Review

Special issue: "Methods and Advances"

Keywords: bioreactors, mesenchymal stem cells, microenvironment, microfluidics, three-dimensional

Title: Regulation of Mesenchymal Stem Cell 3D Microenvironment in Bioreactors

Sébastien Sart [1]

Spiros N. Agathos [2]

Yan Li [3]

Teng Ma [3]

[1]Hydrodynamics Laboratory, CNRS UMR7646, Ecole Polytechnique, Palaiseau, France

[2]Laboratoty of Bioengineering, Catholic University of Louvain, Louvain-la-Neuve, Belgium

[3]Department of Chemical and Biomedical Engineering, FAMU-FSU College of Engineering, Florida State University, Tallahassee, Florida, USA.

Correspondence: Dr. Teng Ma, Department of Chemical and Biomedical Engineering, FAMU-FSU College of Engineering, Florida State University, 2525 Pottsdamer St., Tallahassee, Florida, 32310, USA.

E-mail: teng@eng.fsu.edu

Abbreviations: BMP, bone morphogenetic protein; **C/EBP**, CCAAT/enhancer binding protein; **Col2a**, Collagen type II isoform a; **DKK-1**, Dickkopf-1; **ECM**, extracellular matrix; **eNOS**, endothelial nitric oxide synthase; **ERK**, extracellular signal-regulated kinase; **FAK**, Focal adhesion kinase; **FGF**, fibroblast growth factor; **FHL2**, familial hemophagocytic

lymphohistiocytosis-2, **hMSC**: human mesenchymal stem cells; **MB**, microfluidic bioreactor; **MSC**, mesenchymal stem cells; **PC**, perfusion chamber; **Pe**, Peclet number; **PPAR**, peroxisome proliferator-activated receptor; **OSE2**, Osteocalcin-Specific Element 2; **PGE2**, prostaglandin E2; **Re**, Reynolds number; **RhoA**, Ras homolog gene family member A; **ROCK**, Rho-associated protein kinase; **RUNX2**, Runt-related transcription factor 2; **RWV**, Rotating wall vessel; **SF**, spinner flasks; **SOX9**, sex determining region Y-box 9; **TAZ**, transcriptional coactivator with PDZ-binding motif; **TGF**, transforming growth factor; **VEGF**, vascular endothelial growth factor; **YAP**, Yes-associated protein.

Abstract

Background: Human mesenchymal stem cells (hMSCs) have emerged as an important cell type in cell therapy and tissue engineering. In these applications, maintaining the therapeutic phenotype of hMSCs requires tight control of the culture environments and the structural cell organizations. **Purpose and scope:** Bioreactor systems including stirred tanks, perfusion chambers, or rotating wall vessels are essential tools to achieve these goals in the clinical-scale expansion and tissue engineering applications. **Summary of new synthesis and conclusions reached in the review:** This review summarizes how different bioreactors provide cues to regulate the structure and the chemico-mechanical microenvironment of hMSCs with a focus on 3D organization. In addition to conventional bioreactors, recent advances in microfluidic bioreactors as a novel approach to better control the hMSC microenvironment are also discussed. **Conclusion:** These advancements highlight the key role of bioreactor systems in preserving hMSC's functional properties by providing dynamic and temporal regulation of *in vitro* cellular microenvironment.

1. Introduction

Human mesenchymal stem cells (hMSCs) are important cell sources in cell therapy and regenerative medicine in the last two decades. Up to now, more than 400 clinical trials (www.clinicaltrials.org) have tested hMSCs for the treatment of various diseases such as graft versus host disease, osteogenesis imperfecta, and infarcted hearts etc. The most commonly used MSCs in the trials are extracted from bone marrow stroma (BM-MSCs) [1], although MSCs can also be isolated from various adult and fetal tissues including adipose tissue, cartilage, synovial membrane, Wharton's jelly etc. [2]. The minimal criteria to define MSCs include plastic adhesion, expression of a set of specific surface markers, and the ability of differentiation along osteogenic, adipogenic and chondrogenic lineages [3]. MSCs also bear broad regenerative and trophic activities, including the secretion of extracellular matrix (ECM), pro-mitotic and pro-angiogenic factors, anti-inflammatory and immune-regulatory factors, and other bioactive molecules that stimulate tissue regeneration by reconstructing a pro-regeneration microenvironment and by modulating immune and inflammatory responses [4, 5]. Thus, harnessing these unique properties is central to the success of hMSC-based therapeutic applications.

Bioprocessing of hMSCs using bioreactors plays several crucial roles in their translation to clinical applications. First, to fulfill the potential for cell therapy, the bioreactors are the key to produce a large amount of hMSCs that have the required functional properties in tissue regeneration and repair. Second, to harness MSC's capacity to differentiate into tissues of mesenchymal lineages, the bioreactor systems can provide temporally and spatially controlled physiological and

biomechanical microenvironments that significantly influence hMSC fate. An important approach to achieve these goals is the reconstruction of an artificial *ex vivo* functional microenvironment, which relies on understanding and recapitulating the niche factors in an engineered system *in vitro*. A case in point is hMSC's role in bone marrow, where BM-MSCs have been identified as adventitial reticular cells, a subset of which are musculoskeletal stem cells [6]. BM-MSCs regulate hematopoiesis and the trafficking of immune cells through the secretion of large amounts of growth factors and cytokines as well as through direct cell-cell contacts with hematopoietic cells [7]. BM-MSCs also have extensive capacity to produce and interact with a complex ECM network, forming an interactive microenvironment that influences cell fate [8]. During fracture healing or skeletogenesis, the ECM of bone marrow is remodeled and endogenous MSC aggregates condense to initiate osteogenic differentiation [9]. The natural movements or pathological conditions can mechanically stimulate MSCs through the modulation of intra-medullary pressure, the blood flow shear stress, and the marrow viscosity, which lead to bone formation and the decreased bone resorption [10]. Hence, the bioreactor systems are important tools to recreate the complex interactions between MSC and their microenvironments [11].

To date, three major types of bioreactors have been used for the culture of MSCs: stirred tank bioreactors, perfusion bioreactors, and rotating wall vessels (**Figure 1**) [12], which provide various ranges of mechanical cues and enable the controlled delivery of biochemical molecules to regulate the microenvironment of MSCs *in vitro*. While these bioreactors have been well characterized and widely used in 3D hMSC culture, they are not designed *a priori* to regulate the

microenvironment at cellular levels and have limited capacity to finely control and regulate the reciprocal interactions between hMSCs and their immediate extracellular surroundings (**Table 1**). Thus, novel experimental systems are required to further understand, explore and modulate the dynamic and temporal relation between hMSC fate and their microenvironment. Microfluidic bioreactors (MBs) emerge as suitable systems to better control the MSC microenvironment *in vitro* because of their capacity to precisely control fluid flow, gradients of regulatory molecules, and mechanical stress at the single cell level (**Table 1**) [13, 14]. Thus, MBs are able to reveal the dynamic interactions between hMSCs and their microenvironment.

Current advances in bioreactor design for stem cell bioprocessing and clinical grade cell expansion for cell therapy have been reviewed in recent articles [11, 15]. This review summarizes our present understanding of how the bioreactor systems regulate the microenvironment and properties of hMSCs with a focus on 3D culture systems. The limitations of current systems for effective regulation of hMSC 3D microenvironment are also discussed. Finally, the advances and challenges of using microfluidic bioreactors to modulate MSC microenvironment, to understand the temporal and spatial factors, and to refine MSC culture conditions are reviewed here.

2. MSC organization and properties in 3D cultures

Major types of 3D MSC culture systems are developed recently, including cultures on porous scaffolds, encapsulation using hydrogels, and scaffold-free spheroids or their combinations (**Figure 2**). Different from 2D cultures, 3D culture of MSCs within scaffolds or hydrogels impacts the adhesion pattern (i.e., the focal and the

fibrillar adhesion) and regulates cell polarity and migration [16]. In addition, 3D MSC culture enhances endogenous ECM expression (*e.g.*, collagen type I, vitronectin, laminin etc.), modulates integrin expression profile, and promotes the secretion of trophic factors, resulting in the enhanced cell function [17].

2.1. 3D culture of MSCs within scaffolds or encapsulated in hydrogels

As an example of 3D cultures based on biomaterials, MSC encapsulation can be integrated in the culture device due to the high biocompatibility of hydrophilic hydrogel materials (*e.g.*, agarose, gelatin, alginate, Matrigel etc.). In addition, the gelation process is generally based on temperature modulation of the monomers composing the hydrogels, which only minimally influences cell viability and function. The degree of *in-situ* cross-linking of the hydrogels can tune the mechanical environment using cross-linkers (*e.g.*, Darocur®, Irgacur®, and glutaraldehyde) or by exposure to UV light [18]. The MSC cellular organization and function depend on the mechanical properties as well as the surface properties and spatial distribution of ECM molecules on the scaffolds or within the hydrogels (*e.g.*, spread cells *vs.* spheroids) (**Figure 2A**) [19]. Indeed, modulation of the topography and the Young's modulus of 3D scaffolds has been found to regulate MSC morphology, proliferation, and multi-lineage differentiation potential [20, 21]. For instance, MSC proliferation and osteogenic differentiation were promoted on stiffer (40 kPa) 3D matrices compared to softer matrices (6 kPa) in a similar manner to 2D cultures [20, 22]. The MSC trophic functions (*e.g.*, pro-angiogenic and neurotrophic factor secretion) are also shown to be regulated by the mechanical properties of 3D structures [23].

2.2. 3D culture of MSCs as spheroids

In contrast to 3D organizations formed in porous scaffolds or gels, the formation of MSC spheroids free of exogenous materials can also be integrated in different culture systems (*e.g.*, low adhesion vessels, mechanical agitation, thermal lifting etc.) (**Figure 2B**). MSC spheroids display the enhanced functional properties by increasing the levels of osteogenic, adipogenic and chondrogenic differentiation, as well as the secretion of trophic factors and pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [24-26]. The efficiency of MSC aggregate formation is closely linked with the activation of Ras homolog gene family member A (RhoA)/Rho-associated protein kinase (ROCK) signaling and homodimeric cadherin-cadherin interactions [27]. Compared to 2D cultures, MSC aggregates enhanced N-cadherin expression which activated canonical/non-canonical Wnt signaling and promoted osteogenic and chondrogenic differentiation [25].

Combining spheroid formation and mechanical signaling of biomaterial scaffolds, microencapsulation of MSC aggregates may provide signaling for enhanced differentiation [28]. Aggregate encapsulation enabled the control of spheroid size and prevented their agglomeration [29]. Although further investigation on the effect of capsule stiffness is required, evidence indicated positive regulation of adipogenesis, chondrogenesis, and osteogenesis by encapsulation of MSC aggregates [30, 31].

In 3D cultures, controlled delivery of biochemical and biomechanical cues cannot be readily achieved under static culture conditions. Instead, spatially and temporally controlled biochemical and biomechanical cues require bioreactor systems in which nutrient delivery, biomolecule gradients, and physical forces (*e.g.*, generated from the fluid motion, or from the controlled delivery of cross-linker for hydrogel encapsulation) can be tightly regulated [11, 32].

