substrate rigidities and insufficient knowledge of the native stiffness of most human tissues pose important limitations for successful research in this field. Despite natural polymers like collagen, gelatin and fibrin are readily implantable and present important cell binding domains, they can only be modulated in order to display a small range of soft rigidities.

The most commonly used materials for stiffness-related studies are polyacrylamide and polydimethylsiloxane (PDMS), two synthetic polymers that can be easily cross-linked to achieve an extensive range of physiologically relevant stiffness. However, the cytotoxicity of the former and the non-biodegradability of the latter hinder their use as implantable devices and restrict their application as *in vitro* cell culture surfaces. Therefore, novel biomaterials or fabrication technologies are imperative for future advances in the field. Further, material stiffness is not only dependent on its intrinsic mechanical properties, but also on the methodology chosen to measure it. Stiffness measurements performed with different techniques (indentation, rheometry, tensile and compression testing) and under different testing conditions (temperature, hydration of the material, tensile and compressive test strain rates) result in different Young's modulus values for the same material sample. Although atomic force microscopy (AFM) indentation is considered the most accurate technique to measure the matrix micro-compliance sensed by cells, different techniques used across the literature lead to misconceptions. Ultimately, comprehensive studies on human tissue rigidities that would strongly potentiate the design of biomimetic materials remain elusive.

In summary, matrix rigidity offers great promise as a biophysical tool for controlling stem cell phenotype and differentiation. Its use for *in vitro* cell culture substrates could potentially overrule phenotypic drift of multipotent stem cells associated with long-term cultures, facilitating cell expansion for cellular therapies. Precise substrate stiffness cues incorporated into smart scaffolds potentially driving lineage-specific stem cell differentiation, improving tissue engineering approaches for implantable devices.

4. Mechanical loading

Myriad forces regulate tissue physiology and homeostasis in living bodies. Commonly, they operate in form of compressive loading, hydraulic pressure, shear stress and tensile forces. Differentiated cells,

such as endothelial cells, fibroblasts, osteocytes, tenocytes chondrocytes and even stem cells are mechano-sensitive, constituting key players in the body's responses to mechanical forces. Cells can react to mechanical stimuli through conformational or organisational changes in cellular molecules, such as integrins at focal adhesions, cadherin complexes in cell–cell adhesions and mechanosensitive ion channels. Thus, they regulate signalling pathways involved in cell growth, differentiation, cell survival or programmed cell death. Hence, for *in vitro* engineering of functional tissues, it is essential to mimic their mechanical *in vivo* microenvironment. Additionally, mechanical stimuli are crucial for the regulation of stem cell behaviour by influencing cell proliferation, self-renewal capacity and differentiation. Externally applied forces have been extensively described to modulate BMSC lineage commitment. For example, BMSCs seeded on non-woven scaffolds differentiated into endothelial-like cells, expressing markers such as van Willebrand factor (vWF), CD31, and laminin, after physiological shear stress stimulation. Further, many studies have reported that mechanical stress promoted osteogenic differentiation of BMSCs. Stimulation of BMSCs with cyclic tensile stretch for 6 hours led to an increased expression of the osteogenic markers osteocalcin and Runx2. Similarly, mechanical stretch facilitated osteogenesis in human jaw bone marrow mesenchymal stem cells by blocking nuclear factor-kB activity. Further, mechanical loading promoted osteogenic differentiation of goat BMSCs cultured on PLGA scaffolds. Accumulating evidence increasingly suggests that mechanical loading positively affects tenogenic differentiation of BMSCs. In one study, human jaw bone marrow BMSCs were subjected to cyclical uniaxial stretching of 4 %, 8 % and 12 % strain. A strain of 8 % led to increased collagen production and expression of the tendon-associated markers tenascin-C, scleraxis and tenomodulin, mediated by stretch-activated calcium channels. However, a similar study considered 10 % strain as optimal to induce tenogenic differentiation of BMSCs. Both, gene expression and protein levels were strongly correlated with cell orientation. Finally, in a dynamic 3D model, tenogenic differentiation of BMSCs was mediated by Wnt4/Wnt5 signalling.

