APPROACHES TO MIMIC THE COMPLEXITY OF THE SKELETAL MESENCHYMAL STEM/STROMAL CELL NICHE *IN VITRO*

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Abstract

Mesenchymal stem/stromal cells (MSCs) are an essential element of most modern tissue engineering and regenerative medicine approaches due to their multipotency and immunoregulatory functions. Despite the prospective value of MSCs for the clinics, the stem cells community is questioning their developmental origin, *in vivo* localization, identification, and regenerative potential after several years of far-reaching research in the field. Although several major progresses have been made in mimicking the complexity of the MSC niche *in vitro*, there is need for comprehensive studies of fundamental mechanisms triggered by microenvironmental cues before moving to regenerative medicine cell therapy applications. The present comprehensive review extensively discusses the microenvironmental cues that influence MSC phenotype and function in health and disease – including cellular, chemical and physical interactions. The most recent and relevant illustrative examples of novel bioengineering approaches to mimic biological, chemical, and mechanical microenvironmental signals present in the native MSC niche are summarized, with special emphasis on the forefront techniques to achieve bio-chemical complexity and dynamic cultures. In particular, the skeletal MSC niche and applications focusing on the bone regenerative potential of MSC are addressed. The aim of the review was to recognize the limitations of the current MSC niche *in vitro* models and to identify potential opportunities to fill the bridge between fundamental science and clinical application of MSCs.

Keywords: Mesenchymal stem/stromal cells, skeletal progenitor cells, niche, *in vitro* models, bone, tissue engineering.

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List of abbreviations

2D	two dimensional
3D	three dimensional
4D	four dimensional
ALP	alkaline phosphatase
BM	bone marrow
BMP	bone morphogenetic protein
CFU-Fs	colony-forming unit fibroblasts
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C chemokine receptor type 4
DCCs	disseminated cancer cells
DKK1	Dickkopf-related protein 1
ECs	endothelial cells
ECM	extracellular matrix
FasL	Fas ligand
FDA	Food and Drug Administration
Flt3	fms like tyrosine kinase 3
HCs	hematopoietic cells
HLA	human leucocyte antigene
HSCs	hematopoietic stem cells
HSPCs	hematopoietic stem and progenitor cells
IL	interleukin
iNOS	inducible nitric oxide synthase
MAPK	mitogen-activated protein kinases
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage-colony-stimulating factor
miRNA	microRNA

MMPs	matrix metalloproteinases
MSCs	mesenchymal stem/stromal cells
Mϕ	macrophages
NG2	neural/glial antigen 2
OC	osteoclasts
OPG	osteoprotegerin
PDGFRβ	platelet-derived growth factor receptor beta
piRNA	piwi-interacting RNA
PCL	polycaprolactone
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PMMA	polymethylmethacrylate
RANKL	receptor activator of NF-κB ligand
RGD	arginylglycylaspartic acid
RNAi	RNA interference
ROS	reactive oxygen species
Runx2	Runt-related transcription factor 2
SCF	stem cell factor
SCID	severe combined immunodeficiency
SDF-1	stromal cell-derived factor-1
STAT3	signal trnasduces and activator of trancription 3
TRP	transient receptor potential
VEGF	vascular endothelial growth factor

Introduction

Friedenstein and co-workers originally identified a rare sub-population of cells in the BM with the potential to proliferate in plastic-adherent colonies with a fibroblastic appearance, first designated as CFU-Fs (Friedenstein *et al.*, 1974). Later *in vivo* experiments have revealed the potential of BM-isolated adherent cells to generate *de novo* the BM stroma and its environment upon serial transplantation to heterotopic anatomical sites (Caplan, 1991; Owen, 1988), leading to the current concept of BM-derived MSCs.

Although the BM is the most widely recognized source of MSCs, further research has suggested the presence of MSC-like cells in other tissues, including adipose tissue (Zuk *et al.*, 2002), peripheral blood (Tondreau *et al.*, 2005), dental pulp (Gronthos *et al.*, 2000), pancreatic islets (Carlotti *et al.*, 2010), synovial membrane (Hermida-Gómez *et al.*, 2011), periodontal ligament (Seo *et al.*, 2004), anterior cruciate ligament (Prager *et al.*, 2018), endometrium (Schwab *et al.*, 2008), *bursa subacromialis* (Steinert *et al.*, 2015), placenta (Fukuchi *et al.*, 2004), umbilical cord (Baksh *et al.*, 2007), and umbilical cord blood (Sarugaser *et al.*, 2005). It has been further proposed that MSCs may be present in any vascularized tissue at perivascular sites (Crisan *et al.*, 2008).

Due to their multipotency and wide dispersion in the body, MSCs are an essential element of most modern tissue engineering and regenerative medicine approaches. There are extensive reviews on the biology of the MSCs, elucidating their nature and unique properties (Bronckaers *et al.*, 2014; Méndez-Ferrer *et al.*, 2010; Phinney and Prockop, 2007; Prockop and Oh, 2012). In the present review, the different microenvironmental cues influencing the MSC phenotype and function either in health and disease – including cellular, chemical, and physical interactions – are discussed. In addition, the most recent *in vitro* culture strategies addressing the complexity of the *in vivo* MSC environment are summarized.

The MSC identity relies on their localization

The characterization of MSCs either *ex vivo* or *in vivo* remains difficult since there is neither a distinct definition nor a robust assay to identify MSCs in a mixed population of cells. However, the International Society of Cellular Therapy has established three main criteria that should be fulfilled by genuine MSCs *in vitro*. These cells should (1) exhibit plastic adherence; (2) express a set of surface markers – *i.e.*, CD73, CD90, CD105, and lack the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR; (3) have the ability to differentiate *in vitro* into mesenchymal lineages, namely adipocyte, chondrocyte, and osteoblast (Dominici *et al.*, 2006). These criteria are applied for the *in vitro* characterization and validation of putative MSCs isolated from different tissue sources; however, differences exist in MSCs isolated from various tissue origins for what concerns the clonogenecity level, proliferation rate, differentiation potential, cell surface marker expression, and, most importantly, their regenerative potential *in vivo* (Bianco *et al.*, 2008; Hass *et al.*, 2011; Raicevic *et al.*, 2011). In fact, only cells isolated from the BM reestablish the marrow stroma upon serial transplantation in mice (Méndez-Ferrer *et al.*, 2010; Sacchetti *et al.*, 2007). $CD146^+$ pericytes, firstly isolated from the BM (Bianco *et al.*, 2008) and later from multiple vascularized human organs (Crisan *et al.*, 2008), exhibit long-term proliferation and trilineage differentiation potential in *in vitro* cultures. However, pericytes, identified by the expression of the transcription factor Tbx18, maintain their identity during aging and diverse pathological *in vivo* settings and do not contribute to tissue regeneration (Guimarães-Camboa *et al.*, 2017), suggesting that the plasticity of these cells observed *in vitro* can be in fact the result of artificial cell manipulations. Regardless of the controversy, these findings provide evidence that MSCs should not be classified as a uniform population of theoretically multipotent cells, but rather a super-family of tissue-specific committed progenitors, possibly even with a distinct developmental origin, as proposed by Robey (2017) and Sacchetti *et al.* (2016). Noteworthy, isolated MSCs seem to reflect an organ-specific potency and a mechanical memory from past physical

environments, which can influence cell fate – *e.g.*, MSCs originated from the BM are more predisposed to give rise to the skeleton, MSCs from adipose tissue to adipocytes and those from other organs to the respective native connective tissues (Sacchetti *et al.*, 2016; Yang *et al.*, 2014).