3. Regulation of MSC biomechanical microenvironment in bioreactors

While MSC expansion is preferred in a homogeneous environment with low shear stress and minimized turbulence, differentiation into specific lineages requires an exquisite control of biomechanical environment. 3D scaffolds adapted for the bioreactor culture of MSCs can provide extrinsic signaling to drive MSCs towards specific phenotype through the modulation of RhoA/ROCK and Yes-associated protein (YAP)/ transcriptional coactivator with PDZ-binding motif (TAZ) signaling pathways (**Figure 3A**). Bioreactors also provide mechanical forces which can modulate MSC phenotype. Under certain situations, the effect of mechanical stress could dominate the effect of 3D scaffold topography to modulate cell shape and thus the differentiation potential of MSCs [33].

3.1. Mechanical stress in spinner flasks

Stirred tank bioreactors, with the prototype of spinner flasks (SF), are commonly used for 3D MSC culture on different types of scaffolds or as spheroids [26, 34]. The long-term cultivation of MSC spheroids in stirred bioreactors prevents aggregate agglomeration and improves cell viability compared to static cultures [24]. The encapsulation of MSCs has also been shown to enhance cell viability, potentially through protection from heterogeneous shear stress and by controlling the aggregate size [35]. The culture agitation mediated by the impeller in SF regulates MSC organization and promotes homogeneous cell distribution following Poisson law compared to static cultures [36]. Moreover, SF is found to enhance the secretion of endogenous ECM for MSCs cultivated in 3D scaffolds [37].

Spinner flasks provide moderate turbulent flow with a Reynolds number (Re) ranging between 1,400 and 2,300 [38]. The average shear stress is about 2.9

to 7.5×10^{-3} Pa and the Kolmogorov eddy sizes range between 160 and 230 μm [38]. However, the shear stress distribution inside spinner flasks is heterogeneous with a broad range from 0.004 to 0.2 Pa [39]. Fixation of the scaffolds at a defined location of the spinner flask enables more predictable shear stress compared to free floating scaffolds [40]. Moreover, in spinner flasks, the flow acts predominantly at the edge of the scaffolds, with no or minimal penetration inside the scaffolds [40]. Modifications of SF geometry (*e.g.*, using baffles or wavy walls) have demonstrated better shear stress distribution or mixing dispersion within the vessels [41]. Novel spinner flask designs using biaxial agitation significantly increase the homogeneity of fluid shear stress and its diffusion within the scaffolds [40]. Such new stirred bioreactors have been shown to improve mechanical signaling on MSCs compared to unidirectional flow [42].

3.2 Mechanical stress in perfusion chambers

Perfusion chamber (PC) bioreactors can increase the homogeneity of MSC distribution and promote cell alignment in 3D scaffolds in a flow magnitude-dependent manner [43-45]. Classical PC bioreactors provide parallel laminar flow (around the scaffolds). For a flow rate between 0.01 and 0.5 mL/s, the average shear stress magnitude is about 10^{-4} Pa [46, 47]. MSCs grown in PC demonstrate more elongated cell morphology compared to static condition when increasing flow rate [48]. In addition, flow configuration can affect the endogenous ECM secretion and organization of MSCs [44, 49]. However, the shear stress distribution inside a 3D scaffold is highly heterogeneous and strongly depends on the flow direction and scaffold architecture [50]. For instance, transverse flow has been reported to enhance mechanical signaling compared to parallel flow [51],

presumably due to a higher percentage of cells exposed to interstitial flow induced in the transverse flow mode.

Designs of novel biomaterial architecture (*e.g.*, scaffolds with fibers parallel to the flow) or flow configurations have been tested to enhance the shear stress-mediated diffusion inside scaffolds [52]. The highest shear stress is found on the surface of the pores in the scaffolds as a function of the number and the size of the pores [50, 51]. Conversely, the fluid shear stress is lower inside the scaffolds (almost by 20-fold), where the fluid flow provides convective transport over the mechanical stresses. It was demonstrated that reducing the angle between the fibers or increasing both the porosity or the diameter of the scaffolds significantly enhanced the diffusion of hydrodynamic stresses, and consequently the biological activities of MSCs [53].

3.3. Mechanical stress in rotating wall vessels

The rotating wall vessels (RWV) provide low shear stress and promote the round shape of MSCs, which have shown similar viability and proliferation compared to 2D cultures [54]. The cell distribution inside 3D scaffolds is enhanced in RWV compared to the static culture due to the hydrodynamic environment with high mass transfer rate [55]. In addition, microgravity in RWV can induce the formation of spheroids and is suitable to expand MSC aggregates [56]. For differentiation, MSC spheroids cultured in adipogenic and osteogenic medium in RWV exhibit oil red O staining and von Kossa staining respectively, demonstrating the ability of the aggregated cells for lineage-specific differentiation [56].

Microgravity-based bioreactors with the prototype of RWV reduce the effects of normal gravity and provide laminar flow with lower magnitude of shear

stress compared to other types of bioreactors [57, 58]. However, computational fluid dynamics analysis reveals the variations of shear stress and fluid velocity inside scaffolds. For instance, for cylindrical scaffolds the shear stress is predicted at 6×10^{-3} Pa at the bottom, 9×10^{-3} Pa at the top, and 7×10^{-3} Pa at the lateral part of the scaffolds [58].

3.4. Incorporation of hydrostatic pressure, compression and tensile force

While scalability is a major criterion for the bioreactors designed for hMSC expansion, the ability to recreate and control the 3D microenvironment is an important consideration for the applications in regenerative medicine. Compression bioreactors (CC) provide direct uniaxial compressive forces by hydrostatic pressure or by mechanical stimulators [59]. CC can enhance MSC migration and promote cytoskeletal reorganization, which is reinforced by the scaffold mechanical properties [60]. In addition, CC upregulate the synthesis of several ECM proteins (*e.g.*, collagen) compared to static cultures [61]. As a consequence, CC can induce changes in MSC morphology: from highly spreading cells to the formation of intercellular connections, depending on the initial status of RhoA or Ras-related C3 botulinum toxin substrate 1 activation [59]. The regulation of MSC shape in CC consequently affect the differentiation into osteogenic and chondrogenic lineages.

Tensile strain bioreactors (TS) provide stretching forces to MSCs through mechanical stimulators. Application of tension stress to MSCs promotes elongated cell shape and increases ECM secretion [62]. TS can change MSC mechanical properties such as increasing cellular Young's modulus through the induction of actin stress fiber formation [62]. In addition, TS promotes MSC alignment

perpendicularly to the strain axis [63]. While uniaxial static stretching can induce local alignment of actin fibers, dynamic stretching leads to significant cytoskeletal alignment and repolarization of hMSCs through entire cell network [64].

3.5. Limitation of the conventional bioreactor systems

The bioreactors discussed so far allow the cultivation of MSCs at relatively large scale (~cm^3) (**Table 1**). Among several types of bioreactors, a better MSC colonization of 3D scaffolds has been observed in biaxial spinner flasks compared to PC, RWV and uniaxial spinner flasks [42], while MSC spheroids cultivated in spinner flasks and RWV have shown similar cellular structure and viability [24]. Although spinner flasks are convenient for increasing scale of MSC production at the undifferentiated state, they may be less useful than PC or RWV for investigating the effects of mechanical stress on MSC differentiation (**Table 1**). The limitation of RWV and PC is the difficulty to regulate and control metabolite or morphogen diffusion and mechanical forces within the scaffolds.

More importantly, SF, PC, RWV, CC and TS have restricted throughput for the modulation of culture conditions, as well as a limited resolution for the dynamic analysis of cell behavior, which is usually performed at the population level and or by sampling (**Table 1**). Moreover, the regulation of MSC environment in conventional bioreactors is often performed by trial and error. While the use of computational models combined with the bioreactor systems allows to better direct MSC priming, novel devices are still required for the dynamic, spatial and temporal tight control MSC fate in 3D culture [65].

3.6. Microfluidic bioreactors for a better control of mechanical stress

MBs combine the features of conventional bioreactors (i.e., hydrodynamic stress, capability of 3D culture etc.) but exceed their performance by a high degree of throughput for analysis and the capability for complex combinatorial regulation of MSC microenvironment (**Table 1**) [65]. Indeed, MBs are designed with micro-scale control of the geometry (*e.g.*, by photolithography-based molding) which can precisely control the fluid flow (**Table 2**) [66]. In addition, gradients of mechanical stress can be generated in MBs, enabling the tight control of various shear magnitudes inside the same vessel for the same MSC population [67, 68]. Therefore, the influence of shear stress on single MSCs or individualized spheroids can be assessed in MBs [69-71]. MBs also enable the tuning of physico-chemical properties of the scaffolds or capsules [72]. Therefore, MBs can accurately define combinatorial mechanical parameters for optimal MSC priming *in vitro*. Besides the mechanical stress, other types of forces generated from electromagnetic field, tensile tension, and compression in bioreactors can provide additional signaling to regulate the differentiation of MSCs along specific (*e.g.*, chondrogenic and osteogenic) lineages (**Table 2**) [73-77].

To investigate the effects of mechanical stress distinct from biochemical cues, a novel application of droplet-based microfluidic chips has enabled the development of biological compartmentalization methods, reducing the interference of MSC paracrine signaling on the specific cues provided by the bioreactors [78, 79]. MBs enable the accurate investigation of the specific contribution from the diffusion of biochemical molecules versus the mechanical forces resulting from fluid flow, through the generation of Peclet number (Pe) gradients [80]. Hence, MBs emerge as powerful dynamic culture tools to tightly

refine MSC culture conditions, through the specific combination of biochemical and biomechanical cues that can lead to enhanced functions of MSCs.

4. Regulation of MSC biochemical environment in bioreactors

Bioreactors not only provide mechanical signaling regulating proliferation and differentiation of MSCs, but also modulate mass transport and thus the dynamic and temporal distribution of the nutrients and growth factors (**Table 3**). The biochemical environments are shaped by essential nutrients such as glucose and oxygen and by the molecules that can induce MSC differentiation, playing a synergistic role in a bioreactor environment.

4.1. Glucose and oxygen levels for regulating MSC metabolism

Proliferation of MSCs relies mainly on glycolysis while the differentiation along adipogenic and osteogenic lineages requires a metabolic switch from glycolysis to oxidative phosphorylation [81, 82]. In contrast, MSC chondrogenic differentiation is accompanied by enhanced glycolytic metabolism [81]. During expansion, the specific glucose consumption of MSCs was reported to be 0.2-0.4 pmol/cell/h [83] and the oxygen uptake rate was 98 fmol/cell/h [81]. During osteogenic differentiation, the glucose consumption rate was reduced by 2-fold (about 100 fmol/cell/h), while no change was observed for chondrogenic differentiation [83]. As the major metabolic substrates, both glucose and oxygen levels influence MSC fate. Low concentration of glucose was shown to promote MSC expansion and chondrogenic differentiation [84], while high concentration of glucose was reported to enhance adipogenic and osteogenic differentiation [85]. Oxygen requirements also change upon MSC differentiation. For example, MSC proliferation and chondrogenic differentiation were found to be enhanced under

hypoxia, which promotes glycolytic metabolism [86]. In contrast, adipogenic and osteogenic differentiations are reduced under hypoxia which prevents the metabolic switch to oxidative phosphorylation [87].

The fluid motion in different bioreactors influences oxygen and glucose delivery, the biomolecule gradients, and the accessibility to the cells. For example, a parallel flow perfusion bioreactor is insufficient to sustain 3D bone construct development due to increased oxygen demand, while the increased interstitial flow in transverse flow pattern can enhance oxygen delivery to the cells [51]. In addition, well-instrumented bioreactors (*e.g.*, stirred tank vessels) equipped with oxygen and pH probes as well as sensors of metabolites enable the feedback control of oxygen tension and glucose concentration inside the culture vessel [88].

