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Specificity of 3D MSC Spheroids Microenvironment: Impact on MSC Behavior and Properties

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Abstract

Mesenchymal stem cells (MSC) have been considered the promising candidates for the regenerative and personalized medicine due to their self-renewal potential, multilineage differentiation and immunomodulatory capacity. Although these properties have encouraged profound MSC studies in recent years, the majority of research has been based on standard 2D culture utilization. The opportunity to resemble in vivo characteristics of cells native niche has been provided by implementation of 3D culturing models such as MSC spheroid formation assesed through cells self-assembling. In this review, we address the current literature on physical and biochemical features of 3D MSC spheroid microenvironment and their impact on MSC properties and behaviors. Starting with the reduction in the cells' dimensions and volume due to the changes in adhesion molecules expression and cytoskeletal proteins rearrangement resembling native conditions, through the microenvironment shifts in oxygen, nutrients and metabolites gradients and demands, we focus on distinctive and beneficial features of MSC in spheroids compared to cells cultured in 2D conditions. By summarizing the data for 3D MSC spheroids regarding cell survival, pluripotency, differentiation, immunomodulatory activities and potential to affect tumor cells growth we highlighted advantages and perspectives of MSC spheroids use in regenerative medicine. Further detailed analyses are needed to deepen our understanding of mechanisms responsible for modified MSC behavior in spheroids and to set future directions for MSC clinical application.

Keywords Mesenchymal stem cells · 3D spheroids microenvironment · Morphological changes · Cell survival · Energy metabolism · Epigenetic regulation · Improved functionality

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Introduction

The presence of mesenchymal stem cells (MSC) in multiple tissue types as well as their multi-lineage differentiation and immunomodulatory capacity has led to an increased interest in their clinical application. Notably, since transplantation of MSC requires large cell numbers, culture expansion in vitro has been considered as necessary step in order to obtain sufficient quantities of cells for the intended therapeutic application. However, upon the extraction of the cells from their 3D native niche and further long-term in vitro culture expansion in two-dimensional (2D) conditions, MSC were found to accumulate chromosomal aberrations [1]. Moreover, a large body of evidence suggests that prolonged 2D cultivation leads to a loss of multipotency and induces cellular senescence in MSC [2]. Therefore, conventional 2D culture has been considered as bottleneck in a broader use of MSC-based therapeutics and the upscaling of MSC-culture by development of 3D strategies has become a current technological and economic challenge.

Spheroids are three-dimensional (3D) cell aggregates that can be formed in in vitro conditions through cells self-assembling. These cell aggregates have been considered to better mimic structural and functional features of the native tissue microenvironment and can be generated by more than one type of cells, when termed as multicellular spheroids. Cultivation of cells in form of cell spheroids have a long history. Indeed, the term spheroids was firstly used for the structures that Moscona described for tissue-like aggregates generated by cells of the early chick embryo [3]. Later on, different cell types have been shown as capable of forming spheroids, such as cancer cells [4] and mesenchymal stem cells [5]. Although there are still some challenging problems related to MSC spheroid formation and their maintenance in vitro, the spheroid formation of MSC is one of the most widely used 3D techniques for stem cell-based research. In this review, we summarized the data on specific physical and biochemical features of 3D MSC spheroid microenvironment and their impact on MSC properties and behaviors. Particularly, differences in MSC-environment interactions distinctive for 3D spheroids were discussed with respect to their influence on MSC survival, metabolism, gene expression, differentiation and immunomodulatory potential highlighting advantages and perspectives of MSC spheroids use in regenerative medicine.

Methods for MSC Spheroids Formation

In the body, the MSC are under impact of the particular conditions of the local microenvironment, which determinate their stem cell characteristics. The MSC microenvironment includes soluble factors, extracellular matrix constituents, signaling molecules, physical cues such as oxygen tension and pH [6, 7]. To mimic this natural cell environment and to reveal cell biology a number of 3D cell culture systems have been developed. These systems facilitate both the cell–cell and the cell–matrix interactions, helping cells to resume their natural morphology, and influence their differential capacity and functions.

In the literature, various techniques for generating and culturing of spheroids are described (Table 1). They can be generally divided into two groups: static and dynamic [20]. The static culture systems are attractive for many research studies due to simplicity of the setup. They prompt the aggregates formation by static physical force. For example, the hanging drop technique prevents the cell surface attachment by placing a cell suspension inside a droplet of culture media [21, 22], in which MSC concentrate and spontaneously aggregate at the bottom of the drop and a single spheroid is formed [8, 22]. This technique, traditionally inconvenient for long-term culturing or drug testing, has been significantly improved by creating well plates with perforated arrays in which hanging drops are formed [23, 24]. Recently, a simple, low-cost method utilizing a low-binding plate and seeding MSC at high density (14,000–60,000 cells per well in 96-well plate) to culture 3D spheroids for 5–6 days was also described by Redondo-Castro et al. [25].

Another technique that promotes spontaneous aggregation is liquid overlay. This is one of the simplest and less costly methods that can be used for spheroids production from MSC [26]. In liquid overlay, the cell attachment is avoided by seeding a cell suspension onto a non-adhesive surface that can be prepared from a thin coating of agar [26, 27], agarose [26] or poly (2-hydroxyethyl methacrylate) (poly-HEMA) [28]. Recently, several non-adhesive biomaterials as polycationic chitosan membranes, photosensitized polyethyleneglycol have been reported to stimulate MSC spheroid formation.