The nomenclature debate

Due to already identified functional and anatomical diversity of putative MSCs and the lack of robust assays, the nomenclature of these cells has been extensively debated in the field since their discovery. First named as 'colony-forming-unit fibroblasts' by Friedenstein in 1974 (Friedenstein *et al.*, 1974), quickly their name was changed to 'marrow stromal cells' when their *in vivo* potential to generate *de novo* the complete BM structural components was proven (Owen, 1988). Caplan (Caplan, 1991) has proposed to introduce the term 'mesenchymal stem cells' due to their clonability and multilineage potential as well as their loose architecture of randomly organized cells surrounded by large amounts of ECM, which is a characteristic of mesenchymal tissues. However, the International Society for Cellular Therapy (Horwitz *et al.*, 2005) has decided to change the term back to 'mesenchymal stromal cell', due to the inappropriate and misleading use of the term 'stem', especially in the context of cell therapy applications. Also, the term 'mesenchymal' has been lately involved in controversy since it can be easily misinterpreted with the differentiated lineages derived from the mesoderm germinal layer – *i.e.*, skeletal muscle, bone, connective tissue, heart, and urogenital system – which do not have the same MSC differentiation potential. Given that, the scientific community has widely accepted that, for terms of clarity, the tissue of origin of the isolated MSCs should always be stated in their name (Robey, 2017; Sipp *et al.*, 2018). Other suggested terminologies have emerged, not based on the cell's anatomical location nor their differentiation potential but on their function and application for clinics – *e.g.*, Caplan proposal to change the name to 'medicinal signaling cells' because of their *in vivo* secretory function (Caplan, 2017).

The dynamics of the MSC niche

There is abundant evidence suggesting that the MSC ability to maintain themselves or to give rise to differentiated progeny is strictly governed by complex interactions within their close environment – first proposed for HSCs as stem cell niche (Schofield, 1978). The concept of a stem cell niche has later been established as an interactive structural unit, organized to facilitate cell-fate decisions in a proper spatiotemporal manner, comprising the structural and functional components of the ECM, the cellular signaling with stem adjacent cells and other environmental cues, such as gradients of hypoxia (Fig. 1) (Li and Xie, 2005; Scadden, 2006). *In vivo* remodeling of the stem cell niche occurs constantly during development, *e.g.*, during skeletal development and epithelial branching morphogenesis (Rozario and DeSimone, 2010) or in adults during wound healing (Schultz and Wysocki, 2009), where both inflammatory, angiogenic, and morphogenetic factors are present, culminating in an adjustment of the mechanochemistry and cellular composition of the tissue.

Age and diseases

The dynamics of the BM niche vary strongly with age and disorder phenotypes. During aging, a quiescence-to-senescence transition occurs in niche-residing MSCs, which strongly impairs the interactive signaling network of all niche-residing cells and ultimately the complete regenerative activity (Herrmann *et al.*, 2019). This phenomenon is driven either by the age-associated BM fat tissue expansion (Ambrosi *et al.*, 2017) and/or by the inherent modulation of number and type of vessels in bone and BM (Watson and Adams, 2017; Zimmermann *et al.*, 2011). The signature and proliferation capacity of MSCs is also compromised in ECM-related disorders, such as tissue fibrosis – where an excessive deposition of ECM is observed (Usunier *et al.*, 2014) – or osteoarthritis (Maldonado and Nam, 2013) – which is linked to a resilient ECM degradation mediated primarily by MMPs and to an over-activation of osteoclast activity (Maldonado and Nam, 2013). Disruption of such control mechanisms generates aberrant ECM, both structurally and mechanically altered, leading to abnormal behaviors of cells residing in the niche and, ultimately, to enormous repercussions on the overall tissue homeostasis and functionality (Bonnans *et al.*, 2014; Cox and Erler, 2011).

BM niches can also be targeted by metastasizing cancer cells (discussed in more detail below), where a malignant vicious cycle between niche and tumor cells is created, adapting the ECM dynamics to each step of tumor progression (Herrmann *et al.*, 2019; Lu *et al.*, 2012).

Cellular interactions

MSCs gather in niches in distinct location within the BM – namely endosteal (Nakamura *et al.*, 2010), stromal (Herrmann *et al.*, 2019; Pereira *et al.*, 1998), and perivascular (Winkler *et al.*, 2010). The phenotypical similarities of MSCs within their respective niches are currently not completely known. However, the interplay with cells of different maturation and activation states from each niche must surely play a pivotal role in adult tissue dynamics.

MSC communication with ECs and HCs in the BM, which drives the BM niche integrity and bone tissue homeostasis and repair, is discussed in this section. The proper reproduction of these interactions in experimental approaches is mandatory for revealing fundamental properties of the regenerative process and creating rational cell-based therapeutic

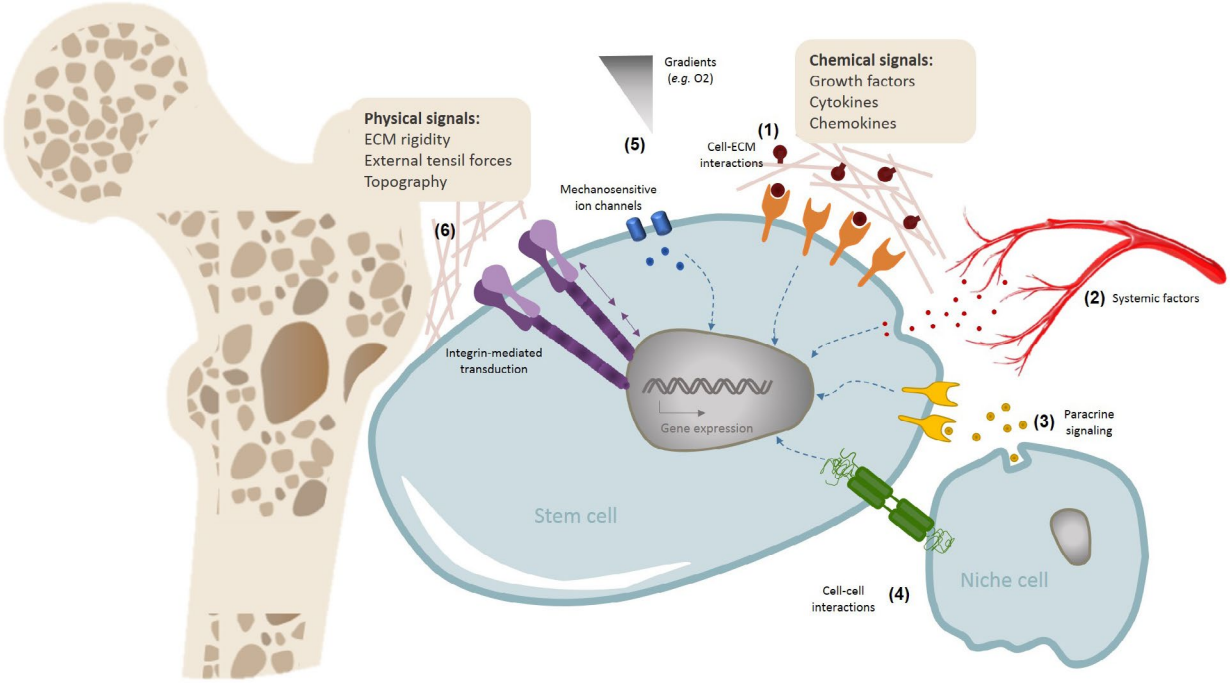


Fig. 1. MSCs' biochemical and physical interactions within the BM niche. A bidirectional synergetic cross-talk is present in the MSC niche, which is ultimately responsible for the modulation of the dynamic state of multicellular tissues – *e.g.*, external signals can change cell DNA transcription, while, in turn, signal transduction from the interior of the cell can modify the extracellular chemistry and mechanics (Bottaro *et al.*, 2002). These interactions may comprise: (1) receptor recognition of insoluble and soluble ECM components – such as cytokines, growth factors, morphogenetic proteins, collagenous proteins, proteoglycans; (2) systemic factors through the vascular system; (3) paracrine and endocrine signals from local or distant sources, *e.g.*, small-molecule agonists, steroid hormones, cytokines, peptides, ions; (4) cell-cell interactions with the neighbor cells, such as niche-supporting cells, immune cells, ECs, or nerve cells; (5) environmental cues, including shear forces, pH effects, oxygen tension; (6) ECM mechanotransduction based on matrix elasticity and geometry.

strategies. The understanding of the relationships between these cells is loaded by difficult definitions of certain cell phenotypes and functionalities, as they might share cell origin, *i.e.*, mesenchyoangioblast and hemangioblats, where progenies possess endothelial and hematopoietic signatures (Angelos *et al.*, 2018; Breitbach *et al.*, 2018; Guibentif *et al.*, 2017). By protecting the primitive stem cells from exhaustion and, on the other side, supporting extensive progenitor activation and differentiation when needed (Ramasamy *et al.*, 2016), the spatial arrangement of the BM hubs is responsible for governing heterogeneity within cell populations (Crisan and Dzierzak, 2016). Cell-to-cell communication includes interaction between membrane and cytoplasm and production of growth factors and cytokines. Extracellular vesicles containing proteins, lipids, miRNA, piRNA (De Luca *et al.*, 2016), or mitochondria transfer (Mahrouf-Yorgov *et al.*, 2017) are important mechanisms of cell communication between MSCs and HCs (De Luca *et al.*, 2016), as well as ECs (Gong *et al.*, 2017; Qin and Zhang, 2017) and cancer cells (Lin *et al.*, 2016), regulating their differentiation, migration, and survival. MSCs provide an instructive environment for angiogenesis, hematopoiesis, and osteopoiesis but also functional assistance to local and disseminated unhealthy or malignant cells (Dhawan *et al.*, 2016; Lee *et al.*, 2012; Roccaro *et al.*, 2014; Xu *et al.*, 2018).