4.2. Biochemical molecules and synergistic effects with biomechanics

Biochemical molecules can act synergistically with mechanical stress in bioreactors, leading to efficient osteogenic differentiation [89]. For example, osteogenic differentiation of MSCs can be enhanced by combining the mechanical stress in bioreactors (*e.g.*, cyclic strains and perfusion) and osteo-inductive soluble factors (*e.g.*, dexamethasone, ascorbic acid, and β-glycerolphosphate) [90, 91]. Dexamethasone and bone morphogenetic protein (BMP)-2 were found to promote the expression of TAZ which led to better osteogenic differentiation (**Figure 3A**) [92]. Dexamethasone also up-regulates familial hemophagocytic lymphohistiocytosis-2 (FHL2), which increases nuclear translocation of β-catenin and thus the osteogenic differentiation of MSCs via activation of RUNX2 [93, 94]. Ascorbic acid might activate the osteoblastic *cis*-acting element (OSE2) promoter to promote osteocalcin expression [95]. β-glycerol phosphate, a source of

inorganic phosphate (substrate for alkaline phosphatase), was shown to stimulate mineralization and regulate osteopontin expression [96].

Similarly, adipogenic differentiation is promoted by combining microgravity in RWV and soluble adipo-inductive factors such as dexamethasone, insulin, indomethacin etc. [97]. While microgravity mainly regulates the Hippo signaling to modulate adipogenic differentiation, the soluble adipogenic factors influence other distinct signaling pathways. Indeed, insulin and glucocorticoids can act on the upstream of adipogenic differentiation, leading to the activation of CCAAT/enhancer binding protein (C/EBP)-β and C/EBP-δ (early stage of adipogenesis) [98]. The treatment of MSCs with indomethacin or a selective repressor of COX-2, parecoxid, has been shown to increase adiponectin expression of MSCs (late stage of adipogenesis), while suppressing osteogenic differentiation [99]. In addition, thiazolidinedione can act as a direct agonist of PPAR-γ at the late stage of adipogenesis [100].

Chondrogenic differentiation of MSCs can be enhanced through the synergistic effects of mechanical forces from bioreactors (such as compression) in the presence of biochemical factors, *e.g.*, dexamethasone, transforming growth factor (TGF)-β, and ascorbic acid [101, 102]. Indeed, dexamethasone was found to induce the expression of sex determining region Y-box 9 (SOX9) during chondrogenesis [103]. TGF-β and TGF-β receptor-regulated SMAD-3 and p300 cooperatively activated SOX9-dependent transcription of collagen type II [103]. Ascorbic acid may also contribute to the MSC expression of Col2a (collagen type II) at the transcriptional and the post-transcriptional level [104].

4.3. Dynamic and temporal regulation of biochemical signaling

Dynamic and temporal regulation of the differentiation through cellular metabolism and various biochemical inducers may be required to improve MSC proliferation and differentiation. Bioreactors can provide sufficient level of throughput to accurately define the biochemical microenvironment for MSC fate decision. In addition, bioreactors allow continuous monitoring of metabolic and phenotypic status, and help to define feeding strategies. Indeed, different types of bioreactors can be equipped for various spectroscopic measurements (*e.g.*, Raman, near infrared, impedance etc.) which enable the on-line monitoring of cell phenotype and metabolic status during MSC expansion or differentiation [105]. Moreover, bioreactors enable the combinational delivery of multiple metabolic, physiological, and mechanical cues, which can hardly be achieved in 2D static cultures.

Bioreactors allow the rational design of culture processes to dynamically meet the metabolic and physiological requirements for MSC proliferation. For instance, batch, fed-batch, and continuous cultures can be operated in bioreactors. While batch culture does not provide sufficient nutrients for MSC growth [12, 106], fed-batch culture based on the monitoring of glucose or growth factors was shown to promote MSC proliferation [106]. The continuous delivery of medium was also found to enhance MSC proliferation independent of flow shear stress [107].

Dynamic and temporal regulation of the biochemical microenvironment in bioreactors also modulates MSC differentiation. For instance, dynamic regulation of oxygen tension in perfusion bioreactors was shown to promote adipogenesis (under 20% O_2) or chondrogenesis (under 5% O_2) depending on oxygen level [108]. In contrast, batch bioreactor culture can promote adipogenic differentiation through autocrine signaling [109]. The continuous delivery of dexamethasone

during both the expansion and the differentiation stages was found to promote osteogenic differentiation compared to the dexamethasone treatment during the differentiation stage only [110]. The transient delivery of serum at the early stage of differentiation promoted both osteogenic and chondrogenic differentiation [111]. Moreover, for chondrogenic differentiation, it was reported that short-term exposure to TGF-β1 enhanced proteoglycan expression and maintained total Col2a expression while the specific productivity of Col2a was decreased compared to the constant exposure to TGF-β1 [112]. Despite these observations, the dynamic and temporal regulation of the differentiation inducers in bioreactors has not been investigated in detail to date. Hence, a tightly controlled microenvironment in appropriately designed bioreactor is indispensable for the dynamic and temporal regulation of MSC biological function.

Using conventional bioreactors, precisely controlled spatial and temporal biomolecule gradients are hardly achievable at microscopic levels. In this regard, MBs have a unique capacity to generate the gradients of metabolites, growth factors, or cytokines [66], via T-sensors, premixer gradient generators, and universal gradient generators etc. [113]. Indeed, due to the micro-size geometry of the channels and thus low Reynolds number in MBs, the mixing of miscible solutions of different concentration or composition never occurs. In the absence of inertial force, viscous force (including diffusion and pressure-driven convection) dominates in MBs for the dragging of the culture medium. Consequently, while a high Peclet number (Pe) does not enable the mixing of different miscible solutions, a low Pe number allows the formation of inter-diffusion zone for the precisely controlled generation of long-term stable gradients [114]. Moreover, due to the

small scale of the culture chamber, steady state occurs within a short time period in MBs, enabling the temporally controlled molecular gradients [115]. Furthermore, MBs can be equipped with multiple gradient generators for the combinatorial regulation of the MSC biochemical environment [116]. Thus, MBs provide better control for nutrient and growth factor delivery at single cell or individual spheroid levels.

However, the applications of MBs for the dynamic and temporal regulation of the MSC microenvironment are still emerging. Indeed, limited studies on biomolecule concentration gradients (*e.g.*, fetal bovine serum, chemotactic agents, and oxygen) have been evaluated in 2D and 3D systems for the regulation of MSC migration or proliferation (**Table 3**) [117-119]. These proof-of-principle studies indicate the feasibility of dynamically refining the 3D environment by combining MBs and live cell imaging (*e.g.*, using reporter gene expression systems) towards the enhanced functional properties of MSCs.

4.4. Regulation of endogenous ECM secretion, paracrine signaling, and trophic activities

MSCs are a signficant source of endogenous macromolecues that have essential signaling function. Regulation of the spatial and temproal distribution of the cell-secreted macromolecules is an important means to regulate MSC trophic effects and control tissue regeneration. In parallel flow, MSCs secrete more ECM proteins compared to static culture or transverse flow [51]. In addition, MSCs in perfusion bioreactors were reported to secrete high levels of growth factors. For instance, autocrine fibroblast growth factor (FGF)-2 expression was enhanced in parallel flow, which promoted the proliferation of MSCs [120]. Moreover, the

culture of MSCs in perfusion bioreactors or under mechanical loading promoted the secretion of VEGF and other pro-angiogenic factors such as FGF-2 and TGF-β1 through the activation of endothelial nitric oxide synthase (eNOS) and PGE2 [121, 122]. MSCs in perfusion bioreactors enhanced the expression of placental growth factor, a pro-osteogenic molecule, through actin polymerization-mediated mechanism (*e.g.*, RhoA/ROCK) [123]. In spinner flasks, MSC spheroids upregulated the expression of VEGF and IL-24 [24], a molecule reducing the viability of cancer cells. Under microgravity, MSCs enhanced the secretion of neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, and ciliary neurotrophic factor [99].

However, the precise impact of the bioreactor-mediated cues remains unclear especially at the single MSC level. Due to the intrinsic heterogeneity of MSC populations, understanding the variability of the responses of MSCs at the single cell level to micro-environmental changes is critical for the rational design of MSC differentiation protocols [124]. Contrasting to other types of bioreactors, MBs enable a time-resolved single cell characterization without the need for cell processing (*e.g.*, cells need to be harvested for flow cytometry analysis) [109, 125, 126]. Moreover, the tight control of flow rate in MBs can precisely balance the equilibrium between the accumulation and the wash-out of cell-secreted factors; thus, MBs allow the assessment on the contribution of paracrine factors to the regulation of MSC behavior [109]. Moreover, droplet-based MBs enabling 3D biological compartmentalization combined with tightly controlled microenvironment are especially promising for new applications [127]. For instance, using a microsphere-conjugated antibody, MB enabled the single cell

detection of the transcriptome by encapsulating individual cells into droplets for nanoliter reverse transcription reaction [69], which can be combined with a cell sorting system [128]. In addition, droplet-based MBs can be used for the high-throughput controlled MSC aggregate formation and encapsulation within tunable hydrogels [129]. Thus, MB systems should ultimately enable the combination of the accurate screening of inductive culture conditions, and the enrichment of homogeneous primed MSC population for a dedicated clinical application.

5. Convergence of intracellular signaling pathways in bioreactors

5.1. Regulation of MSCs through the RhoA/ROCK and Hippo signaling

The mechanical properties of 3D scaffolds (*e.g.*, Young's modulus) and the MSC aggregate formation regulate cellular behavior through integrin-mediated signaling, the increased cellular traction, and the activation of RhoA/ROCK signaling [130, 131]. In addition, RhoA/ROCK also regulates the MSC alignment along the nanofibers in 3D polymeric scaffolds [132]. The viscoelastic properties of biomaterials can affect the expression of cadherins, which in turn modulates the activation of RhoA/ROCK signaling [133]. Transduction of the mechanical signals in most bioreactors was found to act through integrins and focal adhesion kinase (FAK) auto-phosphorylation which activated RhoA/ROCK and promoted MSC proliferation and osteogenic differentiation [97, 134].

Hippo signaling can be regulated through 3D cell organization and shear stress in the bioreactors [135]. The activation of RhoA/ROCK induced by shear stress in perfusion bioreactors, the biomaterials stiffness and 3D cell organization leads to the formation of actin fibers and the activation of Hippo signaling [20, 135]. In this pathway, activation of RhoA/ROCK results in the un-phosphorylated

forms of YAP/TAZ potentially through the inhibition of Lats1/2, a specific kinase of YAP/TAZ [136]. The un-phosphorylated YAP/TAZ is translocated to the nucleus and acts as a co-activator of Runt-related transcription factor 2 (RUNX2) and a co-repressor of peroxisome proliferator-activated receptor (PPAR)-γ that promotes osteogenic differentiation but inhibits adipogenesis (**Figure 3A**) [137].