Mechanically-generated signals also showed effects on the chondrogenic differentiation potential of BMSCs. Regarding cartilage tissue engineering and mechanobiology, so far, dynamic compressive loading has been one of the most utilised model systems for mechanical stimulation. Dynamic loading of BMSC-laden constructs increased aggrecan promoter activity and accumulation of sulphated glycosaminoglycans (sGAG). The combination of growth factor supplementation, especially TGF-β1, and mechanical loading increased gene expression of aggrecan and collagen type II. Further, intermittent hydrostatic pressure has been reported to increase gene expression of aggrecan, collagen type II and Sox9 in human BMSCs, compared to untreated controls in the absence of TGF-β1. Multifactorial approaches involving the combination of shear and dynamic compression have been shown to increase gene expression of chondrogenic markers, gene expression of TGF-β1 and TGF-β3 and protein synthesis in BMSCs. In addition, studies targeting muscle tissue regeneration have assessed the potential of BMSCs to differentiate into skeletal muscle cells. Uniaxial cyclic loading initiated myogenic differentiation without the use of growth factors by increasing mRNA levels of myogenic regulatory factors MyoD and MyoG. Furthermore, the combination of uniaxial loading and insulin-like growth factor 1 (IGF-1) resulted in increased expression of myogenic markers. Similarly, cyclic stretch caused BMSCs to differentiate into smooth muscle cells, by directing fibre alignment and enhancing α-smooth muscle actin expression.

Bioreactors represent one of the key technologies developed in order to induce mechanical stimuli *in vitro*. The use of bioreactors allows for the implementation of mechanical stimulation and concomitant regulation of nutrient and gas exchange during the whole culture period, potentially contributing to the maturation of functional tissue substitutes *in vitro*. In recent years, more sophisticated technologies have been developed in order to precisely recreate physiological microenvironments *in vitro*. Among them, organ-on-a-chip technologies gained considerable success by recapitulating multicellular architectures, physicochemical microenvironments and vascular perfusion. By closely imitating

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physiological conditions, these systems allow for assessing synergistic effects of stiffness, strain, shear forces and additional features in a high throughput format, potentially directing stem cell differentiation more precisely.

A significant number of studies demonstrated the value of mechanical loading for modulating BMSC differentiation *in vitro*. However, further studies need to investigate the underlying mechanisms by unravelling mechanosensitive pathways involved in BMSC lineage commitment.

5. Oxygen tension

Oxygen tension is considered to be an integral component regulating developmental processes, cell fate and tissue function. *In vivo*, tissues experience a wide range of oxygen tensions, depending on their location and capillary supply, which are notably different from the inhaled oxygen tension of ~20 %. The levels of oxygen pressure steadily decrease after entering the lungs and travelling in the bloodstream through the body. Having reached the respective organs, oxygen levels have dropped to approximately 2 % to 9 %. Generally, BMSC-niches are located in regions of low oxygen tension, ranging from 1 % to 6 %. Recent evidence has identified hypoxia to contribute to maintaining a stable phenotype and undifferentiated state of BMSCs. Moreover, various levels of low oxygen tension (1 % – 6 %) are known to affect cell proliferation, cell-fate commitment and cell differentiation, frequently in combination with other microenvironmental cues. Therefore, considerable research effort has been conducted in order to investigate a wide range of oxygen tensions with respect to different tissue engineering applications.

Oxygen tension at low, physiological ranges has been shown to affect the proliferation rates of different cell types. A plethora of studies have highlighted the beneficial effects of hypoxia-inducible factor (HIF-1α), which is activated under low oxygen conditions, on proliferation and expansion rates of BMSCs. HIF-1α enhanced BMSC-proliferation through the stimulation of Twist-related protein, which

in turn downregulated the cyclin-dependent kinase inhibitor 1 (p21) and increased proliferation, thereby bypassing cell senescence. Activation of HIF-1α at 1 % and 5 % oxygen tension promoted the expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and downregulated the pro-apoptotic proteins Bcl-2-associated X protein (BAX) and cleaved caspase-3 in rat BMSCs *in vitro*. Furthermore, HIF-1α upregulated the ratio of phosphorylated extracellular-signal regulated kinase 1/2 (ERK), which plays a significant role in intracellular signalling for cellular proliferation and survival. Similarly, 1 % oxygen tension inhibits alterations in cell morphology and cell size and delayed the expression of senescence-associated β-galactosidase, retaining the expression of multipotency markers and chemokine-related genes, such as OCT4 and C-X-C chemokine receptor type 7 (CXCR7). Nevertheless, preconditioning of BMSCs under hypoxia in the presence of fibroblast growth factor (FGF) - 2 increased cell proliferation and migration rates, while affecting multipotency by increasing chondrogenic and osteogenic differentiation. One study investigated porcine BMSCs in two- and three-dimensional culture systems under 2 % oxygen tension. After 40 days of culture, BMSC proliferation was increased; however, the osteogenic differentiation potential was reduced, when compared with cells cultured under 20 % oxygen tension conditions.