MSCs and ECs

Stem cell behavior, tissue formation and regeneration as well as survival of bone grafts are under the control of the blood vessels, which supply oxygen and nutrients to the cells (Böhrnsen and Schliephake, 2016; Ramasamy *et al.*, 2016). Controlled diffusion of ROS, BM blood-vessel-forming ECs and vascular integrity determine and regulate HSPC as well as MSC localization and functionality (Fehrer *et al.*, 2007; Gomariz *et al.*, 2018; Langen *et al.*, 2014; Xu *et al.*, 2018). Low permeable endosteal vessels with high integrity (H-type) differ from sinusoidal vessels with low integrity (L-type) and provide a poor ROS microenvironment, favoring HSPC maintenance, while fenestrated L-type vessels allow for HSPC respiration and mobilization (Itkin *et al.*, 2016). Sca-1$^+$ and Nestin$^+$ MSCs are likewise associated with H-type vessels and sensitivity to ROS with HSPCs (Itkin *et al.*, 2016). During aging, reduction of H-type vessels

results in decreased levels of SCF and PDGFRβ$^+$ or NG2$^+$ perivascular stromal cells, which is associated with a decrease in the HSPC population in the BM (Kusumbe *et al.*, 2016). Endothelial to mesenchymal transition, an example of cell plasticity, generating pro-inflammatory ECs (Al-Soudi *et al.*, 2017; Chen *et al.*, 2015), is often observed in adult pathologies (Erba *et al.*, 2017; Medici and Olsen, 2012), musculoskeletal injury (Agarwal *et al.*, 2016), and heterotopic bone ossification (Sun *et al.*, 2016), but is also recognized as a developmental process connecting maturation and fate of MSCs and ECs.

MSC-EC cross-talk leads to the modulation of the angiogenic response, with MSCs behaving as pericyte-like cells in the stabilization of newly formed blood vessels (Duttenhoefer *et al.*, 2013; Herrmann *et al.*, 2014; Loibl *et al.*, 2014). However, current data are conflicting. MSCs attenuate activation, proliferation and angiogenesis of ECs, through the production of MMP-1 (Zanotti *et al.*, 2016) and ROS, leading to EC apoptosis, capillary degeneration (Marfy-Smith and Clarkin, 2017; Otsu *et al.*, 2009), and, finally, disease (Cipriani *et al.*, 2007). In contrast, MSC-EC crosstalk stimulates proliferation and osteogenesis in MSCs and angiogenesis in ECs (Bidarra *et al.*, 2011; Böhrnsen and Schliephake, 2016). While BM endothelial progenitors, considered to be CD34$^+$ or CD133$^+$ cells, downregulate osteogenesis in MSCs (Duttenhoefer *et al.*, 2015), EC progenitor-derived growth factors are of critical importance for MSC engraftment, stemness, and repopulation in secondary grafts and osteogenesis (Lin *et al.*, 2014).

MSCs and HCs

Crosstalk of MSCs and HSPCs is one of the most studied issues in physiological homeostasis and adult tissue regeneration (Chan *et al.*, 2015; Raggatt *et al.*, 2014), where progenies of these cells are major participants in immune response, inflammation resolution, and tissue repair. Coherency of the skeletal system and hematopoiesis maintenance (Visnjic *et al.*, 2004) contributes to the BM as stem cell niche environment, as described above. Many mechanisms of HSPC activation by infections or various cytokines have been revealed, while the major pathways involved in steady state and emergency hematopoiesis, generating the full repertoire of immune cells, are still not understood (Boulais and Frenette, 2015; Crisan and Dzierzak, 2016). In case of an altered MSC contribution to osteoblast or adipocyte pool in the BM, biased hematopoiesis occurs through disbalanced myelo-/lymphopoiesis. Distinct stromal cell factors – such as SCF, CXCL12, Flt3 ligand, Wnt3a, angiopoietin-like proteins, thrombopoietin, and fibroblast growth factor 1 – control HSPC quiescence, survival, proliferation, self-renewal, and mobilization or retention in their niche (Crisan and Dzierzak, 2016; Wohrer *et al.*, 2014). Deletion of CXCL12 from perivascular stromal cells or osteoblasts depletes HSPCs and early lymphoid progenitors, respectively (Ding and Morrison, 2013). Leptin receptor$^+$ perivascular stromal cells are the main source of SCF and CXCL12 in the BM (Ding and Morrison, 2013; Zhou *et al.*, 2014) and conditional deletion of SCF leads to the depletion of quiescent HSPCs (Zhou *et al.*, 2014), while deletion of CXCL12 leads to HSPC mobilization (Ding and Morrison, 2013). Since the fast onset of HSPC differentiation in culture complicates the *ex vivo* amplification of HSCPs for their clinical application, development of improved HSPC-amplifying strategies where HSPCs retain their stem cell capacity are still in progress. MSCs support the proliferation of *ex-vivo*-expanded committed hematopoietic progenitors (Hammoud *et al.*, 2012) and their co-culture in 3D macroporous hydrogel scaffolds, mimicking the spongy architecture of trabecular bone, results in higher CD34$^+$ frequency (Raic *et al.*, 2014). However, the impact of MSCs on HSPC stemness during different *in vitro* cultivation and repopulating activity in SCID remains unclear. MSC effects on mature or differentiated HCs are widely studied, particularly in order to reveal the immunobiology of MSCs, where their immunosuppressive capacity is attempted to be harnessed in clinical settings (Galleu *et al.*, 2017; Simonson *et al.*, 2015; Trento *et al.*, 2018). On the contrary, functional adjustment of MSCs in hematologic malignancies, including acute lymphoblastic or myeloid leukemia, multiple myeloma, lymphomas, chronic myeloid leukemia, and myelodysplastic syndromes are described (Civini *et al.*, 2013; de la Guardia *et al.*, 2017), while it is still unknown whether malignant hematopoietic progenitors modify MSCs or if leukemia-triggering changes occur first in MSCs and the healthy marrow niche (Schroeder *et al.*, 2016).

The murine Lin$^-$Sca-1$^+$cKit$^+$ population, referred to as HSPCs, controls MSC differentiation, stimulating osteogenesis through the production of BMP-2 and -6, while, in aged and osteoporotic mice, HSPCs fail to generate BMPs (Jung *et al.*, 2008). Also, *in vitro* co-culturing demonstrates that murine HSPCs impact clonogenicity and favor an osteogenic gene expression profile in MSCs (Jung *et al.*, 2008). On the other hand, differentiated HCs may also affect MSC features, directly through the modulation of their properties as constitutive cells of the mutual niche or indirectly through paracrine activity and feedback effects (Vasandan *et al.*, 2016).