5.2. Regulation of MSCs through the Wnt/β-catenin signaling

Besides Hippo signaling, the Wnt/β-catenin pathway is also influenced by MSC organization and especially by the shear stress in bioreactors (**Figure 3B**). Decreased expression of Dickkopf-1 (DKK-1), an inhibitor of Wnt signaling, has been observed in MSC spheroids [5]. Due to the enhanced N-cadherin expression and β-catenin signaling in MSC spheroids, Wnt pathway might be the key for the enhanced ostegeonic differentiation of MSC spheroids [24]. In bioreactors, fluid shear stress was found to activate non-canonical Wnt pathway and enhance the liberation of β-catenin from N-cadherin, which promoted osteogenesis through RUNX2 activation and reduced adipogenic differentiation [138]. Fluid flow from perfusion bioreactors was reported to act mostly at the early stage of osteogenic differentiation [139], because the sustained β-catenin signaling may have negative effects on the later stages of osteogenic differentiation [140].

Chondrogenic differentiation is also enhanced in PC and SF mediated by Wnt/β-catenin pathway. Perfusion flow was found to promote chondrogenic differentiation over osteogenic differentiation depending on cell density and condensation stage [141, 142]. Fluid shear stress can increase the level of the activated extracellular signal-regulated kinase (ERK) 1/2, which is necessary for chondrogenesis. On the other hand, TGF-β continuously activates the canonical

Wnt/β-catenin and SMAD-3 signaling, which antagonize the late osteogenic differentiation and promoted chondrogenic differentiation [140, 143]. Hence, bioreactor-mediated mechanical stress increases β-catenin signaling and enhances chondrogenic differentiation in combination with the presence of TGF-β (**Figure 3B**). These observations indicate the close cooperation between biochemical and mechanical signals to regulate MSC fate in bioreactors.

While the β-catenin signaling and the Hippo pathway cooperate to enhance MSC differentiation in a context-dependent manner, other signaling may also act in concert to promote MSC differentiation. For instance, the regulation of ion channels, calcium signaling, and the secretion of nitric oxide and prostaglandin E2 (PGE2) also contributes to the enhanced osteogenic differentiation under mechanical stresses [144].

Conclusions and future perspectives

Bioreactors provide culture environment in which molecular signaling from biomaterials, physical forces, and biochemical molecules are integrated and modulated. These functions are especially important in regulating 3D biochemical and biomechanical microenvironments to provide reproducibility and scalability required in MSC bioprocessing and tissue regeneration for clinical application. While the conventional bioreactor systems have been extensively studied, the limitations of these systems may be addressed by microfluidic bioreactors that enable tighter control of 3D culture microenvironment. Microfluidic bioreactors should enable systematic screening of different combinations of mechanical cues and address dynamic delivery of biomolecules for the temporally controlled molecular induction of MSC priming. Moreover, the assessment of heterogeneities

within MSC population should be accurately addressed for better understanding of the non-responding cells. To date, the potential of microfluidic devices for MSC culture *in vitro* has not been fully exploited. Hence, there are avenues towards the refinement of MSC culture processes and better understanding of stem cell niche using microfluidic bioreactors.

Acknowledgement

This work is supported by IN-Wallonia-Brussels International bursary (to S. Sart), FSU start up fund and FSU GAP award (to Y. Li), and the James King Biomedical Research Program (4KB09 and 3KF05 to T. Ma).

Conflict of interest

The authors declare no financial or commercial conflict of interest.

References:

[1] Bianco, P., Robey, P. G., Simmons, P. J., Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008, *2*, 313-319.
[2] Pozzobon, M., Piccoli, M., De Coppi, P., Sources of mesenchymal stem cells: current and future clinical use. *Adv Biochem Eng Biotechnol* 2013, *130*, 267-286.
[3] Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I.*, et al.*, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006, *8*, 315-317.
[4] Bianco, P., Cao, X., Frenette, P. S., Mao, J. J.*, et al.*, The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013, *19*, 35-42.
[5] Bartosh, T. J., Ylostalo, J. H., Mohammadipoor, A., Bazhanov, N.*, et al.*, Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proc Natl Acad Sci U S A* 2010, *107*, 13724-13729.
[6] da Silva Meirelles, L., Caplan, A. I., Nardi, N. B., In search of the in vivo identity of mesenchymal stem cells. *STEM CELLS* 2008, *26*, 2287-2299.
[7] Shi, C., Recent progress toward understanding the physiological function of bone marrow mesenchymal stem cells. *Immunology* 2012, *136*, 133-138.

[8] Kim, J., Ma, T., Autocrine fibroblast growth factor 2-mediated interactions between human mesenchymal stem cells and the extracellular matrix under varying oxygen tension. *J Cell Biochem* 2013, *114*, 716-727.
[9] Ferguson, C. M., Miclau, T., Hu, D., Alpern, E., Helms, J. A., Common molecular pathways in skeletal morphogenesis and repair. *Ann N Y Acad Sci* 1998, *857*, 33-42.
[10] Gurkan, U. A., Akkus, O., The mechanical environment of bone marrow: a review. *Ann Biomed Eng* 2008, *36*, 1978-1991.
[11] Tandon, N., Marolt, D., Cimetta, E., Vunjak-Novakovic, G., Bioreactor engineering of stem cell environments. *Biotechnol Adv* 2013, *31*, 1020-1031.
[12] King, J. A., Miller, W. M., Bioreactor development for stem cell expansion and controlled differentiation. *Curr Opin Chem Biol* 2007, *11*, 394-398.
[13] Vunjak-Novakovic, G., Scadden, D. T., Biomimetic platforms for human stem cell research. *Cell Stem Cell* 2011, *8*, 252-261.
[14] Blagovic, K., Kim, L. Y., Voldman, J., Microfluidic perfusion for regulating diffusible signaling in stem cells. *PLoS One* 2011, *6*, e22892.
[15] Dos Santos, F. F., Andrade, P. Z., da Silva, C. L., Cabral, J. M., Bioreactor design for clinical-grade expansion of stem cells. *Biotechnol J* 2013, *8*, 644-654.
[16] Maia, F. R., Fonseca, K. B., Rodrigues, G., Granja, P. L., Barrias, C. C., Matrix-driven formation of mesenchymal stem cell-extracellular matrix microtissues on soft alginate hydrogels. *Acta Biomater* 2014, *10*, 3197-3208.
[17] Grayson, W. L., Ma, T., Bunnell, B., Human mesenchymal stem cells tissue development in 3D PET matrices. *Biotechnol Prog* 2004, *20*, 905-912.
[18] Kumar, D., Gerges, I., Tamplenizza, M., Lenardi, C.*, et al.*, Three-dimensional hypoxic culture of human mesenchymal stem cells encapsulated in a photocurable, biodegradable polymer hydrogel: a potential injectable cellular product for nucleus pulposus regeneration. *Acta Biomater* 2014, *10*, 3463-3474.
[19] Chien, H. W., Fu, S. W., Shih, A. Y., Tsai, W. B., Modulation of the stemness and osteogenic differentiation of human mesenchymal stem cells by controlling RGD concentrations of poly(carboxybetaine) hydrogel. *Biotechnol J* 2014, *9*, 1613-1623.
[20] Li, Z., Gong, Y., Sun, S., Du, Y.*, et al.*, Differential regulation of stiffness, topography, and dimension of substrates in rat mesenchymal stem cells. *Biomaterials* 2013, *34*, 7616-7625.
[21] Lee, E. A., Im, S. G., Hwang, N. S., Efficient myogenic commitment of human mesenchymal stem cells on biomimetic materials replicating myoblast topography. *Biotechnol J* 2014, *9*, 1604-1612.
[22] Pek, Y. S., Wan, A. C., Ying, J. Y., The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel. *Biomaterials* 2010, *31*, 385-391.
[23] Silva, N. A., Moreira, J., Ribeiro-Samy, S., Gomes, E. D.*, et al.*, Modulation of bone marrow mesenchymal stem cell secretome by ECM-like hydrogels. *Biochimie* 2013, *95*, 2314-2319.
[24] Frith, J. E., Thomson, B., Genever, P. G., Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. *Tissue Eng Part C Methods* 2010, *16*, 735-749.
[25] Hsu, S. H., Huang, G. S., Substrate-dependent Wnt signaling in MSC differentiation within biomaterial-derived 3D spheroids. *Biomaterials* 2013, *34*, 4725-4738.

[26] Sart, S., Tsai, A.-C., Li, Y., Ma, T., Three-dimensional aggregates of mesenchymal stem cells: cellular mechanisms, biological properties, and applications. *Tissue Engineering Part B Reviews* 2014, *20*, 365-380.
[27] Hong, S., Song, S. J., Lee, J. Y., Jang, H.*, et al.*, Cellular behavior in micropatterned hydrogels by bioprinting system depended on the cell types and cellular interaction. *J Biosci Bioeng* 2013, *116*, 224-230.
[28] Hwang, N. S., Varghese, S., Li, H., Elisseeff, J., Regulation of osteogenic and chondrogenic differentiation of mesenchymal stem cells in PEG-ECM hydrogels. *Cell Tissue Res* 2011, *344*, 499-509.
[29] Bauwens, C., Yin, T., Dang, S., Peerani, R., Zandstra, P. W., Development of a perfusion fed bioreactor for embryonic stem cell-derived cardiomyocyte generation: oxygen-mediated enhancement of cardiomyocyte output. *Biotechnol Bioeng* 2005, *90*, 452-461.
[30] Weber, C., Pohl, S., Portner, R., Wallrapp, C.*, et al.*, Cultivation and differentiation of encapsulated hMSC-TERT in a disposable small-scale syringe-like fixed bed reactor. *Open Biomed Eng J* 2007, *1*, 64-70.
[31] Ziane, S., Schlaubitz, S., Miraux, S., Patwa, A.*, et al.*, A thermosensitive low molecular weight hydrogel as scaffold for tissue engineering. *Eur Cell Mater* 2012, *23*, 147-160; discussion 160.
[32] Rodrigues, C. A., Fernandes, T. G., Diogo, M. M., da Silva, C. L., Cabral, J. M., Stem cell cultivation in bioreactors. *Biotechnol Adv* 2011, *29*, 815-829.
[33] Doroudian, G., Curtis, M. W., Gang, A., Russell, B., Cyclic strain dominates over microtopography in regulating cytoskeletal and focal adhesion remodeling of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2013, *430*, 1040-1046.
[34] Sart, S., Agathos, S. N., Li, Y., Engineering stem cell fate with biochemical and biomechanical properties of microcarriers. *Biotechnol Prog* 2013, *29*, 1354-1366.
[35] Elseberg, C. L., Leber, J., Salzig, D., Wallrapp, C.*, et al.*, Microcarrier-based expansion process for hMSCs with high vitality and undifferentiated characteristics. *Int J Artif Organs* 2012, *35*, 93-107.
[36] Frauenschuh, S., Reichmann, E., Ibold, Y., Goetz, P. M.*, et al.*, A microcarrier-based cultivation system for expansion of primary mesenchymal stem cells. *Biotechnol Prog* 2007, *23*, 187-193.
[37] Stiehler, M., Bunger, C., Baatrup, A., Lind, M.*, et al.*, Effect of dynamic 3-D culture on proliferation, distribution, and osteogenic differentiation of human mesenchymal stem cells. *J Biomed Mater Res A* 2009, *89*, 96-107.
[38] Kaiser, S., Jossen, V., Schirmaier, C., Eibl, D.*, et al.*, Fluid flow and cell proliferation of mesenchymal adipose-derived stem cells in small-scale, stirred, single-use bioreactors. . *Chemie Ingenieur Technik* 2013, *85*, 95-102.
[39] Ismadi, M. Z., Gupta, P., Fouras, A., Verma, P.*, et al.*, Flow characterization of a spinner flask for induced pluripotent stem cell culture application. *PLoS One* 2014, *9*, e106493.
[40] Singh, H., Teoh, S. H., Low, H. T., Hutmacher, D. W., Flow modelling within a scaffold under the influence of uni-axial and bi-axial bioreactor rotation. *J Biotechnol* 2005, *119*, 181-196.
[41] Liu, Y., Teoh, S. H., Chong, M. S., Yeow, C. H.*, et al.*, Contrasting effects of vasculogenic induction upon biaxial bioreactor stimulation of mesenchymal stem