A rationale of using BMSCs for tissue engineering approaches is their ability to migrate to the site of tissue damage. HIF-1α promotes the expression of CXCR4 and CX3CR1 in BMSCs, which stimulate cell migration and engraftment after transplantation. Similarly, hypoxia increased phosphorylation of cell migration related proteins c-Jun N-terminal kinases (JNK), focal adhesion kinase (FAK) and ERK1/2, as well as signal transducer and activator of transcription 3 (STAT3). Moreover, hypoxia-activated HIF-1α significantly increased BMSCs migration via downregulation of integrin α4 and upregulation of Rho associated kinase ROCK1 and serine/threonine kinase (Rac1/2/3) pathways. Stemness is characterised by the ability of stem cells to self-renew and to differentiate into multiple lineages, which is a prerequisite for cell-based therapies. Under ambient oxygen tension, BMSCs have

shown to undergo senescence, whereas hypoxic conditions (1 %) have promoted multipotency, which was attributed to the downregulation of p16 expression. Moreover, subjecting BMSCs to 5 % oxygen tension during expansion from passage 0 (p0) up to passage 3 (p3) preserved an undifferentiated and multipotent state. Additionally, cells subjected to 5 % hypoxia contained less mitochondria and exhibited an undifferentiated morphology, compared to cells grown under normoxic conditions. Furthermore, 2 % oxygen tension in combination with macromolecular crowding, a biophysical phenomenon known to accelerate extracellular matrix deposition, resulted in a microenvironment capable of maintaining the phenotype of BMSCs and their multilineage potential. In summary, low oxygen tension is of great importance for maintaining BMSC plasticity.

With regards to multilineage differentiation potential of BMSCs, several studies have compared hypoxic preconditioning of cells versus a continued hypoxic culture. Interestingly, chondrogenesis was promoted when BMSCs were isolated and expanded at an oxygen tension of 3 %, compared to cells cultured in normoxia. Similarly, an increased chondrogenic potential of ovine BMSCs was observed after isolation, expansion and differentiation in hypoxia (3 %), when seeded on collagen and hyaluronan (HA) scaffolds. Another study illustrated elevated chondrogenic marker expression when BMSCs were cultured at a low oxygen tension (5 %); however, this effect was attributed to the type of scaffold used. Thus, 5 % oxygen tension was able to induce chondrogenesis of BMSCs grown on PCL, HA and on collagen type I scaffolds. Additionally, BMSCs cultured at 2 % oxygen tension with chondrogenic induction media, showed that increased expression of HIF-1a led to phosphorylation of both protein kinase B and mitogen activated protein kinase p38, which in turn resulted in an upregulation of chondrogenic markers (collagen type II, Sox-9) and increased proteoglycan deposition. The combination of 3 % oxygen tension, collagen scaffolds, and bone morphogenetic protein 2 (BMP-2) and TGF-ss1 supplementation strongly increased chondrogenic differentiation of equine BMSCs,

compared to normoxic control conditions, resulting in the *in vitro* synthesis of hyaline-like neocartilage.

Hypoxia has been shown to also play a role in osteogenic differentiation of BMSCs. Hypoxic (1 %) preconditioning of BMSCs enhanced both osteogenesis and chondrogenesis *in vitro*, while it promoted osteogenesis in an *in vivo* mouse ectopic model. Furthermore, hypoxia enhanced the formation of a stable ECM *in vivo*, as revealed by the increased soluble and insoluble collagen production and collagen type I and III expression. In another study, expansion of BMSCs under low oxygen tension (5 %) promoted osteogenesis. Similarly, 5 % oxygen tension was found to promote osteogenic and angiogenic responses of BMSCs more effectively compared to cells in normoxia, when cultured on bone-derived scaffolds. These effects were attributed to the activation of the ERK1/2 and p38 pathway, induced by a preceding HIF-1α activation. Arg-Gly-Asp (RGD peptides) incorporated into biomaterials have previously been shown to upregulate osteoblastic differentiation in MSCs. Interestingly, stabilisation of HIF-1α resulted in an enhanced osteogenic and angiogenic potential of BMSCs seeded in RGD hydrogels after low oxygen preconditioning. Additionally, reduced osteogenic and adipogenic differentiation of BMSCs was observed in a low oxygen culture (1 %) supplemented with platelet lysate. Interestingly, increasing the oxygen tension to 3 % allowed for a recovery of the cells' osteogenic potential. In addition, the effects of hypoxia have been studied in different osteonecrosis models. Stem cells extracted from the bone marrow of osteonecrotic rabbits exhibited a decreased proliferation ability, loss of multipotency, reduced osteoblastic differentiation and increased adipogenic potential. Notably, when exposed to a hypoxic environment, extracted BMSCs showed enhanced proliferation and osteogenic potential, highlighting a beneficial effect of hypoxia for osteonecrosis-related therapies. Besides chondrogenesis and osteogenesis, different studies explored the influence of low oxygen tension on the differentiation of BMSCs towards other mesenchymal lineages. Hence, combined low oxygen tension (2 %) and endothelial growth medium were used in order to stimulate

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endothelial differentiation. Further, 1 % oxygen tension combined with epidermal growth factor (EGF) and basic fibroblast growth factor supplementation was used to generate neural progenitors. In another study, transplantation of hypoxic preconditioning BMSCs into rat Achilles tendon defects improved the healing outcome, compared to normoxic BMSCs.