Increased megakaryocyte numbers in the BM are associated with elevated BMP-2, -4, and -6 in mice and are followed by stimulation of MSC osteogenesis (Garimella *et al.*, 2007). While osteoblast maturation and skeletal homeostasis might be supported by megakaryocyte (Alvarez *et al.*, 2018; Kacena *et al.*, 2006), data regarding the effects on osteoclastogenesis suggest inhibitory effects of megakaryocyte on osteoclast development and functions (Beeton *et al.*, 2006; Ciovacco *et al.*, 2010; Kim *et al.*, 2018). *In vitro* studies show that

monocytes can induce osteogenesis in MSCs through cell contact, which leads to the activation of STAT3 signaling followed by upregulation of Runx2, ALP, and Oncostatin M and downregulation of DKK1 in MSCs (Nicolaidou *et al.*, 2012). From these data, it is clear that bidirectional interactions of MSCs and HCs at different developmental stages regulate local tissue functionality and their elucidation particularly contributes to the understanding of normal as well as malignant stem cell biology.

MSCs and Mϕ

Mϕ are phagocytic myeloid cells involved in inflammatory processes through dead cell and foreign material degradation. Mϕ pool contains self-renewable embryonic Mϕ, which are established before the emergence of adult Mϕ which derive from marrow immature myeloid progenitors or circulating monocytes (Gomez Perdiguero *et al.*, 2015; Yona *et al.*, 2014). Mϕ are functionally specialized in lung, liver (Kupffer cells), or bone, where multinucleated OC near the bone surface participate in physiologic or pathologic bone resorption (Kim *et al.*, 2014; Park *et al.*, 2014; Wu *et al.*, 2015). OC dissolve crystalline hydroxyapatite (Wenisch *et al.*, 2003) and degrade the collagen-rich organic bone matrix (Henriksen *et al.*, 2006). Due to their plasticity, Mϕ may have an anti-inflammatory (M2) or pro-inflammatory (M1) profile as well as many intermediate activation states. MSCs can facilitate monocyte to macrophage transition, but attenuate (Vasandan *et al.*, 2016) or favor their pro-inflammatory and osteoclastic activities (Gamblin *et al.*, 2014). MSCs induce a M2 phenotype in BM-Mϕ, increasing their expression of arginase-1, IL-10, IL-4, and CD206 and decreasing the expression of IL-6, MCP-1, and iNOS (Cho *et al.*, 2014; Takizawa *et al.*, 2017). Through the production of major osteoclastogenic [*e.g.*, RANKL (Biswas *et al.*, 2018) and M-CSF (Cappellen *et al.*, 2002)] and anti-osteoclastogenic factors [*e.g.*, OPG (Oshita *et al.*, 2011)], MSCs control bone resorption and remodeling (Sharaf-Eldin *et al.*, 2016). By producing OPG and/or FasL protein, MSCs exert a suppressive effect on osteoclastogenesis (Shao *et al.*, 2015; Varin *et al.*, 2013) and are proposed to be suitable cell candidates for controlling inflammation-associated bone destruction, such as rheumatoid arthritis (Oshita *et al.*, 2011). However, the absence of osteoclastogenesis may be associated with reduced osteoblastic commitment of MSCs, endosteal osteoblast loss, and impaired homing (Mansour *et al.*, 2012) or clonogenicity of HSPCs (Lymperi *et al.*, 2011).

MSCs and DCCs

MSC and their progeny may facilitate neoplastic growth (Doron *et al.*, 2018). Communication between MSCs and bone-metastatic DCCs is unclear, but it is possible that MSCs control DCC settlement in the BM as competition for niche space may exist (Dhawan *et al.*, 2016; Gordon *et al.*, 2014; Rossnagl *et al.*, 2018; Shiozawa *et al.*, 2015). Prostate cancer cells induce an osteoblastic-type lesion, while breast cancer and myeloma cells form osteolytic-type of bone lesions (Hashimoto *et al.*, 2018). Human BM biopsies show higher $CD271^+$ MSCs and $CD31^+$ frequencies in the absence of DCCs in the BM of prostate cancer patients in comparison with breast cancer (Rossnagl *et al.*, 2018). SDF-1 chemokine gradient is one of the most described explanations for tumor-to-BM homing and MSC-derived osteoblasts produce SDF-1, creating a chemo-attractant gradient for CXCR4-expressing cancer cells (Amend *et al.*, 2016; Devignes *et al.*, 2018). *In vitro* and *in vivo* migration assays have revealed that MSCs have tropism toward multiple myeloma cells, where MSCs promote multiple myeloma progression (Xu *et al.*, 2012). Although tumor-homing ability of MSCs suggests their utilization in anti-tumor strategies, it is still unknown how MSCs in the metastatic niche of the BM contribute to graft *versus* tumor reaction, one of the currently most investigated anti-tumor approaches. Exosomal transfer of miRNAs from MSCs to breast DCCs (Ono *et al.*, 2014) induces MSCs dormancy in the BM niche. Moreover, multiple-myeloma-cell-derived exosome miRNA promotes a phenotype switch of MSCs towards a cancer-associated fibroblast state (Cheng *et al.*, 2017). Concerning tumor persistence, there is a complex bidirectional crosstalk of MSCs and cancer cells involving various mechanisms which are still unclear but important for the understanding of peculiarities of normal and stem cell niche in tumors.

ECM dynamics

Although the crucial importance of cellular interactions with surrounding elements is recognized, a major challenge is still to understand how the chemical composition and mechanical properties of the ECM can functionally influence tissue homeostasis under physiological and pathological conditions.

Particularly, a better understanding on how disruption of ECM dynamics, *i.e.*, both biochemical signaling and physical cues, contributes to progression of complex diseases will be important towards the development of new therapeutic targets in regenerative medicine.

Biochemical interactions

Microenvironmental cues, such as cellular interactions, the paracrine environment and ECM-associated proteins, critically influence MSC behavior *via* biochemical pathways. This is suggested by plenty of studies demonstrating that MSCs acquire tissue-specific characteristics when co-cultured with mature cells types (Csaki *et al.*, 2009; Deng *et al.*, 2008; Plotnikov *et al.*, 2008; Schneider *et al.*, 2011; Strassburg *et al.*, 2010) or in complex biological substrates *in vitro* (Bosnakovski *et al.*, 2006; Datta *et al.*, 2005; Hoch *et al.*, 2016; Suzuki *et al.*, 2010).

The oxygen tension applied to the *in vitro* culture also significantly influences both MSC proliferation and differention potential in a lineage-specific matter.

Although the published literature is quite diverse and occasionally contraditory, some studies have reported that reduced oxygen tension attenuates the MSC differentiation capacity into the osteogenic lineage (D'Ippolito *et al.*, 2006) whilst promoting adipogenic (Fink *et al.*, 2004) and chondrogenic differentiation (Kanichai *et al.*, 2008; Robins *et al.*, 2005). This correlates with the *in vivo* situation where chondrocytes and adipoctes reside in more hypoxic environments. Low oxygen tension also regulates MSC paracrine activity. An induced hypoxic *in vitro* culture stimulates an upregulation of angiogenic genes, such as VEGF and IL-6 (Hu *et al.*, 2008). Furthermore, in hypoxia, large amounts of SDF-1, along with its receptor CXCR4, are expressed and secreted by MSCs, stimulating their mobilization and, thus, promoting MSC-homing toward damaged tissue (Liu *et al.*, 2012; Rosenkranz *et al.*, 2010). Likewise, tissue-specific ECM proteins have an important role as a supportive scaffold, exposing binding sites for growth factors, cell receptors ligands, proteases, *etc.*. In the context of bone, a type I collagen-rich ECM is required to regulate local availability of BMPs in a spatio-temporal manner and, therefore, controls osteoblast lineage progression. Specifically, integrin binding of ECM-released BMPs to osteoblastic precursor cells initiates a MAPK-dependent signaling cascade that leads to the phosphorylation and activation of Runx2 (Yang *et al.*, 2003), the master transcriptional regulator of the osteoblastic differentiation. Noteworthy, the ECM-MSC dynamic is rather a bidirectional system, where the lineage commitment process of MSCs also induces a remodeling of the microenvironment's chemical and mechanical characteristics. Manduca *et al.* (2009) have investigated the role of MMPs in osteogenic differentiation, demonstrating that preosteoblastic cells sense the microenvironment through binding of β1 integrins to fibronectin and collagen type I in the ECM, resulting in the formation of a complex with MMP-1. This complex initiates the expression of proMMP-2, required for type-I collagen and ALP proteolysis, which is involved in mineral deposition during osteogenic differentiation.