cells and endothelial progenitor cells cocultures in three-dimensional scaffolds under in vitro and in vivo paradigms for vascularized bone tissue engineering. *Tissue Eng Part A* 2013, *19*, 893-904.
[42] Zhang, Z. Y., Teoh, S. H., Teo, E. Y., Khoon Chong, M. S.*, et al.*, A comparison of bioreactors for culture of fetal mesenchymal stem cells for bone tissue engineering. *Biomaterials* 2010, *31*, 8684-8695.
[43] Dong, J. D., Gu, Y. Q., Li, C. M., Wang, C. R.*, et al.*, Response of mesenchymal stem cells to shear stress in tissue-engineered vascular grafts. *Acta Pharmacol Sin* 2009, *30*, 530-536.
[44] Zhao, F., Grayson, W. L., Ma, T., Irsigler, A., Perfusion affects the tissue developmental patterns of human mesenchymal stem cells in 3D scaffolds. *J Cell Physiol* 2009, *219*, 421-429.
[45] Dahlin, R. L., Meretoja, V. V., Ni, M., Kasper, F. K., Mikos, A. G., Design of a high-throughput flow perfusion bioreactor system for tissue engineering. *Tissue Eng Part C Methods* 2012, *18*, 817-820.
[46] Porter, B., Zauel, R., Stockman, H., Guldberg, R., Fyhrie, D., 3-D computational modeling of media flow through scaffolds in a perfusion bioreactor. *J Biomech* 2005, *38*, 543-549.
[47] Zhao, F., Chella, R., Ma, T., Effects of shear stress on 3-D human mesenchymal stem cell construct development in a perfusion bioreactor system: Experiments and hydrodynamic modeling. *Biotechnol Bioeng* 2007, *96*, 584-595.
[48] Grayson, W. L., Marolt, D., Bhumiratana, S., Frohlich, M.*, et al.*, Optimizing the medium perfusion rate in bone tissue engineering bioreactors. *Biotechnol Bioeng* 2011, *108*, 1159-1170.
[49] Bancroft, G. N., Sikavitsas, V. I., van den Dolder, J., Sheffield, T. L.*, et al.*, Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc Natl Acad Sci U S A* 2002, *99*, 12600-12605.
[50] Bartnikowski, M., Klein, T. J., Melchels, F. P., Woodruff, M. A., Effects of scaffold architecture on mechanical characteristics and osteoblast response to static and perfusion bioreactor cultures. *Biotechnol Bioeng* 2014, *111*, 1440-1451.
[51] Kim, J., Ma, T., Perfusion regulation of hMSC microenvironment and osteogenic differentiation in 3D scaffold. *Biotechnol Bioeng* 2012, *109*, 252-261.
[52] Pedersen, J. A., Lichter, S., Swartz, M. A., Cells in 3D matrices under interstitial flow: effects of extracellular matrix alignment on cell shear stress and drag forces. *J Biomech* 2010, *43*, 900-905.
[53] Maes, F., Van Ransbeeck, P., Van Oosterwyck, H., Verdonck, P., Modeling fluid flow through irregular scaffolds for perfusion bioreactors. *Biotechnol Bioeng* 2009, *103*, 621-630.
[54] Zhang, X., Nan, Y., Wang, H., Chen, J.*, et al.*, Model microgravity enhances endothelium differentiation of mesenchymal stem cells. *Naturwissenschaften* 2013, *100*, 125-133.
[55] Jin, F., Zhang, Y., Xuan, K., He, D.*, et al.*, Establishment of three-dimensional tissue-engineered bone constructs under microgravity-simulated conditions. *Artif Organs* 2010, *34*, 118-125.

[56] Cerwinka, W. H., Sharp, S. M., Boyan, B. D., Zhau, H. E.*, et al.*, Differentiation of human mesenchymal stem cell spheroids under microgravity conditions. *Cell Regeneration* 2012, *1*, 2.
[57] Vunjak-Novakovic, G., Searby, N., De Luis, J., Freed, L. E., Microgravity studies of cells and tissues. *Ann N Y Acad Sci* 2002, *974*, 504-517.
[58] Cinbiz, M. N., Tigli, R. S., Beskardes, I. G., Gumusderelioglu, M., Colak, U., Computational fluid dynamics modeling of momentum transport in rotating wall perfused bioreactor for cartilage tissue engineering. *J Biotechnol* 2010, *150*, 389-395.
[59] Zhao, Y. H., Lv, X., Liu, Y. L., Zhao, Y.*, et al.*, Hydrostatic pressure promotes the proliferation and osteogenic/chondrogenic differentiation of mesenchymal stem cells: The roles of RhoA and Rac1. *Stem Cell Res* 2015, *14*, 283-296.
[60] Steward, A. J., Wagner, D. R., Kelly, D. J., Exploring the roles of integrin binding and cytoskeletal reorganization during mesenchymal stem cell mechanotransduction in soft and stiff hydrogels subjected to dynamic compression. *J Mech Behav Biomed Mater* 2014, *38*, 174-182.
[61] Ye, R., Hao, J., Song, J., Zhao, Z.*, et al.*, Microenvironment is involved in cellular response to hydrostatic pressures during chondrogenesis of mesenchymal stem cells. *J Cell Biochem* 2014, *115*, 1089-1096.
[62] Khani, M. M., Tafazzoli-Shadpour, M., Goli-Malekabadi, Z., Haghighipour, N., Mechanical characterization of human mesenchymal stem cells subjected to cyclic uniaxial strain and TGF-beta1. *J Mech Behav Biomed Mater* 2015, *43*, 18-25.
[63] Ghazanfari, S., Tafazzoli-Shadpour, M., Shokrgozar, M. A., Effects of cyclic stretch on proliferation of mesenchymal stem cells and their differentiation to smooth muscle cells. *Biochem Biophys Res Commun* 2009, *388*, 601-605.
[64] Goli-Malekabadi, Z., Tafazzoli-Shadpour, M., Rabbani, M., Janmaleki, M., Effect of uniaxial stretch on morphology and cytoskeleton of human mesenchymal stem cells: static vs. dynamic loading. *Biomed Tech (Berl)* 2011, *56*, 259-265.
[65] Ertl, P., Sticker, D., Charwat, V., Kasper, C., Lepperdinger, G., Lab-on-a-chip technologies for stem cell analysis. *Trends Biotechnol* 2014, *32*, 245-253.
[66] Young, E. W., Beebe, D. J., Fundamentals of microfluidic cell culture in controlled microenvironments. *Chem Soc Rev* 2010, *39*, 1036-1048.
[67] Zhong, W., Tian, K., Zheng, X., Li, L.*, et al.*, Mesenchymal stem cell and chondrocyte fates in a multishear microdevice are regulated by Yes-associated protein. *Stem Cells Dev* 2013, *22*, 2083-2093.
[68] Toh, Y. C., Zhang, C., Zhang, J., Khong, Y. M.*, et al.*, A novel 3D mammalian cell perfusion-culture system in microfluidic channels. *Lab Chip* 2007, *7*, 302-309.
[69] Li, Z., Zhang, C., Weiner, L. P., Zhang, Y., Zhong, J. F., Molecular characterization of heterogeneous mesenchymal stem cells with single-cell transcriptomes. *Biotechnol Adv* 2013, *31*, 312-317.
[70] Zheng, W., Xie, Y., Zhang, W., Wang, D.*, et al.*, Fluid flow stress induced contraction and re-spread of mesenchymal stem cells: a microfluidic study. *Integr Biol (Camb)* 2012, *4*, 1102-1111.
[71] Ong, S. M., Zhang, C., Toh, Y. C., Kim, S. H.*, et al.*, A gel-free 3D microfluidic cell culture system. *Biomaterials* 2008, *29*, 3237-3244.
[72] Salerno, A., Levato, R., Mateos-Timoneda, M. A., Engel, E.*, et al.*, Modular polylactic acid microparticle-based scaffolds prepared via microfluidic

emulsion/solvent displacement process: fabrication, characterization, and in vitro mesenchymal stem cells interaction study. *J Biomed Mater Res A* 2013, *101*, 720-732.
[73] Kang, K. S., Hong, J. M., Kang, J. A., Rhie, J. W.*, et al.*, Regulation of osteogenic differentiation of human adipose-derived stem cells by controlling electromagnetic field conditions. *Exp Mol Med* 2013, *45*, e6.
[74] Xu, B., Song, G., Ju, Y., Li, X.*, et al.*, RhoA/ROCK, cytoskeletal dynamics, and focal adhesion kinase are required for mechanical stretch-induced tenogenic differentiation of human mesenchymal stem cells. *J Cell Physiol* 2012, *227*, 2722-2729.
[75] Pelaez, D., Arita, N., Cheung, H. S., Extracellular signal-regulated kinase (ERK) dictates osteogenic and/or chondrogenic lineage commitment of mesenchymal stem cells under dynamic compression. *Biochem Biophys Res Commun* 2012, *417*, 1286-1291.
[76] Moraes, C., Wang, G., Sun, Y., Simmons, C. A., A microfabricated platform for high-throughput unconfined compression of micropatterned biomaterial arrays. *Biomaterials* 2010, *31*, 577-584.
[77] Gao, X., Zhang, X., Tong, H., Lin, B., Qin, J., A simple elastic membrane-based microfluidic chip for the proliferation and differentiation of mesenchymal stem cells under tensile stress. *Electrophoresis* 2011, *32*, 3431-3436.
[78] Chabert, M., Viovy, J. L., Microfluidic high-throughput encapsulation and hydrodynamic self-sorting of single cells. *Proc Natl Acad Sci U S A* 2008, *105*, 3191-3196.
[79] Joensson, H. N., Andersson Svahn, H., Droplet microfluidics--a tool for single-cell analysis. *Angew Chem Int Ed Engl* 2012, *51*, 12176-12192.
[80] Hsu, Y. H., Moya, M. L., Abiri, P., Hughes, C. C.*, et al.*, Full range physiological mass transport control in 3D tissue cultures. *Lab Chip* 2013, *13*, 81-89.
[81] Pattappa, G., Heywood, H. K., de Bruijn, J. D., Lee, D. A., The metabolism of human mesenchymal stem cells during proliferation and differentiation. *J Cell Physiol* 2011, *226*, 2562-2570.
[82] Hofmann, A. D., Beyer, M., Krause-Buchholz, U., Wobus, M.*, et al.*, OXPHOS supercomplexes as a hallmark of the mitochondrial phenotype of adipogenic differentiated human MSCs. *PLoS One* 2012, *7*, e35160.
[83] Higuera, G., Schop, D., Janssen, F., van Dijkhuizen-Radersma, R.*, et al.*, Quantifying in vitro growth and metabolism kinetics of human mesenchymal stem cells using a mathematical model. *Tissue Eng Part A* 2009, *15*, 2653-2663.
[84] Lo, T., Ho, J. H., Yang, M. H., Lee, O. K., Glucose reduction prevents replicative senescence and increases mitochondrial respiration in human mesenchymal stem cells. *Cell Transplant* 2011, *20*, 813-825.
[85] Li, Y. M., Schilling, T., Benisch, P., Zeck, S.*, et al.*, Effects of high glucose on mesenchymal stem cell proliferation and differentiation. *Biochem Biophys Res Commun* 2007, *363*, 209-215.
[86] Kanichai, M., Ferguson, D., Prendergast, P. J., Campbell, V. A., Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha. *J Cell Physiol* 2008, *216*, 708-715.