The above described static culture systems are between the most prominent technique for production and cultivation of MSC spheroids. They are suitable for producing of homogenous aggregates, do not generate shear stress damages, show high reproducibility, but have some limitations such as unsuitability for large scale production of spheroids and lack of mixing that brings concentration alteration in culture conditions (Table 1). Unlike them the dynamic 3D culture systems provide high spheroid generation efficiency, homogeneous culture physicochemical environment and enhanced the mass transfer. These methods force cellular aggregation by applying of the external forces such as centripetal forces, magnetic or electric fields and ultrasounds. However, the main disadvantages of almost all dynamic systems are requirement of specialized equipment and production of non-uniform aggregates.

Table 1 Size of MCS spheroids in dependence on cell origin, cell number and method of 3D culturing

Method	Advantages	Disadvantages	MSC source	Starting cell density	Initial cell number per aggregate	Aggregate size Diameter µm	Cultivation period	Volume/ Speed	Reference
Hanging drop	Production of uniform-size and shape aggregates.	Spheroids cannot be cultured for long period.	Human bone marrow--derived MSC (hBM-MSC)	6×10^5 cells/ml 12×10^5 cells/ml 24×10^5 cells/ml	15,000/ 30,000 60,000	352 ± 16–394 ± 53 502 ± 24- ~ 700 ± 30 706 ± 36–1049 ± 196	48 h	25 µL	[8, 9]
			hMSC	4×10^5 cells/mL	4000	~256 ± 10 at 24 h ~228 ± 10 at 48 h ~232 ± 14 at 72 h ~216 ± 14 at 96 h	24 to 96 h in low (2%) and normal oxygen (20%).	10 µL	[10]
			hBM-MSC	5×10^4 cells/mL	5000	~410 ± 100 at 24 h ~290 ± 100 at 120 h	7 days	100 µL	[11]
			Gingiva-Derived Mesenchymal Stem Cells (GMSC)	2×10^5 cells/mL	ND	GMSC 20–100	3D spheroid formation for up to 3 days	ND	[12]
Liquid overlay									
Chitosan membranes + surface--bound calcium	Suitable for long-term cultivation, allows production of spheroids without size and shape limitations, as well as an easy assessment of spheroid parameters and kinetics.	Variations in concentration at different apea of cell culture.	Adipose derived adult stem cells (AT-MSC)	2×10^4 cells/cm^2		from 95.3 ± 19.6 to 51.2 ± 7.7 with thickness of chitosan	12 days	ND	[13]
Chitosan membranes			Subcutaneous adipose tissue from abdomen	1.50×10^3 cells/cm^2 3.13×10^3 cells/cm^2 6.25×10^3 cells/cm^2 1.25×10^4 cells/cm^2 2.50×10^4 cells/cm^2 5.00×10^4 cells/cm^2		76.7 ± 41.2 85.9 ± 43.0 106.1 ± 43.8 136.0 ± 69.5 144.5 ± 55.1 194.6 ± 43.3	14 days		[14]
Chitosan (C) membranes or those further modified by hyaluronan (HA)			human adipose (hAT-MSC) placenta (hPDMC)	1.50×10^4 cells/cm^2		on chitosan hADAS 22 ± 5, hPDMC 25 ± 7 C + HA0.1 mg/cm^2 hADAS 30 ± 15, hPDMC 37 ± 16	7 days		[15]

Table 1 (continued)

Method	Advantages	Disadvantages	MSC source	Starting cell density	Initial cell number per aggregate	Aggregate size Diameter µm	Cultivation period	Volume/ Speed	Reference
						C + HA0.5 mg/ cm^2 hADAS 41 ± 15, hPDMC59 ± 21 C + HA2.5. mg/ cm^2 hADAS 54 ± 18, hPDMC78 ± 35			
Micropattern substrates coated with photosensitized poly(ethylene glycol)			MSC	2.5 10^3/cm2	500,000	100	26 days		[16]
Pellet culture system	A cost--effective and rapid formation of 3D MSC spheroids with displayed homogeneous and stable morphology.	Produce a low oxygen tension environment with very high cell density, subsequently a poor nutrient delivery and cell apoptosis in central part of spheroids	hBM-MSC	5×10^5 cells/ml	250,000 cells	~500		500 g for 5 min	[11]
Spinner flasks	The technique Improves nutrition diffusion and cell viability.	lack the ability to control critical process parameters such as pH and dissolved oxygen, and raise high level of shear stress	hAT-MSCs adipose--derived stromal cells	6.0×10^5 cells/mL		100 to 350	72 h	stirring at 70 rpm	[17]
				1×10^6 cells/mL.			11 days	80 rpm	[18]

Table 1 (continued)

Method	Advantages	Disadvantages	MSC source	Starting cell density	Initial cell number per aggregate	Aggregate size Diameter µm	Cultivation period	Volume/ Speed	Reference
			Umbilical cord tissue derived UC-MSC, UCX®			143 ± 9.78 at day 2309.5 ± 9.38 from day 4 to day 11			
Non-adherent plate + spinner flask or + rotating wall vessel (RWV)			hBM-MSC	2.0×10^5 cells/mL	50,000	physioxia (2% oxygen) ~380–680 or hyperoxia (20% oxygen) ~350–500	14 days	500×g for 5 min	[19]

Pellet culture system, a high-density culture system, uses centripetal forces to enforce the cell assembly at the bottom of the tube. It is a gold standard for both MSC differentiation studies in vitro as well as chondrocyte re-differentiation studies.