These studies emphasize the crucial role of cell-matrix interactions as highly instructive elements for stem cell biologic functions including growth, differentiation, apoptosis, and, ultimately, tissue remodeling.

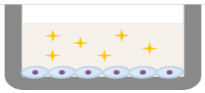
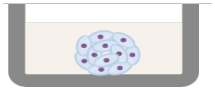
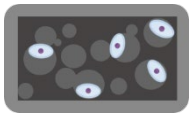
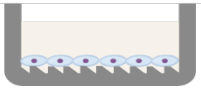
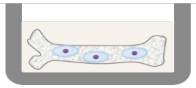
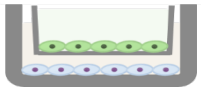
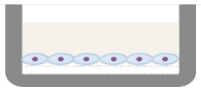
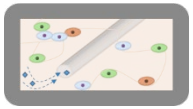
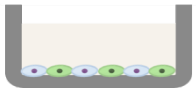
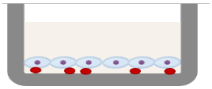
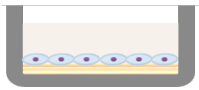
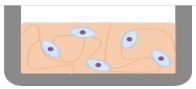
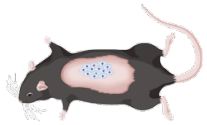
The importance of the environmental mechanical properties

In the context of bone, collagen fibrils comprise binding sites for mineral deposition while still keeping the structural flexibility for a tissue that would otherwise be overly rigid. Alongside, there is evidence showing that ECM physical cues not only provide support and anchorage for the cells but strongly elicit changes in gene expression and, ultimately, affect cell fate and tissue development (Chen, 2008; Engler *et al.*, 2006; Lutolf and Hubbell, 2005). Biologically, osteocytes trapped within the matrix are the principal sensors of mechanical forces applied to the bone, with a crucial role in local mineral deposition regulation (Klein-Nulend *et al.*, 2013). Likewise, for tissue engineering approaches, MSCs are sensitive to their substrate stiffness and able to detect its nano- and micro-topography or porosity (reviewed by Sun *et al.*, 2012a). The transduction of these mechanical stimuli into cellular processes, otherwise known as a mechanotransduction, is accomplished through direct or indirect processes (described in detail by Sun *et al.*, 2012a; Yim and Sheetz, 2012). Briefly, direct mechanotransduction occurs when forces applied to integrins, which are linked to the nucleus through focal adhesion interactions with the cytoskeletal protein filaments (*e.g.*, actin and vimentin), lead to changes in gene expression through chromatin remodeling. Indirect mechanotransduction occurs either through mechanosensitive ion channels, mainly from TRP family (Ranade *et al.*, 2015), or through integrin-mediated signal pathways (Jalali *et al.*, 2001; Schwartz, 2010), which internally couple with other growth factor pathways to regulate stem cell fate. Even with all the recent discoveries on how MSC behavior can be tailored by artificial mechanic features (refer to the next section for MSC environment modelling applications), there is still a poor understanding of the underlying mechanisms of biophysically-induced stem cell differentiation and how these dynamic complex feedbacks can be manipulated towards a therapeutic application.

Modelling MSC niche complexity

The extensive presence of MSC-like progenitor cells throughout the vascularized organs raises a wide range of possible therapeutic strategies intending to accelerate the tissue regenerative capacity following injury. The up-to-date therapeutic applications, either based on the stem-properties or on the paracrine and immunomodulatory competence of these cells, are highlighted in recent reviews (Matsumoto *et al.*, 2016; Park *et al.*, 2011; Peired *et al.*, 2016; Yousefi *et al.*, 2016). Nevertheless, in most cases, MSC-based clinical trials occur in an early phase (phase I or II) according to FDA guidelines, where the long-term safety and treatment efficacy is not yet conclusively established (Squillaro *et al.*, 2016). Regardless of the extensive effort and advances made in MSC identity and experimental handling, there are still substantial ambiguities about their integrative functions *in vivo* and long-term safety, which continues to pose a major limitation on their envisioned therapeutic use. An extensive scientific knowledge of each MSC subpopulation and their interaction with the environment is still necessary to successfully translate them to the clinic. The more recent and relevant illustrative examples of novel bioengineering approaches to mimic biological, chemical, and mechanical microenvironmental signals present in

Table 1. Research models to study MSC behavior and their interactions with the surrounding biochemical and physical cues.

Model	Features and relevance	Model	Features and relevance	Model	Features and relevance
Soluble factors 	Low cost Simple output analysis	Spheroids culture 	Low cost Simple output analysis Cell-cell adhesion Three-dimensionality Diffusion gradients	3D scaffolds 	Cell-ECM interactions Tunable chemistry Tunable mechanical properties Three-dimensionality Diffusion gradients
Conditioned media 	Low cost Simple output analysis Biochemical complexity	Topography-patterning 	Simple output analysis Spatial control Cell-ECM interactions Pseudo-3D arrangement	Decelluarized tissue 	Native ECM arrangement Cell-ECM interactions Three-dimensionality Diffusion gradients
Indirect co-culture 	Low cost Simple output analysis Biochemical complexity	Substrate stiffness 	Simple output analysis Cell-ECM interactions Tunable chemistry Tunable mechanical properties	Dynamic bioreactors 	Continuous perfusion Tunable chemistry Tunable mechanical properties Biochemical complexity Cell-cell and cell-ECM interactions Three-dimensionality
Direct co-culture 	Low cost Biochemical complexity Cell-cell adhesion	Surface protein-immobilization 	Spatial control Simple output analysis Cell-ECM interactions Pseudo-3D arrangement Tunable chemistry	Microfluidic chips 	Spatial-temporal control Continuous perfusion Accessible live tracking Highly customizable
Protein coating 	Low cost Simple output analysis Tunable chemistry Cell-ECM interactions	3D gel matrices 	Tunable chemistry Tunable mechanical properties Cell-ECM interactions Diffusion gradients Three-dimensionality	*In vivo* models 	Native environment

the MSC niche are summarized in this section (see Table 1). Furthermore, the current limitations are highlighted and potential opportunities to fill the bridge between fundamental science and clinical application discussed.

Monolayer culture: an *in vitro* mechanistic tool

Culture of an adherent cell monolayer on flat and rigid 2D substrates is a well-established straightforward technique by which cells of interest can be maintained outside the body and observed over time with a good viability of cells in culture.

Paracrine factors

To date, the simplest models for examining biological behavior of MSCs in response to microenvironmental factors are conduced by direct exposition to soluble factors (Celil and Campbell, 2005; Indrawattana *et al.*, 2004; Kratchmarova *et al.*, 2005; Luo *et al.*, 2010) and conditioned media from either other cell type cultures (Chowdhury *et al.*, 2015; Menon *et al.*, 2007; Siciliano *et al.*, 2015) or from tissue extracts (Chen *et al.*, 2002). In the strictest sense, the conditioned medium refers to the cell secretome, which encompasses proteins shed from the cell surface and intracellular proteins released through non-classical secretion pathway or exosomes, including numerous enzymes, growth factors, cytokines and hormones, or other soluble mediators (Veronesi *et al.*, 2017). Therefore, conditioned medium approaches offer the possibility of studying the paracrine interactions of complex combinations of factors, in a specific physiological or pathological environment.

Co-cultures

Co-culture techniques find countless applications in biology for studying interactions between cell populations. Overall, the co-culture systems can be set-up either by direct co-culture of both cell types or using compartmented systems, such as trans-wells or microfluidic chambers, to study solely the paracrine crosstalk and not the cell-cell signaling pathways that may occur. Many studies have explored this approach by co-culturing MSCs with mature cells in direct (Aguirre *et al.*, 2010; Ball *et al.*, 2004; Csaki *et al.*, 2009; Deng *et al.*, 2008; Strassburg *et al.*, 2010; Takigawa *et al.*, 2017; Wang *et al.*, 2007) and indirect contact (Li *et al.*, 2011; Luo *et al.*, 2009; Wei *et al.*, 2010). When comparing cultures of MSCs and osteoblasts alone with MSC/osteoblast co-cultures, for example, an increase in calcification over time is observed in co-culture. These results suggest the idea of a positive augmentation of the MSC differentiation process by osteoblast-secreted factors (Glueck *et al.*, 2015).