[87] Hsu, S. H., Chen, C. T., Wei, Y. H., Inhibitory effects of hypoxia on metabolic switch and osteogenic differentiation of human mesenchymal stem cells. *STEM CELLS* 2013, *31*, 2779-2788.
[88] Sart, S., Agathos , S. N., Li, Y., Process engineering of stem cell metabolism for large scale expansion and differentiation in bioreactors. *Biochem Eng J* 2014, *84*, 74-82.
[89] Wu, C. C., Chao, Y. C., Chen, C. N., Chien, S.*, et al.*, Synergism of biochemical and mechanical stimuli in the differentiation of human placenta-derived multipotent cells into endothelial cells. *J Biomech* 2008, *41*, 813-821.
[90] Park, S. H., Sim, W. Y., Min, B. H., Yang, S. S.*, et al.*, Chip-based comparison of the osteogenesis of human bone marrow- and adipose tissue-derived mesenchymal stem cells under mechanical stimulation. *PLoS One* 2012, *7*, e46689.
[91] Jagodzinski, M., Drescher, M., Zeichen, J., Hankemeier, S.*, et al.*, Effects of cyclic longitudinal mechanical strain and dexamethasone on osteogenic differentiation of human bone marrow stromal cells. *Eur Cell Mater* 2004, *7*, 35-41; discussion 41.
[92] Hong, D., Chen, H. X., Xue, Y., Li, D. M.*, et al.*, Osteoblastogenic effects of dexamethasone through upregulation of TAZ expression in rat mesenchymal stem cells. *J Steroid Biochem Mol Biol* 2009, *116*, 86-92.
[93] Hamidouche, Z., Hay, E., Vaudin, P., Charbord, P.*, et al.*, FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/beta-catenin signaling-dependent Runx2 expression. *Faseb J* 2008, *22*, 3813-3822.
[94] Gaur, T., Lengner, C. J., Hovhannisyan, H., Bhat, R. A.*, et al.*, Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* 2005, *280*, 33132-33140.
[95] Xiao, G., Cui, Y., Ducy, P., Karsenty, G., Franceschi, R. T., Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. *Mol Endocrinol* 1997, *11*, 1103-1113.
[96] Beck, G. R., Jr., Zerler, B., Moran, E., Phosphate is a specific signal for induction of osteopontin gene expression. *Proc Natl Acad Sci U S A* 2000, *97*, 8352-8357.
[97] Huang, Y., Dai, Z. Q., Ling, S. K., Zhang, H. Y.*, et al.*, Gravity, a regulation factor in the differentiation of rat bone marrow mesenchymal stem cells. *J Biomed Sci* 2009, *16*, 87.
[98] Muruganandan, S., Roman, A. A., Sinal, C. J., Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cell Mol Life Sci* 2009, *66*, 236-253.
[99] Kellinsalmi, M., Parikka, V., Risteli, J., Hentunen, T.*, et al.*, Inhibition of cyclooxygenase-2 down-regulates osteoclast and osteoblast differentiation and favours adipocyte formation in vitro. *Eur J Pharmacol* 2007, *572*, 102-110.
[100] Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O.*, et al.*, An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* 1995, *270*, 12953-12956.
[101] Mauck, R. L., Nicoll, S. B., Seyhan, S. L., Ateshian, G. A., Hung, C. T., Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. *Tissue Eng* 2003, *9*, 597-611.

[102] Campbell, J. J., Lee, D. A., Bader, D. L., Dynamic compressive strain influences chondrogenic gene expression in human mesenchymal stem cells. *Biorheology* 2006, *43*, 455-470.
[103] Sekiya, I., Koopman, P., Tsuji, K., Mertin, S.*, et al.*, Dexamethasone enhances SOX9 expression in chondrocytes. *J Endocrinol* 2001, *169*, 573-579.
[104] McNulty, A. L., Vail, T. P., Kraus, V. B., Chondrocyte transport and concentration of ascorbic acid is mediated by SVCT2. *Biochim Biophys Acta* 2005, *1712*, 212-221.
[105] Lourenco, N. D., Lopes, J. A., Almeida, C. F., Sarraguca, M. C., Pinheiro, H. M., Bioreactor monitoring with spectroscopy and chemometrics: a review. *Anal Bioanal Chem* 2012, *404*, 1211-1237.
[106] Sart, S., Schneider, Y. J., Agathos, S. N., Influence of culture parameters on ear mesenchymal stem cells expanded on microcarriers. *J Biotechnol* 2010, *150*, 149-160.
[107] Li, D., Tang, T., Lu, J., Dai, K., Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. *Tissue Eng Part A* 2009, *15*, 2773-2783.
[108] Lovett, M., Rockwood, D., Baryshyan, A., Kaplan, D. L., Simple modular bioreactors for tissue engineering: a system for characterization of oxygen gradients, human mesenchymal stem cell differentiation, and prevascularization. *Tissue Eng Part C Methods* 2010, *16*, 1565-1573.
[109] Hemmingsen, M., Vedel, S., Skafte-Pedersen, P., Sabourin, D.*, et al.*, The role of paracrine and autocrine signaling in the early phase of adipogenic differentiation of adipose-derived stem cells. *PLoS One* 2013, *8*, e63638.
[110] Oshina, H., Sotome, S., Yoshii, T., Torigoe, I.*, et al.*, Effects of continuous dexamethasone treatment on differentiation capabilities of bone marrow-derived mesenchymal cells. *Bone* 2007, *41*, 575-583.
[111] France, L. A., Scotchford, C. A., Grant, D. M., Rashidi, H.*, et al.*, Transient serum exposure regimes to support dual differentiation of human mesenchymal stem cells. *J Tissue Eng Regen Med* 2012.
[112] Buxton, A. N., Bahney, C. S., Yoo, J. U., Johnstone, B., Temporal exposure to chondrogenic factors modulates human mesenchymal stem cell chondrogenesis in hydrogels. *Tissue Eng Part A* 2011, *17*, 371-380.
[113] Keenan, T. M., Folch, A., Biomolecular gradients in cell culture systems. *Lab Chip* 2008, *8*, 34-57.
[114] Fung, W. T., Beyzavi, A., Abgrall, P., Nguyen, N. T., Li, H. Y., Microfluidic platform for controlling the differentiation of embryoid bodies. *Lab Chip* 2009, *9*, 2591-2595.
[115] Kim, S., Kim, H. J., Jeon, N. L., Biological applications of microfluidic gradient devices. *Integr Biol (Camb)* 2010, *2*, 584-603.
[116] Ye, N., Qin, J., Shi, W., Liu, X., Lin, B., Cell-based high content screening using an integrated microfluidic device. *Lab Chip* 2007, *7*, 1696-1704.
[117] Rexius-Hall, M. L., Mauleon, G., Malik, A. B., Rehman, J., Eddington, D. T., Microfluidic platform generates oxygen landscapes for localized hypoxic activation. *Lab Chip* 2014, *14*, 4688-4695.

[118] Park, J. Y., Hwang, C. M., Lee, S. H., Gradient generation by an osmotic pump and the behavior of human mesenchymal stem cells under the fetal bovine serum concentration gradient. *Lab Chip* 2007, *7*, 1673-1680.
[119] Pagano, G., Ventre, M., Iannone, M., Greco, F.*, et al.*, Optimizing design and fabrication of microfluidic devices for cell cultures: An effective approach to control cell microenvironment in three dimensions. *Biomicrofluidics* 2014, *8*, 046503.
[120] Kim, J., Ma, T., Regulation of autocrine fibroblast growth factor-2 signaling by perfusion flow in 3D human mesenchymal stem cell constructs. *Biotechnol Prog* 2012, *28*, 1384-1388.
[121] Bassaneze, V., Barauna, V. G., Lavini-Ramos, C., Kalil, J.*, et al.*, Shear stress induces nitric oxide-mediated vascular endothelial growth factor production in human adipose tissue mesenchymal stem cells. *Stem Cells Dev* 2010, *19*, 371-378.
[122] Kasper, G., Dankert, N., Tuischer, J., Hoeft, M.*, et al.*, Mesenchymal stem cells regulate angiogenesis according to their mechanical environment. *STEM CELLS* 2007, *25*, 903-910.
[123] McCoy, R. J., Widaa, A., Watters, K. M., Wuerstle, M.*, et al.*, Orchestrating osteogenic differentiation of mesenchymal stem cells - identification of placental growth factor as a mechanosensitive gene with a pro-osteogenic role. *STEM CELLS* 2013, *31*, 2420-2431.
[124] Wu, J., Tzanakakis, E. S., Deconstructing stem cell population heterogeneity: single-cell analysis and modeling approaches. *Biotechnol Adv* 2013, *31*, 1047-1062.
[125] Choi, J., Kim, S., Jung, J., Lim, Y.*, et al.*, Wnt5a-mediating neurogenesis of human adipose tissue-derived stem cells in a 3D microfluidic cell culture system. *Biomaterials* 2011, *32*, 7013-7022.
[126] Ju, X., Li, D., Gao, N., Shi, Q., Hou, H., Hepatogenic differentiation of mesenchymal stem cells using microfluidic chips. *Biotechnol J* 2008, *3*, 383-391.
[127] Brouzes, E., Medkova, M., Savenelli, N., Marran, D.*, et al.*, Droplet microfluidic technology for single-cell high-throughput screening. *Proc Natl Acad Sci U S A* 2009, *106*, 14195-14200.
[128] Konry, T., Dominguez-Villar, M., Baecher-Allan, C., Hafler, D. A., Yarmush, M. L., Droplet-based microfluidic platforms for single T cell secretion analysis of IL-10 cytokine. *Biosens Bioelectron* 2011, *26*, 2707-2710.
[129] Chan, H. F., Zhang, Y., Ho, Y. P., Chiu, Y. L.*, et al.*, Rapid formation of multicellular spheroids in double-emulsion droplets with controllable microenvironment. *Sci Rep* 2013, *3*, 3462.
[130] Huebsch, N., Arany, P. R., Mao, A. S., Shvartsman, D.*, et al.*, Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. *Nat Mater* 2010, *9*, 518-526.
[131] Khetan, S., Guvendiren, M., Legant, W. R., Cohen, D. M.*, et al.*, Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. *Nat Mater* 2013, *12*, 458-465.
[132] Andalib, M. N., Lee, J. S., Ha, L., Dzenis, Y., Lim, J. Y., The role of RhoA kinase (ROCK) in cell alignment on nanofibers. *Acta Biomater* 2013, *9*, 7737-7745.
[133] Cameron, A. R., Frith, J. E., Gomez, G. A., Yap, A. S., Cooper-White, J. J., The effect of time-dependent deformation of viscoelastic hydrogels on myogenic