Many MSC studies used the dynamic culture conditions for MSC spheroids culturing such as spinner flasks and rotating wall vessel bioreactor. These bioreactors stimulate MSC aggregation by applying the intrinsic centripetal forces. The spinner cell cultures are maintained under continuous agitation, thus minimizing the cell adhesion [29]. The rotating wall vessels create an internal microgravity environment maintaining cells that has lower levels of shear stress then spinner flasks [29]. Both spinner flask bioreactors and rotating wall vessels require seeding of preformed MSC aggregates for generating uniform in size aggregates [29].

Other dynamic systems applied for cultivation of MSC multicellular spheroids are pin-on-ball bioreactor systems [30], microgravity bioreactors [31], wave motion bioreactors [32].

Another approach for MSC 3D culturing, nicely reviewed and summarized by Kim et al., refers to encapsulation technologies that produce 3D matrices of various shapes and sizes and are accomplished through the solidification of a cell-suspended liquid material [33]. These methods include micromolding, electrostatic droplet extrusion, microfluidic encapsulation in droplets and microfibers and bioprinting (inkjet, extrusion and laser-assisted bioprinting).

Dynamics of MSC Spheroids Formation

In multicellular aggregates, the morphology, adhesion, cytoskeleton organization and tension of MSC are drastically changed compared to the adherent cells. The cellular morphology is one of the main characteristics used to define the phenotype and fate of MSC [34]. The cell size and morphology are determined by the cytoskeleton organization, which is in direct dependence on biochemical properties of microenvironment [35]. MSC, cultured in standard monolayer conditions for several passages, are heterogeneous population, according to their shape and size. The size of the MSC grown in 2D conditions varies widely, regardless of the tissue source from which they are obtained. Moreover, cells differ in morphology - some have a typical fibroblast form, while others are larger and flat. Bartosh et.al. [22] found that the size of BM-MSC that had been expanded in monolayer varies in range 13-38 µm. Similar results are also reported for MSC derived from placenta; whose diameter varies from 15 to 50 µm [36]. In contrast, in spheroids MSC are smaller and highly homogeneous [18, 22, 36]. MSC in 3D aggregates have more compact cytoplasm and their volume is one-fourth of the volume of MSC in 2D culture [18, 22, 36]. Our study also proved that the nuclear volume of 3D cultured adipose tissue derived MSC was decreased by 75% [37] (Fig. 1).