Protein-coating

The native ECM is essentially a 3D network of fibrillar and non-fibrillar proteins, such as collagens, fibronectin, elastin, laminin, vitronectin, glycosaminoglycans, such as hyaluronan or heparin, and proteoglycans (Bason *et al.*, 2018). Many of these ECM components are commercially available, either as complex mixtures (*e.g.*, matrigel) or as purified proteins and are extensively applied to guide MSC differentiation *in vitro* (Curran *et al.*, 2006; Phillips *et al.*, 2010; Qian and Saltzman, 2004; Rojo *et al.*, 2016). To achieve a high chemical complexity, MSCs are also cultured on decellularized extracellular 2D coatings, *i.e.*, matrix produced by cells *in vitro*, resulting in a composition of cell-secreted components without the potentially antigenic cellular structures or contaminating DNA after collection and processing (Hoshiba *et al.*, 2010). The enhancement of proliferation and stemness maintenance of naïve MSCs is verified in cells cultured on a basal-cultured MSC-derived decellularized ECM; while, when cultured on decellularized ECM deposited by MSCs under osteogenic differentiation, an osteogenic

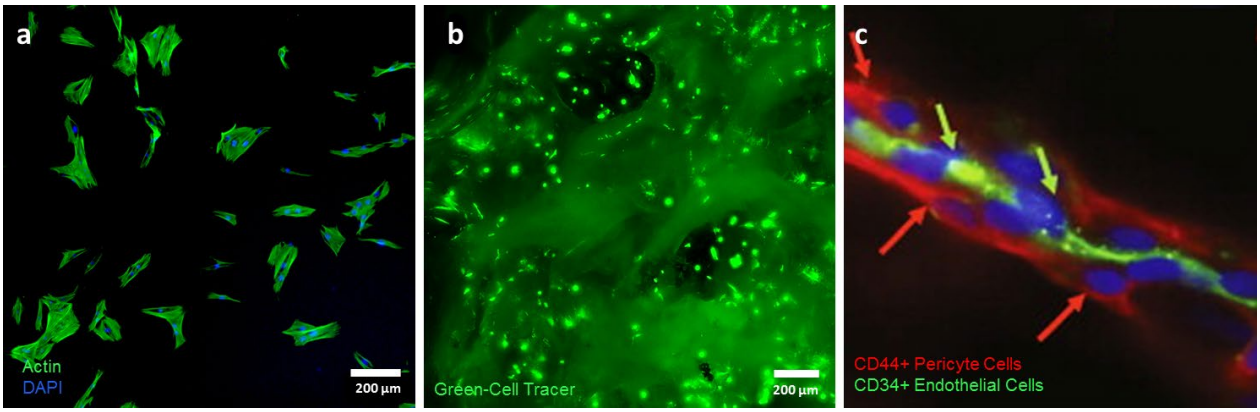


Fig. 2. Comparison of morphology and spatial organization of MSC *in vitro* cultures with the MSC perivascular niche *in vivo*. When cultured on a 2D monolayer culture of standard tissue culture polystyrene (**a**) MSCs acquire a stretched and flattened morphology. On the other hand, when a 3D decellularized bone scaffold is used to physically support MSC culture, (**b**) the cell-matrix interactions induce a different cellular distribution and arrangement which closely mimics the niche organization observed in (**c**) an *in vivo* perivascular niche [600× magnification (with permission of Crisan *et al.*, 2008)].

lineage differentiation is observed in seeded MSCs cultured in the absence of dexamethasone (Rao *et al.*, 2014).

Limitations

Although very convenient and effective for mechanistic purposes, the results from 2D cell culture models may not be representative of essential and complex features of native microenvironments. A main limitation of *in vitro* studies in 2D monolayer cultures is the lack of spatial and temporal control of multiple signals, similar to what happens in a native 3D context. Furthermore, a significant limitation of 2D cell cultures is that diffusion and transport conditions do not reflect the *in vivo* situation – cells grown in monolayers are exposed to a uniform environment with constant supply of oxygen, nutrients or metabolic products, which can lead to significant deviations in cellular function and response (Baker *et al.*, 2012).

Paradigm shift: mimicking MSC niche environment through third dimension

Due to the lack of structural architecture, 2D cell culture models are substantially diverging from the *in vivo* state (Fig. 2). Accordingly, many research groups apply a 3D culture environment that aims to better resemble the native tissue organization.

Spheroids culture

Since the observation that chondrocytes lose their phenotype quickly in monolayer culture (Caron *et al.*, 2012; Thompson *et al.*, 2017), micromasses and pellet *in vitro* cultures have been established, allowing cells to aggregate in high densities and create their own 3D cartilaginous matrix (Cottrill *et al.*, 1987; Johnstone *et al.*, 1998). In addition, using a simple 3D scaffold-free spheroid culture system, Wang *et al.* (2009) have demonstrated that MSC multipotency can be significantly increased for both osteogenic and adipogenic lineage when compared with the conventional 2D monolayer culture. Moreover, using a perivascular-like *in vitro* 3D spheroid co-culture system, Saleh *et al.* (2011) have shown that endothelial cells regulate MSC activity by maintaining quiescence and facilitating niche exit by osteogenic differentiation through activation of endogenous Wnt and BMP signaling. Other studies have reported enhanced anti-inflammatory properties after a short period of spheroid culture by increased expression of genes, such as CXCR4, which promotes adhesion, or IL-24, with tumor-suppressing properties (Bartosh *et al.*, 2010; Potapova *et al.*, 2008; Ylöstalo *et al.*, 2012).

Micropatterning

A different approach being adopted is the creation of a pseudo-3D environment for MSCs using soft lithography techniques to imprint a topography-patterning in the culture substrates. This is rather a bottom-up organization approach, with cells being instructed at the molecular level. Several studies have shown increased mineralization during osteogenesis induction on micro- and/or nano-patterning growth surfaces (McNamara *et al.*, 2010; Oh *et al.*, 2009; Yim *et al.*, 2007; Zhao *et al.*, 2012). Differences in groove size as well as their geometric arrangement dictate the matrix alignment and cell morphology, resulting in a strong effect on cell proliferation and gene expression and eventually induction of bone nodules formation. Dalby *et al.* (2007) have shown that MSC osteogenic differentiation can be initiated with a nanopitted topographical pattern in a square geometry with a moderate level of disorder embossed into PMMA surfaces; while, on the other hand, an ordered square nanopit-pattern is proposed to mediate retention of MSC stemness (McMurray, 2011). Also, Stanciuc *et al.* (2018) have shown an accelerated maturation of human osteoblast maturation on micro-rough surfaces of zirconia-toughened alumina with nano-porosity obtained by selective chemical etching.

Substrate stiffness

Engler *et al.* (2006) have demonstrated for the first time that the substrate stiffness itself can direct MSC lineage fate. Subsequently, Pek *et al.* (2010) have optimized a 3D hydrogel system to guide MSC differentiation either to neural, myogenic, or osteogenic phenotypes depending on whether they are cultured in gels of elastic moduli in the lower (7 Pa), intermediate (25 Pa), or higher range (75 Pa), respectively. Interestingly, the matrix that optimally drives MSC differentiation to specific lineages corresponds to the stiffness of the relevant target tissue. Accordingly, tuning the elasticity of the culture material is a common strategy, adopted to control MSC fate (Du *et al.*, 2016; Huebsch *et al.*, 2015; Kuboki and Kidoaki, 2016; Seib *et al.*, 2009; Wingate *et al.*, 2012).

The influence of the chemical and physical biomaterial properties

Progress in the development of biomimetic materials have lately been chasing the complexity of the mechanical and physical-chemistry arrangement of the biomaterial itself, such as a scaffold, commonly used for tissue engineering applications. Such tissue-engineered constructs not only represent potential therapeutic options for the treatment of bone defects but may also serve as a model system of the MSC naïve environment in the bone and facilitate our understanding of the interactions within the niche.