induction and Rac1 activity in mesenchymal stem cells. *Biomaterials* 2014, *35*, 1857-1868.
[134] Arnsdorf, E. J., Tummala, P., Kwon, R. Y., Jacobs, C. R., Mechanically induced osteogenic differentiation--the role of RhoA, ROCKII and cytoskeletal dynamics. *J Cell Sci* 2009, *122*, 546-553.
[135] Hiemer, S. E., Varelas, X., Stem cell regulation by the Hippo pathway. *Biochim Biophys Acta* 2013, *1830*, 2323-2334.
[136] Liu, H., Jiang, D., Chi, F., Zhao, B., The Hippo pathway regulates stem cell proliferation, self-renewal, and differentiation. *Protein Cell* 2012, *3*, 291-304.
[137] Halder, G., Dupont, S., Piccolo, S., Transduction of mechanical and cytoskeletal cues by YAP and TAZ. *Nat Rev Mol Cell Biol* 2012, *13*, 591-600.
[138] Arnsdorf, E. J., Tummala, P., Jacobs, C. R., Non-canonical Wnt signaling and N-cadherin related beta-catenin signaling play a role in mechanically induced osteogenic cell fate. *PLoS One* 2009, *4*, e5388.
[139] Delaine-Smith, R. M., Reilly, G. C., Mesenchymal stem cell responses to mechanical stimuli. *Muscles Ligaments Tendons J* 2012, *2*, 169-180.
[140] Ling, L., Nurcombe, V., Cool, S. M., Wnt signaling controls the fate of mesenchymal stem cells. *Gene* 2009, *433*, 1-7.
[141] McBride, S. H., Falls, T., Knothe Tate, M. L., Modulation of stem cell shape and fate B: mechanical modulation of cell shape and gene expression. *Tissue Eng Part A* 2008, *14*, 1573-1580.
[142] McBride, S. H., Knothe Tate, M. L., Modulation of stem cell shape and fate A: the role of density and seeding protocol on nucleus shape and gene expression. *Tissue Eng Part A* 2008, *14*, 1561-1572.
[143] Yang, Z., Zou, Y., Guo, X. M., Tan, H. S., *et al.*, Temporal activation of beta-catenin signaling in the chondrogenic process of mesenchymal stem cells affects the phenotype of the cartilage generated. *Stem Cells Dev* 2012, *21*, 1966-1976.
[144] Liu, L., Yuan, W., Wang, J., Mechanisms for osteogenic differentiation of human mesenchymal stem cells induced by fluid shear stress. *Biomech Model Mechanobiol* 2010, *9*, 659-670.
[145] Sim, W. Y., Park, S. W., Park, S. H., Min, B. H., *et al.*, A pneumatic micro cell chip for the differentiation of human mesenchymal stem cells under mechanical stimulation. *Lab Chip* 2007, *7*, 1775-1782.
[146] Ruiz, S. A., Chen, C. S., Emergence of patterned stem cell differentiation within multicellular structures. *STEM CELLS* 2008, *26*, 2921-2927.

Table 1. Comparison of different bioreactors for MSC expansion and differentiation.

Type of bio-reactor	Geometry/ Dimension	Manufac-turing limitations	Sample volume	Micro-environment regulation	Biochemical Micro-environment control	Mechanical Micro-environment control	Resolution level for monitoring	Ref.
Spinner flask (SF)	Cylinder e.g. 145×63.5 mm (125 mL spinner from Corning)	SF requires glass or plastic molding	Up to 5 cm^3	Single environmental condition could be tested per run	SF can be equipped with probes/analy-tic tools (e.g., Raman spectrometry)	Various level of shear stress generated by impeller	Cell Population/ Sample level	Zhang et al., 2010 [42]
Rotating wall vessel (RWV)	Cylinder e.g., 95.3 ×198.1mm (1000 mL, Synthecon)	RWV requires glass or plastic molding	Up to 10 cm^3	Single environmental condition could be tested per run	RWV can be equipped with probes/analytic tools (e.g., Raman spectrometry)	Homogeneous shear stress	Cell Population / Sample level	Cinbiz et al., 2010 [58]
Perfusion chambers (PC)	Cylinder/ Rectangle	No limitation: PC can be custom-fabricated	Up to 5 cm^3	Single environmental condition could be tested per run	PC can be equipped with probes/analy-tic tools (e.g., Raman spectrometry)	Homogeneous shear stress	Cell Population / Sample level	Bartniko wski et al., 2014 [50]
Compress ion chamber (CC)	Cylinder (10 × 5 mm, e.g., CartiGen)	CC requires plastic molding	Up to 1 cm^3	Single environmental condition could be tested per run	Limited	Homogeneous compressive forces	Cell Population / Sample level	Ye et al., 2014 [61]
Tensile stress chamber (TC)	Cylinder (e.g., up to 31 mm^2, Flexcell)	No limitation: TC can be custom-fabricated	cm^3 scale	Single environmental condition could be tested per run	Limited	Homogeneous stretching forces	Cell Population / Sample level	Ghazanfa ri et al., 2009 [63]
Micro-fluidic bio-reactors (MB)	Rectangle High aspect ratio: (height: micron size ; width: centimeter size)	No limitation: MBs are usually custom-fabricated	mm^3 scale	Gradient of biochemicals; Gradient of bio-mechanical forces	Highly controlled/live imaging/can be equipped with probes/ analytic tools (e.g., Raman spectrometry)	Highly controlled hydro-dynamic	Single cell analysis / Live imaging	Pagano et al., 2014 [119]

Table 2. Regulation of MSC mechanical microenvironment in microfluidic bioreactors.

Cell organiza-tion	Type of microfluidic bioreactor	Parameter evaluated	Effect on MSC proliferation	Effect on MSC phenotype	Ref.
2D	Pneumatic based air chamber	Compression forces (1 kPa to 20 kPa) in the same vessel	1.5 fold increase	Moderate compression (5 kPa) enhanced osteogenic differentiation	Sim et al., 2007 [145]
	Diaphragm vacuum pump negative pressure	Tensile stress (2.2 to 3.7 % membrane deformation) in the same vessel	2 fold increase compared to unstressed conditions	3.7% membrane deformation increased osteogenesis and decreased adipogenesis; Comparison with 2D	Gao et al., 2011 [77]
	Pneumatic air chamber	Simultaneous adipose tissue (AT) derived versus bone marrow (BM) derived MSCs osteogenic differentiation upon compression (from 1 psi to 5 psi)	N.D.	BM-MSCs displayed higher osteogenic differentiation compared to AT-MSCs	Park et al., 2012 [90]
	Perfusion plus resistance chamber	Multiple shear stress magnitude (0.009 to 1.089 dyne/cm^2) in the same bioreactor	1.5 fold increase between 0.009 and 1.089 dyne/cm^2	Shear stress (1.089 dyne/cm^2) increased osteogenic differentiation	Zhong et al., 2013 [67]
3D	Syringe pump perfusion	MSC self-crosslinking (i.e., poly-ethyleneimine-hydrazide) (spheroids)	N.D.	Similar von Kossa staining compared to 2D culture	Ong et al., 2008 [71]
	Syringe pump perfusion	Shear stress (flow rate ranging from 0.06–0.2 ml h^{-1}) into 3D scaffolds	N.D.	Enhanced osteogenic differentiation (von Kossa staining) compared to 2D culture	Toh et al., 2007 [68]
	Static cultures	Surface micro-patterning (250-1000 μm) for multicellular structures of MSCs	N.D.	Size of the micropattern regulated adipogenic and osteogenic differentiation	Ruiz et al., 2008 [146]
	Solenoid valve compression device	Compressive strain magnitude (poly ethylene glycol encapsulation), 6 to 26% compressive strain	N.D.	High compressive stain (26%) increased MSC deformation	Moraes et al., 2010 [76]

N.D. not determined.

Table 3. Regulation of MSC physiological microenvironment in microfluidic bioreactors

Cell organiza-tion	Type of bioreactor	Parameter evaluated	Effect on MSC proliferation	Effect on MSC phenotype	Ref.
2D	Osmotic pump perfusion	Gradient of fetal bovine serum (FBS) (0 to 30%) in the same vessel	30% FBS enhance MSC proliferation, attachment viability and spreading compared to lower concentration	N.D.	Park et al., 2007 [118]
	Peristaltic pump perfusion chamber	Series dilution of adipogenic conditioned medium in the same bioreactor	N.D.	Increased homogeneity in differentiation. Paracrine signaling promoted early adipogenic differentiation (C/EBP β and C/EBP δ)	Hemmings en et al., 2013 [109]
	Perfusion	Perfusion of hepatogenic differentiation medium	2 fold increase compared to 96 well plates	Increased hepatogenic differentiation (4 fold increase in low-density lipoproteins uptake)	Ju et al., 2008 [126]
3D	Perfusion	3D perfusion versus 2D cultures	2 fold increase	Enhanced neuronal-like differentiation compared to 2D	Choi et al., 2011 [125]
	Static	MSC spheroid encapsulation by flow-focusing	N.D.	Enhanced osteogenic differentiation of MSC spheroids in alginate-RGD compared to alginate alone	Chan et al., 2013 [129]
	Perfusion	Gradient of bone morphogenetic protein (BMP)-2	N.D.	Enhanced chemotaxis under gradient compared to uniform BMP-2 concentration	Pagano et al., 2014 [119]

N.D. not determined.

Figure legends

Figure 1. Different types of bioreactors to regulate MSC microenvironment. (A) Spinner flask (SF) bioreactor enables MSC culture in 3D scaffolds or as spheroids. (B) Perfusion chamber (PC) enables MSC culture in 3D scaffolds. (C) Rotating wall vessel enables MSC culture on 3D scaffolds or as spheroids (RWV). Microfluidic bioreactor (MB) with flow focuser to generate capsule droplets for MSC encapsulation as single cells or as spheroids.

Figure 2. Culture systems and configurations for 3D expansion and differentiation of MSCs. (A) The 3D MSC encapsulation processes include polymeric hydrogel gelation, ECM-grafting and crosslinking, which can be integrated in the culture system. The degree of hydrogel cross-linking regulates the hydrogel mechanical properties, which in turn modulate MSC biological functions (*e.g.*, the osteogenic differentiation is favored on stiff scaffolds or hydrogels). Alternatively, MSCs can also organize into 3D structure in pre-fabricated porous scaffolds with various surface and structural properties that influence MSC fate through their interactions with ECM proteins and biomechanical signaling. (B) The formation of 3D MSC spheroids free of exogenous materials can be integrated in the culture vessels such as low attachment plates, stirred tanks, culture dishes coated with positively charged membrane or UpCell™ for thermal lifting, adapted from Sart et al., 2014 [26].

Figures 3. Regulation of MSC phenotype by bioreactors through the Hippo/YAP pathway and Wnt/β-catenin pathway. (A) The Hippo/YAP

pathway. The inducers of differentiation (*e.g.*, dexamethasone) regulate the production of TAZ. The pattern and the elasticity of ECM regulate the activation of RhoA/ROCK. Shear stress from bioreactor acts through the focal complex to activate RhoA/ROCK. RhoA/ROCK promotes the formation of stress fibers, which inhibit the phosphorylation of YAP/TAZ. The un-phosphorylated form of YAP/TAZ translocates into the nucleus and acts as a co-repressor of PPAR-γ, inhibiting adipogenic differentiation. On the other hand, YAP/TAZ acts as a co-activator of RUNX2, activating osteogenic differentiation. (B) The Wnt/β-catenin pathway. Upon application of mechanical stress, β-catenin is liberated for N-cadherin. β-catenin translocates to the nucleus and inhibits adipogenesis. In combination with exogenous or autocrine transforming growth factor (TGF)-β, β-catenin promotes chondrogenesis. When associated with T-cell factor (TCF) and lymphoid enhancer factor (LEF), the liberated β-catenin promotes the transcription of RUNX2 and osteogenesis. FAK: focal adhesion kinase; GDP: guanosine diphosphate; GTP: guanosine triphosphate; p: phosphorylated; PPAR: peroxisome proliferator-activated receptor; ROCK: Rho-associated protein kinase; RUNX2: Runt-related transcription factor 2; TAZ: transcriptional coactivator with PDZ-binding motif; YAP: Yes-associated protein.