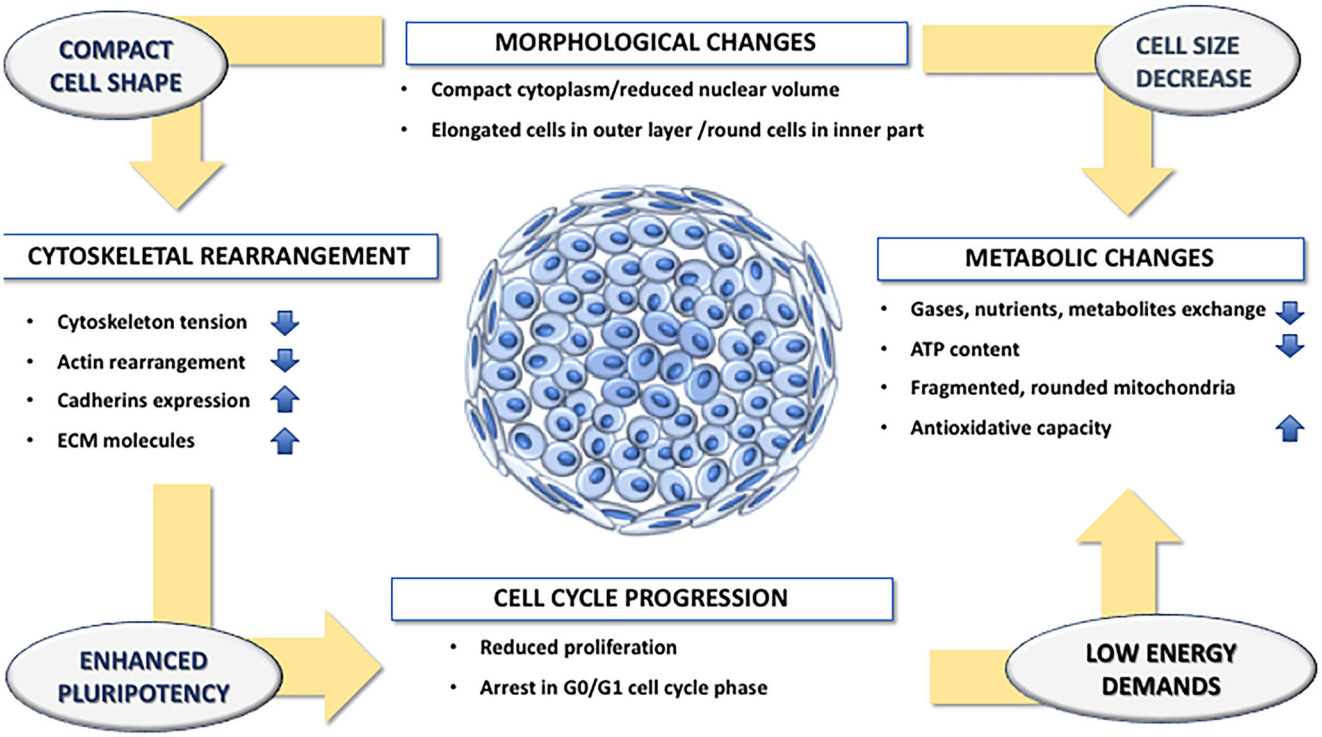

Fig. 1 Schematic presentation of MSCs behavior changes in 3D spheroid culture system. MSCs in 3D aggregates have compact cytoplasm, as well as reduced nuclear volume, while the outer layer is composed of elongated cells packed more densely than round cells in the interior. Compact cell shape induces cytoskeletal rearrangement that is characterized by loose cytoskeleton tension, expression of only weak, parallel orientated actin filaments and increased expression of cell-adhesion proteins, particularly cadherins. Also, increased internal levels of various ECM components are detected. These changes are related with enhanced pluripotency which is accompanied by reduced MSCs proliferation and G0/G1 cell cycle phase arrest in 3D spheroids, supporting the MSCs low-energetic state. In accordance with these modifications, metabolic changes associated with cell size decrease are documented and represented by limited diffusion of gases, nutrients uptake and metabolic wastes release. Along with these features, ATP content of MSCs in spheroids is decreased, while antioxidative capacity increased. The presence of rounded, fragmented mitochondria is reported, altogether indicating low-energetic state of MSCs in 3D spheroids

Morphology Changes during MSC Spheroids Formation

The formation of spheroids is a process of active association and arrangement of MSC in spheres [22]. During multicellular assembly of MSC drastic changes occurred in organization of cellular and extracellular microenvironment [32]. Furthermore, it appears that it includes involvement of cells with defined characteristics such as smaller size, higher CXCR-4 expression, migration and resistance to ischemic stress in vitro [32]. Firstly, BM-MSC form a loose network, followed by a gradually merge of small aggregates into a single central spheroid [22], but others report formation of a single spheroid from the first day [8, 22, 37]. The outer layer of BM-MSC aggregates is composed of elongated BM-MSC that are packed more densely than round cells in the interior [8, 22] (Fig. 1). Moreover, the spheroids compress progressively between 48 and 96 h due to a reduction in the amount of cytoplasm and in cell volume [22]. Additionally, the number of apoptotic or necrotic BM-MSC enlarges hardly with extension of incubation [22] and the detected low levels of apoptosis and caspase activity on day 1 remain unchanged as the large spheroid is formed [5] in contrast to non-MSC spheroids (obtained from tumor cells, hepatocytes, etc.) that formed necrotic cells core.

Adhesive Molecules and Cell-Cell Contacts in MSC Spheroids

During 3D aggregation mono-dispersed MSC experience drastic morphological transformations that are initiated and maintained through cell-cell contacts and cell-ECM interactions [38, 39]. Although precise mechanisms of spheroid formation are not fully understood, in vitro 3D cellular aggregation may be roughly observed as three-phase process. At first, scattered cells start to form weakly bonded aggregates by interacting via long-chain ECM fibers with multiple RGD motifs that attach to integrins on membrane surface. Next, during the delayed phase, these contacts (i.e. integrin activation) induce expression of cadherins and its accumulation on cell surface. Consequently, compact 3D spheroids are formed by homophilic connections between cadherins [39, 40].

Regarding this simplified model for 3D cellular aggregation, here we discuss changes in MSC cell surface molecules expression, their relations with cytoskeleton reorganization and associated functional activities of 3D MSC (Fig. 1).

As described for 3D aggregate formation of other cell types [39], various studies emphasize the role of cadherin expression in 3D MSC condensation [41]. Along with complete human bone marrow MSC aggregation achieved by N-cadherin expression [42], it has been reported that E-cadherin is critical factor for aggregation of umbilical cord blood MSC regulating the proliferative and paracrine activity of cells and promoting their therapeutic potential [43]. Flow cytometry analysis also demonstrated increased N-cadherin expression in MSC derived from multiple myeloma patients when cultured in 3D models [44]. Furthermore, in comparison to 2D human turbinate MSC, cells in cell-only formed spheroids and spheroids formed with cells and incorporated nanofibers (hybrid spheroids) significantly increased expression of cell junction proteins such as connexin 43 and E-cadherin, whereas within hybrid spheroids this effect was additionally promoted. Increased expression of cell-adhesion proteins, especially E-cadherin, was also found to be strongly associated with pluripotent state of mouse ESC [45], altogether indicating the correlation between stemness and compact, 3D spheroidal structure. On the other hand, in the study of Huang et.al. [46], performed with adipose MSC spheroids developed on different surfaces, elevated expression of N-cadherin was reported, which was shown to influence the biomaterial-dependent chondrogenic capacity of spheroids, both in in vitro and in vivo conditions.