3D gel matrices/scaffolds

For tissue engineering applications, the culture substrate should not only provide physical support but also present a functional surface chemistry compliant with the biological purpose. Noteworthy, the chemical composition as well as the fabrication process itself determine the final geometry, porosity, and roughness of the bulk material (Akbarzadeh and Yousefi, 2014; Loh and Choong, 2013; Pina *et al.*, 2016). The macroporosity (pores > 50 μm) of a scaffold

contributes to osteogenesis by facilitating cell and ion transport (Bignon *et al.*, 2003), while microporosity (pores < 20 μm) augments bone growth by providing attachment points for osteoblasts (Bignon *et al.*, 2003) and increasing growth factors retention upon which bone formation depends in ectopic sites (Hing *et al.*, 2005; Woodard *et al.*, 2007).

Commonly used natural polymers for bone tissue engineering are collagen, fibrin, alginate, silk, hyaluronic acid, and chitosan. They provide high biological recognition that may positively support cell adhesion and function, yet often lack the mechanical strength required by bone (reviewed by O'brien, 2011). Synthetic biodegradable polymers, such as PLA, PGA, and PCL are widely used due to their reproducible large-scale production, with controlled properties of strength, degradation rate, and microstructure (reviewed by O'brien, 2011). Calcium-phosphate-based materials, such as hydroxyapatite and beta-tricalcium phosphate, are widely used ceramics that often shape the inorganic-phase of bone graft substitutes. Their non-toxic, non-inflammatory, non-immunogenic properties and their biological affinity (*i.e.*, ability to form direct chemical bonds with the surrounding environment) direct tissue integration when implanted in bone defects (Ambard and Mueninghoff, 2006; Venkatesan and Kim, 2014). Besides, extensive studies of organic modifications of hydroxyapatite-based composites show the enhancement of the osteoconductive properties of the material (review by Swetha *et al.*, 2010). For example, Zhao *et al.* (2006) have investigated two types of biomimetic composite materials, chitosan-gelatin and hydroxyapatite/chitosan-gelatin. They have shown that hydroxyapatite enhances protein and calcium ion adsorption – which in turn improves i) initial cell-adhesion and long-term growth, ii) maintains MSC stemness and iii) upon induction enhances osteogenic differentiation (Zhao *et al.*, 2006).

Engineered substrates

The tissue engineering field provides valuable knowledge for modeling the MSC niche *in vitro*. Moreover, advances in protein engineering and synthetic chemistry of peptide-conjugated polymers allow the fabrication of the so-called artificial ECM constructs, which can respond to cell-secreted signals and enable proteolytic matrix remodeling (Lutolf and Hubbell, 2005). These synthetic networks are typically achieved by crosslinking of specific bioactive components in a structural mesh – *e.g.*, (1) cell-adhesive ligands, such as integrin-binding peptides of the prototypical RGD family, resulting in an increased cell growth efficiency (Chang *et al.*, 2009; Maia *et al.*, 2014); (2) domains with susceptibility to degradation by cell-secreted proteases to facilitate bidirectional cell-matrix interactions (Lutolf *et al.*, 2003); (3) binding sites for growth factor matching the pretended application (Madl *et al.*, 2014; Park *et al.*, 2009). Thevenot *et al.* (2010) have developed a PLGA scaffold with incorporated SDF-1 to enhance the recruitment of endogenous MSCs to the injury site. Likewise, Phillippi *et al.* (2008) have created spatially defined patterns of immobilized BMP-2 using inkjet bioprinting technology to modulate the cell organization and, consequently, their differentiation toward the osteogenic lineage.

As another powerful element, synthetic biology has recently been applied to tissue engineering modeling. Encapsulated modified cells with sophisticated tunable modular genetic switches that couple repressor proteins with an RNAi can be controlled by an external factor or specific microenvironment changes (Saxena *et al.*, 2016; Weber and Fussenegger, 2012).

3D biofabrication

Combined knowledge of material science and 3D fabrication principles results in the advent of additive manufacturing techniques as a complex innovative approach to generate complex 3D environments with a designed and controlled arrangement of tissue morphology features and spatial distribution of cells (Bose *et al.*, 2013; Malda *et al.*, 2013). 3D biofabrication is becoming popular due to the ability to directly print porous scaffolds with designed shape, controlled chemistry, and interconnected porosity. Apart from inorganic scaffold manufacturing, additive manufacturing approaches are also used to explore the possibilities in fabricating scaffolds with live cells and tissues. Levato *et al.* (2014) have shown a combined method where MSC-laden polylactic acid microcarriers are printed by encapsulation in gelatin methacrylamide-gellan. This combined bioprinting approach allows for the improvement of the elastic modulus of the hydrogel construct, facilitating cell adhesion and survival, while supporting osteogenic differentiation and bone matrix deposition (Levato *et al.*, 2014). Alternatively, Gurkan *et al.* (2014) have used another interesting approach where MSCs are encapsulated in a gelatin-based metacrylated hydrogel with addiction of BMP-2 and TGF-β1 mimicking the fibrocartilage phase of the bone. Incorporating bioprinting technology with a nanoliter gel droplet system, this model can induce the upregulation of osteogenesis and chondrogenesis, thus making this approach a functional tissue model system (Gurkan *et al.*, 2014).

Decellularized tissue

Although a variety of different materials and composites are available, to achieve a physiologically relevant protein and structural complexity, whole organ or tissue decellularization techniques are investigated. These natural scaffolds preserve the complex biochemical and biomechanical ultrastructure of the native tissue and can be recellularized to generate a new functional tissue or organ (Crapo *et al.*, 2011; Lund *et al.*, 2017). Particularly, decellularized bone is used as a scaffold for bone

tissue engineering or bone *in vitro* modelling due to its 3D porous structure and its natural biochemical component arrangement, providing osteoinductive properties that are not fully resembled by synthetic polymers or hydrogels (Nyberg *et al.*, 2017). However, the current challenge of working with decellularized matrices and their translation to clinics is to balance the decellularization methods in order to maintain the specific epitopes that will have a positive impact on cell functions but eliminate any component that could cause an immunogenic response (Gilpin and Yang, 2017; Keane *et al.*, 2016).

The dynamic dimension

Biomechanical stimuli caused by physical deformation and fluid shear stress generated by interstitial fluid movement through bone lacunae are recognized as a significant part of *in vivo* bone remodeling (Carter, 1984; Duncan and Turner, 1995). Therefore, to better reassemble the *in vivo* counterpart, *in vitro* models for skeletal progenitors may likewise be integrated in an intrinsic dynamic environment.

Dynamic bioreactors

Dynamic culture of MSCs has expanded greatly in the last 15 years and dynamic optimized bioreactors are now widely used to provide the technological means to achieve both improved nutrient transportation and mechanical stimulation. A variety of dynamic 3D bioreactor concepts mimicking the native microenvironment of bone tissues have been developed – *e.g.*, perfusion chambers (Dahlin *et al.*, 2012; Hosseinkhani *et al.*, 2006; Kleinhans *et al.*, 2015; Porter *et al.*, 2005; Yeatts and Fisher, 2010), stirred tanks (Eibes *et al.*, 2010; King and Miller, 2007), rotating wall vessels (Nishi *et al.*, 2013; Song *et al.*, 2008), mechanical loading chambers (Altman *et al.*, 2002; Baker *et al.*, 2011; Pelaez *et al.*, 2012; Sittichockechaiwut *et al.*, 2009), and, more recently, nanovibrational reactors (Tsimbouri *et al.*, 2017). MSCs cultured under those dynamic cultures are subjected to mechanical shear created by fluid flow, which promotes osteogenesis *via* the ERK1/2 pathway through upregulation of Runx2 (Yeatts *et al.*, 2013) and, therefore, provides the right microenvironmental setup to augment bone formation (David *et al.*, 2007; McCoy and O'Brien, 2010; Stiehler *et al.*, 2009).