Figure 1

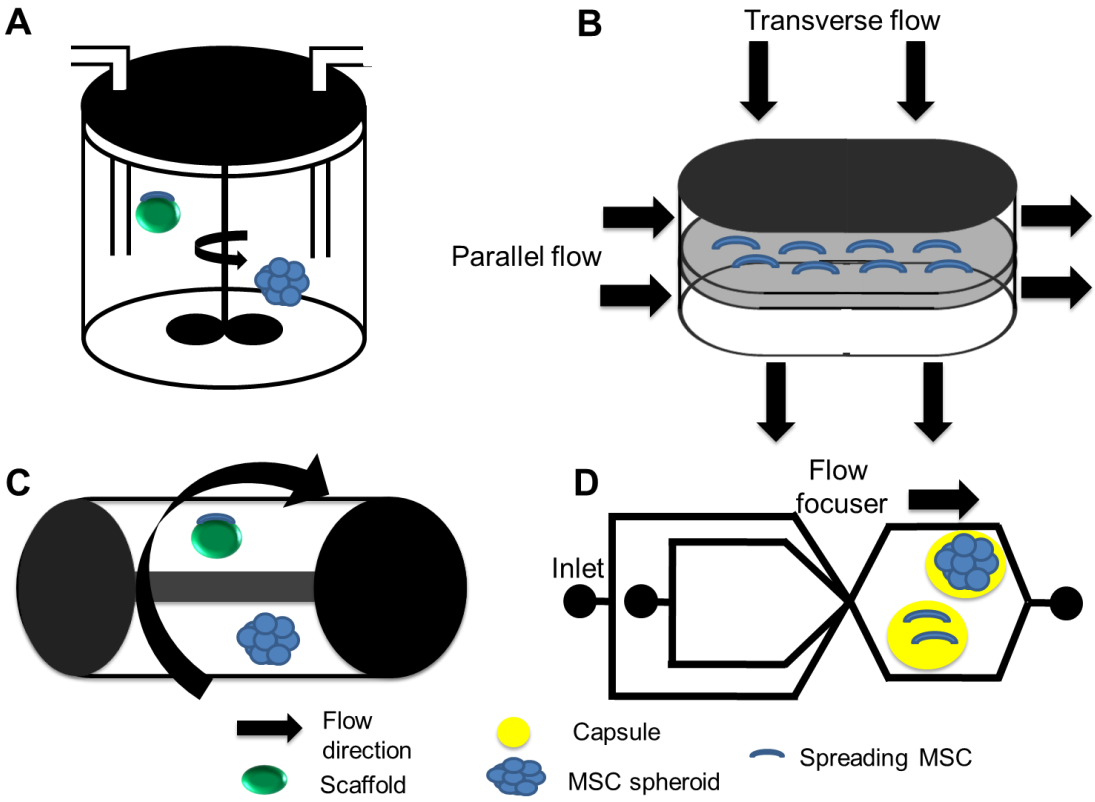

A
B
Transverse flow
Parallel flow
C
D
Flow
focuser
Inlet
Flow
direction
Scaffold
Capsule
MSC spheroid
Spreading MSC

Figure 2

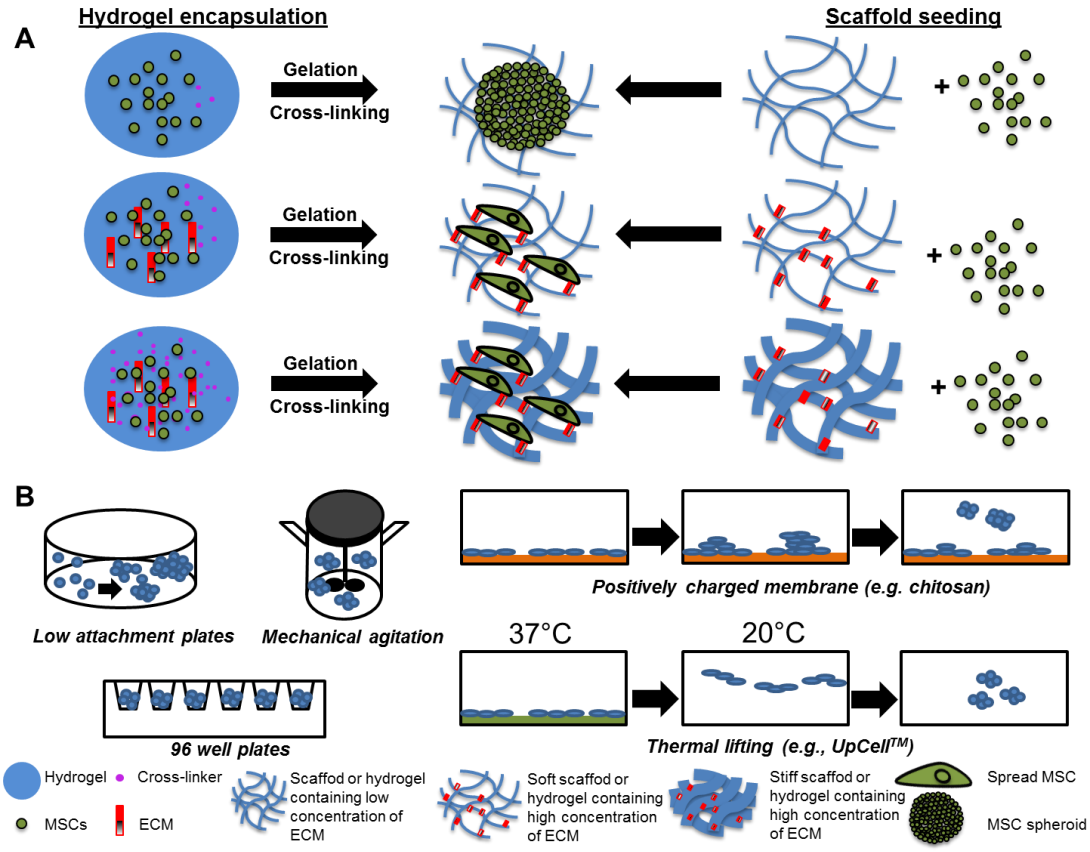
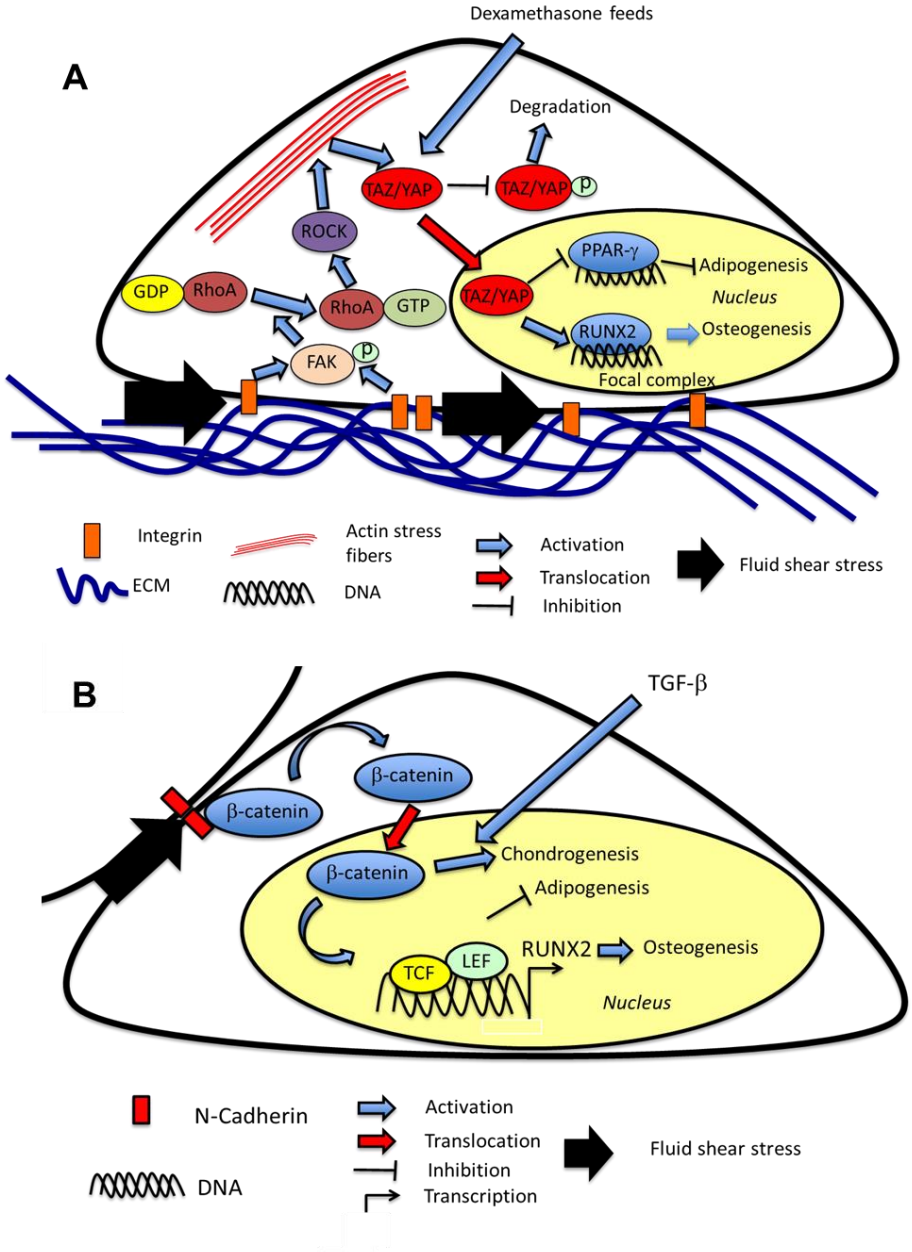

A
Hydrogel encapsulation
Scaffold seeding
Gelation
Cross-linking
Gelation
Cross-linking
Gelation
Cross-linking
B
Low attachment plates
Mechanical agitation
96 well plates
Positively charged membrane (e.g. chitosan)
37°C
20°C
Thermal lifting (e.g., UpCell™)
Hydrogel
Cross-linker
MSCs
ECM
Scaffod or hydrogel containing low concentration of ECM
Soft scaffod or hydrogel containing high concentration of ECM
Stiff scaffod or hydrogel containing high concentration of ECM
Spread MSC
MSC spheroid

Figure 3


A
Dexamethasone feeds
Degradation
TAZ/YAP
TAZ/YAP
P
ROCK
PPAR-γ
Adipogenesis
Nucleus
GDP
RhoA
RhoA
GTP
TAZ/YAP
RUNX2
Osteogenesis
FAK
P
Focal complex
Integrin
Actin stress fibers
Activation
Translocation
Inhibition
Fluid shear stress
ECM
DNA
B
TGF-β
β-catenin
β-catenin
β-catenin
Chondrogenesis
Adipogenesis
RUNX2
Osteogenesis
TCF
LEF
Nucleus
N-Cadherin
Activation
Translocation
Inhibition
Transcription
Fluid shear stress
DNA