Besides cadherins, other adhesive molecules are shown to participate in spheroid formation and maintenance, but the data are conflicting considering the experimental approach. During hanging drop cultivation of MSC derived from human placenta, along with increased N-cadherin expression, down-regulation of integrin-β1 was demonstrated [47]. Unlike adherent cells, cells from the hanging drop spheroids did not express α4-integrin (CD49d), but expression of α2-integrin (CD49b) was detected [48]. These results were supported by Bartosh et.al. [22], who reported that in spheroidal conditions MSC exhibit reduced expression of CD49d and increased expression of CD49b. In contrast to these findings, expression of integrin α2, α5, and β1 was significantly increased in cell-cell spheroids and additionally within cells from hybrid spheroids with incorporated nanofibers [49]. In addition, CD82, protein involved in suppression of cancer cell motility, was increased, while expression of an anti-adhesive protein podocalyxin-like protein (PODXL) was inhibited on the surface of MSC spheroids cultivated in hanging drops [22]. Based on these results, it might be indicated that the promotion of compact bonding is accompanied with the inhibition of cell mobility, which is in accordance with the inhibited cell migratory ability of MSC grown at high density [50]. However, data about migration of 3D MSC are very inconsistent since 3D model studies also demonstrated increased expression of CXCR4 receptor [32, 46, 48] and CXCL12 [44, 46] that assemble important axis in homing/mobilization of cells. Apart from direct cell-cell contacts, structure of spheroids is supported by ECM molecules that interact mutually and/or with cell surface receptors. Namely, as determined by flow cytometry and confocal microscopy analysis, significantly higher expression of ECM molecules including fibronectin, laminin, vitronectin, collagen IV, and collagen I was evident in 3D MSC in comparison to flat MSC [44] (Fig. 1). Similarly, as demonstrated by immunofluorescence staining in study of Ahmad et.al. [49], expression of fibronectin and laminin was markedly upregulated compared to 2D cells, along with their wider distribution, while these changes were more pronounced within hybrid spheroids then in spheroids formed by cells only [49]. Although recent study using hydrogels revealed that 3D culture itself influences ECM gene expression in BM-MSC [50], the mechanisms underlying higher ECM molecules expression in 3D MSC spheroids require intensive further research. According to findings demonstrating the involvement of HIF1 activity in regulation of ECM expression in fibroblast [51, 52], along with TAZ signaling activation found responsible for enhanced Col1a1 expression in hanging drop fibroblasts spheroids [53] it might be assumed that activation of these signaling molecules in 3D MSC spheroids could be relevant for increased ECM production. However, further studies are needed to confirm these assumptions.

Cytoskeleton Reorganization and Biomechanical Forces in MSC Spheroids

Enhanced cell-cell and cell-matrix interactions induce significant modifications in cytoskeletal network within each cell of 3D aggregate [54, 55] (Fig. 1). Namely, activated cadherins transduce signal through intracellular domains by interaction with adhesion complex (comprised of p120-catenin, β-catenin, α-catenin and other molecules involved in cytoskeletal organization) that enables direct physical contact and subsequent signal transfer to the actin filaments [56, 57]. Engagement of integrins also leads to adhesion complex activation and signal transmission inside the cell through actin cytoskeleton remodeling [58]. It has been shown that compact cell shape with reduced size is accompanied by loose cytoskeleton tension due to expression of only weak, parallel orientated actin filaments that contour the cell interior, contrary to adherent, big, spindle shape MSC that express well-spread actin filaments, as well as contractile actin bundles (stress fibers) across the whole cell [47]. Expression of α-tubulin, as constituent of microtubules, was not significantly changed in these models, indicating critical role of actin rearrangement for cell maintenance within 3D aggregates [32, 47]. Moreover, in accordance with Ahmad et.al. [49], increased Nanog

expression was also observed in 3D culture cells, while the correlation between Nanog expression and cytoskeleton tension was additionally confirmed using cytochalasin D to relax actin filaments of adherent cells, when increased Nanog expression was subsequently detected [47]. As for nestin, an intermediate filament of cytoskeleton network, its role in 3D assembly is elusive since both stimulation [59] and inhibition [44] of nestin expression have been detected in 3D models of MSC. Therefore, further examination of intermediate filaments involvement in MSC aggregation and functional properties of formed structures are required.

Besides cell-assembly initiation, adhesive molecules through cytoskeleton reorganization provide mechanical force sensing [60, 61]. Mechanical forces regulate various biological functions of MSC [62–65], suggesting that mechanical properties could be critical for understanding 3D MSC spheroid behavior. Particularly, this is important for considering MSC spheroids as building blocks for tissue engineering, since tissue-engineered scaffolds with appropriate anatomical and mechanical characteristics are necessary to mimic native tissues [66]. To give physical background for multicellular assembly Steinberg's differential adhesion hypothesis (DAH) consider that cells during this process tend to naturally reduce interfacial tension and by strong mutual connections restore thermodynamically stable structure [67]. However, since within cellular interior are present forces due to contractile actin network, Bordland proposed extension of DAH hypothesis termed as differential interfacial tension hypothesis (DITH) suggesting that interfacial tension is produced by cell adhesion and cell contractility tensions [68]. Thus, it has been considered that the overall biomechanical forces represent a result of balance between adhesion tension and cortical tension within individual cell [67, 69]. Moreover, forces affecting the cells can be observed as isotropic or anisotropic mechanical cues depending on their direction. When cultured in aggregates, due to maximized cell-to-cell contact, MSC produce highly isotropic cytoskeletal tension which is in correlation with promotion of pluripotent state and self-renewal, whereas, increased anisotropy of cytoskeletal forces synergistically leads to reduced pluripotency and enhanced MSC lineage commitment [70].