Microfluidic chips

The advances in microfluidic technology brought great progresses in the field of dynamic *in vitro* models, mainly regarding the spatiotemporal control of gradients and the introduction of individual or combination of factors with low volumes and low cell suspension density requirements (Sart *et al.*, 2016; Sun *et al.*, 2012b; Tatárová *et al.*, 2016). Recently, Marturano-Kruik *et al.* (2018) have developed a perivascular model containing ECs and MSCs seeded on a bone matrix, forming a bone perivascular niche-on-a-chip, which allows following slow-cycling metastatic cancer cells in a BM niche.

Smart materials

Meanwhile, advances in the material science field have been made with the development of the so-called 'smart' materials – *i.e.*, biomaterials specifically designed to allow dynamic changes in their structure in response to an external stimulus (Kaliva *et al.*, 2017). These materials can be metals or polymers sensitive either to temperature (Dessì *et al.*, 2013; Roy *et al.*, 2013), pH (Wang *et al.*, 2004), magnetic (Ribeiro *et al.*, 2016) or electrical fields (Balint *et al.*, 2014), light (Muraoka *et al.*, 2009; Zhao, 2012), or lytic-enzymes (Hu *et al.*, 2012; Todd *et al.*, 2007). The concept of a dynamic 4th dimension is also being explored in 3D printing approaches for tissue engineering (reviewed by Gladman *et al.* , 2016; Khoo *et al.*, 2015). The development of new tailored inks capable of adapting their shape or functionalities to external stimuli will surely be a pivotal milestone in achieving reliable and close to *in vivo* MSC niche models.

***In vivo* models**

Animal models are a vital part of MSC biology research and MSC-based therapeutic approaches, enabling investigations at the systemic level in a physiological environment. Nevertheless, the prediction of effectiveness of a therapeutic approach in preclinical models can be highly inaccurate, resulting in hurdles upon translation of results in clinics. This frequent discrepancy happens mainly due to (1) intrinsic divergence of molecular mechanisms between species and the non-human stromal component of the ECM, or (2) anatomic discrepancies particularly in orthopedic applications. These facts, along with high costs of maintenance, need for qualified expertise, limited output analysis, and ethical concerns about animal experimentation are motivating governments and regulatory organizations to limit their use and support the implementation of alternative methods following the 3R's principles – firstly established by Russell in 1959 (Russell *et al.*, 1959). Yet, improvements in modeling the complex bone environment (as discussed in the present review) present promising options to provide tissue grafts for regenerative medicine in large bone fractures and, also, to screen with precision therapeutic agents that may facilitate bone repair.

Final remarks

In vitro models should not be confined to single stationary conditions; *i.e.*, an individual architecture or a particular chemical functionalization with a specific biological function. Instead, it is desirable that the emerging constructs should comprise complex combinatorial signals with tunable cues, to support stemness maintenance or direct stem cell differentiation with spatiotemporal control. Nevertheless, successes in various aspects of the tissue engineering assure a bright future for the development of models that mimic the relevant properties of naïve tissues. The progressive increase in complexity of *in vitro* models that is been

witnessed is surely paving the way towards a better understanding of the detailed biological events involved in tissue homeostasis and related disorders *in vivo*.

Further perspectives

The localization, identification, and regenerative potential of MSCs is under controversial discussion in the stem cell community. This is mainly attributed to the lack of distinct surface markers for the identification and prospective isolation of naïve MSC/tissue-specific progenitor cells *in vivo* in mouse and human, resulting in inconsistences of the studied cell population, and the restriction of many studies to the assessment of the cell regenerative potential *in vitro*. The *in vitro* MSC characterization methods are highly artificial and do not proof the function of MSC/tissue-specific progenitor cells *in vivo*. Indeed, there is an ambiguous distinction between the physiological function of isolated MSCs in culture and their presumed *in vivo* counterpart – *i.e.*, MSCs isolated from the BM give rise to all the mesenchymal cell lineages (Pittenger *et al.*, 1999) and even transdifferentiate into cells from the central nervous system (Wislet-Gendebien *et al.*, 2005), the skeletal muscle system (Ferrari *et al.*, 1998), the hepatic system (Lee *et al.*, 2004), and the cardiac system (Toma *et al.*, 2002) when exogenously stimulated; whereas naïve non-stimulated BM-MSCs do not share the same phenotypic plasticity (Bara *et al.*, 2014). In fact, robust *in vivo* assays of progenitor cells from other tissues, all sharing the *in vitro* characteristics attributed to MSCs (Dominici *et al.*, 2006), suggest that distinct tissue-specific stem/progenitor cells with distinct regenerative capacity exist throughout the body (Robey, 2017; Sipp *et al.*, 2018), specifically settled in a specific environment which control either the maintenance of their stemness or the orchestration of tissue modulation activities. This, along with the increasing amount of data showing a microenvironmental-dependent behavior of MSCs, as reviewed in the present article, highlights the importance of considering and implementing microenvironmental cues upon assessment of the MSC regenerative potential.

Although substantial advances have already been made in the field, the recapitulation of the complex biological recognition and signaling functions, *e.g.*, between cells and ECM, is still crucial and controlling the dynamics and spatial organization of multiple signals remains a current challenge. Substantial testing and optimization is still required to ensure that the 3D constructs realistically mimic the native tissue counterparts. Accordingly, despite the unquestionable value of MSCs for clinical applications, comprehensive studies of fundamental mechanisms triggered by microenvironmental cues are critical before moving to regenerative medicine cell therapy applications.

The future holds great potential for 3D/4D models for studying tissue dynamics in health and disease as well as for tissue engineering applications. Progress in engineering, technology, biomaterials, and imaging will surely be at the forefront of the MSC niche model revolution.

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Discussion with Reviewer

Reviewer: Given the wide distribution of MSCs and their diversity, there are likely to be a multitude of niches. Will each need to be analyzed separately or will there be common features?

Authors: Stem cells, including MSCs, require particular microenvironments to maintain themselves *in vivo*, otherwise known as niches (Schofield, 1978), where their stemness is protected and the stimulus for differentiation is triggered by cellular signaling with either tissue-adjacent cells, paracrine and endocrine signals from local or systemic sources, or external mechanical forces. Given the theoretically wide distribution of MSCs through several connective tissues in the organism (Crisan *et al.*, 2008), it is reasonable to assume that the inherent chemistry, mechanical structure, and function of different tissues may influence the single MSC entity. Since native stem cell niches at distinct anatomical locations and developmental stages have remained a theoretical construct and criteria for the *in vitro* characterization of MSCs weakly delineate MSCs from other cell types, it remains challenging to compare naïve cells from different niches. However, increasing evidences indicate different transcriptome and differentiation capacity of MSCs-like cells obtained from diverse tissues (Sacchetti *et al.*, 2016), while tissue-specific elements involved in MSC lineage decision still have to be revealed. Based on current knowledge, it

can be assumed that both tissue-specific as well as common mechanisms control MSC fate in distinct niches. However, future research will be required to unravel these mechanisms, which will be also critical to resemble specific niche features in *in vitro* models. Recent technical advances in niche *in vitro* modelling will certainly play a pivotal role in understanding and elucidation of MSC physiology and regulation within different locations.

Reviewer: Are MSCs an *in vitro* artefact?
Authors: Most of the knowledge on MSC biology derives from *in vitro* studies, due to the current lack of sophisticated methods allowing to specifically track MSCs *in vivo*. As discussed in the article, *in vitro* cultures, despite being great mechanistic tools, can often manipulate the cell phenotype in favor of specific differentiation events, by exposing them to highly artificial situations, such as the unnatural 2D environment in monolayer cultures or chemical stimulation. These potentially stressful *in vitro* conditions provoke subcultured MSCs to adjust their physiology (Bara *et al.*, 2014), while their stem cell features, inherent to rare cell population only, may disappear. Thus, regenerative properties of *in vitro* described MSCs are required to be validated *in vivo* with appropriate controls and reproducible protocols, which indeed only some studies have demonstrated until now. Nevertheless, *in vitro* amplified MSCs show therapeutic potential for certain clinical application, *e.g.* the treatment of graft-*versus*-host disease (Le Blanc *et al.*, 2008; Ringdén *et al.*, 2006; additional references), indicating therapeutic value of these cells independent of the fact that they might be an *in vitro* artefact.

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Editor's note: The Scientific Editor responsible for this paper was Chris Evans.