Culture of BMSCs under physiologically low oxygen tensions has shown significant benefits with respect to cell proliferation, migration, plasticity and differentiation. However, the ideal combination of microenvironmental cues for controlling stem cell phenotype and differentiation still remain unclear and new approaches need to be explored in order to more closely mimic the native *in vivo* microenvironment of the respective tissues.

6. Co-culture systems

The human body comprises a complexity of multiple, distinct cell types which are in charge of different functions. Due to this heterogeneity, cells interact and communicate closely with each other. The combination of different cell populations using *in vitro* co-culture systems allows for a closer recapitulation of the native *in vivo* microenvironment with the aim to direct *in vitro* stem cell behaviour more accurately. Different methodologies such as direct and indirect co-cultures are used in tissue engineering approaches. In direct co-culture systems the different cell types are in direct contact with each other. This allows for direct cell-cell interactions via gap-junctions and ECM. Distinct cell populations are separated by a permeable membrane and cell interactions occur exclusively via paracrine secretion. Within the emerging stem cell field, co-culture has rapidly become a suitable tool for controlling differentiation, proliferation and phenotype more effectively. A recent review discussed co-culture systems for different tissue engineering applications in detail, with covering tissues such as cartilage, ligament, bone, heart, liver, lung and kidney. Co-culture for bone tissue engineering focusses on inducing vascularisation of the bone constructs, a crucial element for efficient bone regeneration.

Bone morphogenetic proteins (BMPs) have been reported to play a key role in osteogenesis. In one study, BMP-2 facilitated the osteogenic differentiation of BMSCs by upregulating the expression of alkaline phosphatase (ALP) and osteocalcin. More recently, efforts have been directed towards more complex co-culture systems utilising scaffolds or bioreactors. One study reported the formation of an osteoid, when BMSCs and endothelial progenitor cells were co-seeded on a polysaccharide scaffold comprised to pullulan and dextran. In another study, BMSC-derived endothelial cells and BMSCs were co-cultured on tricalcium phosphate scaffolds and transplanted into large segmental bone defects in rabbits. A highly vascularised tissue with improved mechanical properties proved the effectiveness of this co-culture approach for bone tissue engineering. When endothelial progenitor cells derived from umbilical cord blood were co-cultured with BMSCs and subjected to mechanical stimuli in a bioreactor, an increased deposition of calcium with enhanced overall mineralization and vessel infiltration of the constructs were reported.

The discovery of the chondrogenic potential of BMSCs lead to studies investigating co-cultures of chondrocytes and BMSCs. Both in direct and indirect configurations, chondrocyte proliferation was increased and the presence of chondrocytes induced chondrogenic differentiation of BMSCs. Similar results were obtained in three-dimensional co-culture systems using PCL microfibre mats. Additionally, co-culture with synovial fluid or synovial cells triggered chondrogenic differentiation of BMSCs. Generally, a dense ECM is responsible for the specific biomechanical properties of cartilage. Co-culture of meniscus cells and BMSCs under low oxygen tension promoted a cartilage-specific ECM formation with increased expression of aggrecan, collagen type I and collagen type II. The development of bioreactors has contributed to the development of various co-culture approaches for cartilage tissue regeneration. In one study, a dynamic bioreactor induced the spontaneous formation of 3D aggregates of articular chondrocytes when co-cultured with BMSCs. A lower mRNA ratio of

collagen type I / collagen type II was achieved and glycosaminoglycan contents increased more than 2 fold compared to single cultures.