Relative elasticity or stiffness of the surrounding microenvironment which cells sense through adhesive molecules, as mechanical cue, significantly impact stem cell functionality and fate. Namely, MSC grown on the soft substrate differentiate into adipocytes, while with increasing stiffness of the substrate muscle and osteogenic differentiation of MSC are induced [71]. Also, MSC surrounded by other cells within 3D aggregates experience drastically softer environment then MSC grown in standard 2D monolayer cultures. The importance of elasticity-associated signaling in MSC spheroids has been recently indicated by Cesarz et.al. [21], who showed that mechano-signaling induced by soft elasticity increases BMP2 expression and IL1 signaling upstream of both pro- and anti-inflammatory gene expression in hanging drop BM-MSC spheroids. However, additional studies are required since the role of elasticity sensing in regulation of MSC properties when aggregated within spheroids has only begun to be elucidated.

Microenvironment of MSC Spheroids – Impact on Cell Survival and Metabolism

Oxygen Diffusion in MSC Spheroids

Specific microenvironment formed within MSC spheroids has been affected by their architecture which influences limited diffusion of gases and nutrients inwards, as well as metabolic wastes outwards the spheroids. Moreover, various growth factors, cytokines and ECM components produced by MSC accumulate in spheroids resulting in their increased internal levels. All these events contribute to formation of specific heterogenous milieu, featured by spatial and temporal gradients of different soluble molecules [38] more accurately mimicking in vivo microenvironment.

Diffusive mass transport in cell aggregates has been suggested to affect stem cell properties through spatial distribution of soluble factors [72]. Among these molecules, oxygen has been considered as the key factor that influences stem cells, since it is known to directly affects their proliferation and differentiation in vivo and in vitro [73].

According to the studies conducted with cancer cells and hepatocytes spheroids, oxygen gradient formation has been considered as common spheroids property featured by higher oxygen concentration on their surfaces and hypoxic core inside the aggregates [74, 75], which was correlated with the highest cell proliferation rate on spheroid surfaces opposite to the presence of viable quiescent cells in the middle and necrotic cells in the central zone [76] (Fig. 1). Moreover, it has been suggested that hypoxic core formation within spheroids potentiates cell functions and stimulates secretion of various factors [41, 77].

However, it is still under debate whether MSC spheroids even possess hypoxic core and if it could be directly related to the enhanced MSC functions. The existence of hypoxic core within MSC spheroids was actually proposed according to findings obtained for aggregates formed of embryonic stem cells (ESCs), cancer cells and hepatocytes analyzed using predictive mathematical models or oxygen-sensitive microelectrodes that directly measure oxygen levels [72, 78–80]. However, those non-MSC aggregates were built of cells featured by different rate of oxygen and nutrient consumption.

In the recent study, oxygen gradient was measured directly for the first time within BM-derived MSC spheroids of increasing diameter formed by hanging drop technique [8].

Obtained results showed that oxygen levels detected even in the largest spheroids (made of 60,000 cells) differed less than 10% from external levels. Actually, presence of hypoxic core was demonstrated only in spheroids formed from 250,000 cells. Absence of hypoxic environment in BM-MSC spheroids was explained by differences in their packing density, which resulted in formation of smaller, tightly packed and larger, loosely packed spheroids permitting transport of oxygen to the central zone. On the other hand, it was observed that glucose consumption significantly decreased as spheroid size increased demonstrating that oxygen is not responsible for altered function of MSC spheroids.

Oxygen availability is known to regulate activity of hypoxia inducible factors (HIFs), main transcription factors which regulate expression of hypoxia-associated genes involved in regulation of stem cell properties, development and metabolism. These transcription factors are also expressed by MSC [81], in which they play important roles in regulation of MSC stemness and self-renewal, as well as growth factors and cytokines expression [82]. Increased expression of HIF-1 and HIF-2 proteins have been observed in spheroids formed by gingival and adipose tissue MSC (AT-MSC) accompanied by higher antioxidative capacity and increased superoxide dismutase 2 expression [12, 17]. In addition, increased reactive oxygen species (ROS) production was demonstrated in BM-MSC spheroids [83]. Moreover, it has been suggested that HIF-1 stabilization observed within MSC spheroids could be involved in increased cell survival and cytokine secretion. Indeed, mild hypoxic environment formed within MSC spheroids has been thought as responsible for upregulated expression of VEGF, FGF-2, HGF, CXCR4, PGE2, and Bcl-XL [17, 84], as well as for increased expression of ECM constituents [85, 86]. However, direct evidences for these assumptions have been missing and in light of the data demonstrating the absence of hypoxic core within MSC spheroids other mechanisms known to activate and stabilize HIF-1 under normoxic conditions should be further investigated.

Together, all these data indicate that oxygen gradient in spheroids is highly dependent upon the cell type, which affects aggregates packing density and porosity. Besides, they point to the necessity for extensive investigations of oxygen diffusion in relation to cellular adaptation and responses within spheroids formed of different tissue origin MSC.

MSC Survival and Minimization of Energy Demands in Spheroids

As the energetic status of cell determines cell phenotype, here we discuss energy demands of MSC in self-assembled 3D aggregates and its repercussion on survival and MSC functions.

Evidences Indicating Reduced Energy Metabolism in MSC Spheroids

Besides reported decrease of size of spheroids formed by MSC during time in culture [87], change in single cell size and reduction of 3D MSC cell size in comparison to adherent MSC was also documented. As somatic cell reprogramming to pluripotency requires reduction in cell size, it was suggested that reduction of cell size might be one of MSC responses to the new spatial conditions. Similar decrease in cell volume and increase in cell density, followed by reduced adenosine triphosphate (ATP) demands, occurred in lymphocyte population after growth factor withdrawal and it is supposed that proliferating cells possess large cytoplasm for extensive protein synthesis, signaling, and other cellular processes [88]. Analysis of cellular energetics and genomics data indicated that bioenergetic costs of a gene at the DNA, RNA, and protein levels decline with cell volume [89] (Fig. 1). Therefore, it is possible that cell size decrease represents one strategy for saving energy in 3D MSC aggregates.

Findings indicated that proliferation of MSC deceases in 3D spheroid conditions. Namely, it was shown that frequency of cells in S-phase of cell cycle was lower in 3D MSC (1%) (Fig. 1) in comparison to adherent human bone marrow MSC cultivated at low (14.1%) or high density (3.5%) [22]. Similarly, the fraction of actively proliferating cells in spheroids was revealed by BrdU staining, where was shown that <5% murine BM-MSC proliferated. Moreover, EdU and poor Ki67 staining indicated reduced proliferation of human 3D BM-MSC [87]. Near 90% of 3D gingival MSC arrest in G0/G1 phase of cell cycle [12], which is in accordance with recent findings revealing upregulation of *p15* and *p21* gene expression and suggesting cell cycle arrest in 3D BM-MSC [83]. Thus, together with the reduced cell size, cell cycle quiescence found in 3D MSC spheroids supports the trend of MSC towards low-energetic state.

On the other hand, the data regarding MSC survival in 3D spheroids are conflicting. It has been reported that 3D cultivation of MSC did not provoke significant cell death after 1, 3 and 5 days [87]. This was also observed by Bartosh et.al. [22], who showed that almost 90% of MSC seeded in 3D aggregates stayed viable after 3 days, while number of apoptotic or necrotic cells increased when this period was extended to 4 days. Moreover, although Annexin V staining did not reveal any significant presence of apoptotic cells, upregulation of caspase activity in MSC occurred when spheroids of greater diameter were formed [8], thus indicating possible involvement of cell death as regulatory mechanism of 3D MSC physiology.

To define functional state of MSC cultivated in 3D aggregates in detail, recent studies investigated macroautophagy (autophagy). Autophagy is an essential proteostasis and stress response mechanism which controls mitochondria dynamic and cell respiration regulating cell survival and overall cellular

homeostasis. Namely, under basal conditions autophagy enables removal of damaged cellular parts, while this mechanism can be amplified during stress [90, 91]. However, it is not clear how autophagy regulates MSC status in 3D conditions. Namely, in comparison to adherent MSC, 3D MSC showed elevated mRNA expression of Beclin-1, lysosomal associated membrane protein 1 (Lamp1) and transcription factor EB (TFEB) which correlate with lysosomal biogenesis and increased frequency of autophagic vacuoles [83, 87]. Also, it was observed that exogenous inhibition of autophagy decreased 3D aggregates formation [87]. Upregulation of autophagy was also demonstrated in adipose tissue (AT) MSC spheroids cultured on chitosan substrates [92]. Interesting model suggested enhanced stemness in chitosan 3D MSC spheroids via selection of more primitive cells that survive by autophagy increase, while senescent cells undergo apoptosis due to reduced autophagy [93]. This might indicate that MSC sense poor nutrients and oxygen environment in 3D aggregates and adjust their phenotype though metabolic transition, which contributes to their improved survival.

Metabolic Changes of MSC in Spheroids

The environment in which the cell resides, in vivo or in vitro, and its resultant impact on the intracellular metabolite pool, plays a defining role in determining cellular phenotype with long-term impact through regulation of the epigenome [94]. As mitochondrial respiratory chain complex generates approximately 90% of cellular ATP via oxidative phosphorylation, mitochondria play central role in cell metabolism [95]. Quality of mitochondria is essential for the stemness maintenance and asymmetric apportioning during stem cell division [96], while mitochondrial clearance and a metabolic switch to glycolysis have to be achieved to convert large organelle-rich, mature cell type to organelle-poor primitive cell in process of somatic reprogramming [87].