Co-culture studies targeting ligament tissue engineering mainly involve BMSCs and anterior cruciate ligament (ACL) cells in direct and indirect systems. Mechanical loading is essential for ligament tissue engineering, for this reason, studies applied various mechanical loading regimes in direct and indirect co-culture systems. In an indirect co-culture approach, using a trans-well system, ligament cells were seeded in the lower chamber, whereas a gelatine / silk-fibroin scaffold seeded with BMSCs was positioned in the trans-well. Thus, differentiation of BMSC towards ligament-like cells was achieved. Studies demonstrated successful differentiation of BMSCs towards the tenogenic lineage and increased cell proliferation by direct co-culture with tendon derived stem cells (TDSCs), by indirect co-culture with tenocytes and by culturing BMSCs in the presence of tendon tissue fragments. Co-culture of BMSCs and TDSCs enabled the formation of cell sheets, that significantly promoted tendon healing in a rat patellar tendon window defect model, compared to cell sheets generated with a single-cell type. Co-cultured cell-sheets lead to an improved alignment of collagen fibres with more elongated cells and tendons exhibited a higher ultimate load to failure and Young's modulus.

BMSCs have previously been shown to play a pivotal role in cardiac regeneration. In a 3D co-culture system BMSCs were co-seeded with ventricular embryonic cardiomyocyte. BMSCs differentiated into cardiomyocyte-like cells that exhibited spontaneous contraction. Co-culture of BMSCs and transformed lung epithelial cells has been performed in order to direct differentiation towards pulmonary cells. BMSCs in co-culture expressed epithelial markers specific for lung tissue such as cytokeratin 5, 8, 14, 18, 19, pro-surfactant protein C and zonula occludens-1 (ZO1). BMSCs co-cultured with proximal tubular epithelial cells led to improved cell viability and proliferation of the latter cell population. Further, co-culture approaches were used in order to investigate cytoplasm and organelle transfer between different cell types. When BMSCs were directly co-cultured with renal

tubular cells, the formation of intercellular contacts, such as tunnelling nanotubes, was observed. Using fluorescent probes specific to mitochondria, cytosol, plasmalemma, transport of cellular contents through nanotubes was observed in both directions, direct and retrograde. Successful differentiation of BMSCs into renal tubular cells was attributed to this exchange of contents. In tissue engineering applications targeting liver regeneration, direct co-culture of hepatocytes and BMSCs resulted in hepatogenic differentiation and formation of three-dimensional liver spheroids. Further, beneficial effects such as preservation of hepatocyte morphology, improved cell viability and increased ECM deposition was observed in co-cultures of hepatocytes and BMSCs. In a similar approach, serum derived from rats with acute liver failure (ALF) induced secretion of anti-inflammatory molecules when added to co-culture systems, caused by changes in the BMSC secretome. Compared to single cell sources and other co-cultures, liver assisted devices (LAD) containing cocultures of BMSCs and hepatocytes showed the highest cell survival, indicating its potential use for therapies targeting acute liver failure. Liver fibrosis is partially caused by the activation and proliferation of hepatic stellate cells and an increase in ECM deposition. In one study, co-culturing BMSCs and hepatic stellate cells led to a decrease of ECM deposition and an inhibition of hepatic stellate cell activation by BMSCs, posing a potential application to prevent liver fibrosis.

Co-culture systems, in both direct and indirect configurations, have shown great potential for various clinical targets regarding tissue engineering and regeneration. These systems aim to recapitulate the native *in vivo* microenvironment more accurately and therefore contribute to a deeper understanding of cell-cell and cell-tissue interactions. Further, co-cultures pose valuable tools for the development of novel *in vitro* models and tissue engineering strategies.

7. Conclusions

Conventional *in vitro* culture systems fail to imitate native microenvironments of the respective tissues, compromising stem cell viability and proliferation, finally leading to cellular senescence, loss of multipotency and phenotypic drift. The ability of BMSCs to self-renew and differentiate into various lineages has been extensively investigated in emerging fields such as tissue engineering, gene therapy and regenerative medicine. However, the control of stem cell fate *in vitro* and *in vivo* due to the manifold underlying processes is yet to be fully understood. This review emphasises novel *in vitro* approaches employing different microenvironmental cues, such as surface topography, substrate stiffness, mechanical loading, oxygen tension and co-culture, aiming to elucidate the underlying mechanisms involved in cell-substrate interactions.

Other *in vitro* microenvironment modulators, such as macromolecular crowding, which has been shown to enhance ECM deposition and to enable stable expansion of embryonic stem cells are expected to play a pivotal role in stem cell expansion and in accelerated development of tissue equivalents in the years to come. Even though the use of single cues showed promising results, the integration of multiple cues within a system is a relatively unexplored field. It is expected that the combination of different cues and their synergistic effects contribute to a more accurate recapitulation of the complex *in vivo* niche. Moreover, an improved understanding of direct and indirect mechanisms regulating BMSC phenotype maintenance and differentiation will pave the way for novel methods and tools in the fields of tissue engineering and regenerative medicine.

8. Acknowledgements

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