Recent study reported that cultivation of MSC in self-assembled 3D aggregates induces metabolic reconfiguration in BM-MSC, followed by change of mitochondrial shape and organization. Namely, in comparison to MSC cultured in standard adherent conditions, mitochondria of 3D MSC were rounded, fragmented and immature-like, similarly to those observed in embryonic blastomera [83, 87]. These mitochondria exhibited bimodal mitochondrial membrane potential (MMP), while majority of 3D MSC possessed depolarized mitochondria, which suggested altered mitochondrial electron transport activity, with decreased mitochondrial complex I activity [11, 83]. As mitochondria impairment and energy deficiency have been shown to be key factor for loss of attachment of hepatocytes [97], it cannot be excluded that "immature-like" mitochondria state and loss of MMP might be associated with ability of MSC to form spheroids in nonadherent conditions.

Furthermore, 3D MSC were shown to possess lower ATP content [11], higher levels of mitochondrial ROS (mROS), which was followed by elevated levels of total cell ROS. Using of trans-dehydroandrosterone, which stimulates beta-oxidation in mitochondria, it was shown that ATP content was restored in 3D MSC. This was associated with decreased total and mROS levels, diminished upregulation of glycolytic enzyme gene expression, pluripotency-associated as well as autophagy-related genes in 3D MSC. However, it was suggested that changes in mitochondria induced by 3D MSC cultivation were not irreversible, and it is supposed that mitochondrial respiration is only reduced in response to decreased level of oxygen (or reduced oxygen demand) in comparison to its level in adherent MSC [83].

In respect to MSC aggregate size, results indicated that cell ATP content was not dependent on aggregate size and it is suggested that modified cell energetic transition, rather than diffusion limitation is dominant factor regulating reduced cell metabolism [11].

In addition, it was revealed that 3D MSC expressed significantly higher levels of glycolysis and pentose phosphate pathway (PPP)-associated enzymes mRNA, including pyruvate dehydrogenase kinase 1 (*PDK1*), hexokinase 2 (*HK2*), pyruvate kinase M2 *(PKM2*), lactate dehydrogenase A (*LDHA*), glucose-6-phosphate dehydrogenase and 6-Phosphogluconate dehydrogenase enzymes. Treatment with glycolysis inhibitor, 2-deoxyglucose, suggested high dependence of 3D MSC on glycolysis as ATP generation pathway. On the other hand, use of dichloroacetate, inhibitor of PDK1 and pyruvate oxidation only partly restored ATP levels through the inhibition of aerobic glycolysis in 3D MSC in comparison to adherent MSC [83]. Thus, biochemical strategies that 3D MSC use to satisfy their ATP demand further investigations in order to get better clarified.

Using ^{13}C-labeled glucose, difference in citrate as well as aspartate released by adherent MSC and 3D aggregated MSC were found suggesting enhanced activity of the pyruvate/malate dehydrogenase anaplerotic pathway followed by reduced activity of pyruvate dehydrogenase (PDH) in 3D MSC spheroids. Glucose consumption rate and lactate production rate were significantly lower when tested in 3D MSC [83], indicating low oxygen concentration in aggregates related to lower activity of aerobic metabolic pathway. Moreover, large-size aggregates were shown to consume lower amount of glucose and produce lower levels of lactate [8]. Stimulated pyruvate/malate dehydrogenase anaplerotic pathway and reduced PDH expression observed in 3D MSC [83], could be also observed in anchorage-independent cancer cells which are able to escape anoikis. Thus, whether glycolysis or coupled pentose phosphate pathway, are primary metabolic pathways used by 3D MSC to process glucose, remain to be clarified.

Together, these findings indicated that 3D self-assembled aggregates represent environments which predominantly can rescue MSC from exhausting oxidative metabolism, thus possibly contributing to the preservation of their stemness. However, energetic demands of 3D MSC phenotype, described as slow-cycling cells with immature mitochondria and increased pluripotency-associated marker expression, need to be additionally investigated.

Epigenetic Regulation of Gene Expression in MSC Spheroids

Mechanisms by which changes in microenvironment and metabolic states influence stem cells pluripotency and differentiation programs include epigenetic regulatory events. Epigenetic changes are defined as the heritable changes in the pattern of gene expression that occure without a change in the primary nucleotide sequence. The major mechanisms of the epigenetic regulation which can be affected by various microenvironmental stimuli encompass DNA methylation, histone modification, chromatin remodelling and microRNAs. Through modulation of gene programming events all these mechanisms may contribute to stemness maintenance and differentiation decisions.

With aim to evaluate what does it happen with the gene expression systems when MSC were transferred from 2D to 3D culture system, Potapova et.al. [98] had found that in human MSC spheroids 1731 genes were significantly upregulated and 1387 genes were down regulated more than two folds. In line of that Duggal et.al. [99] showed up- and down-regulated genes in the AT-MSC, cultured in alginate beads, through the microarray of 14,500 genes. The 48 up-regulated genes were associated with cell adhesion and metabolic processes and the 39 down-regulated genes were related to early development, intracellular signalling, and cell shape. Moreover, analyses of gene expression in BM-MSC spheroids demonstrated that the gene expression profile differs significantly from the 2D MSC regardless the passage (early or late), they were prepared from [100]. This research data proof that 3D environment provokes drastic changes in the global gene expression and these alterations are in dependence on cell origin, method and material used for the 3D culturing. However, whether these changes were provoked by direct regulation on transcripts levels or were mediated by epigenetic patterns, remains not clear.

Since epigenetic changes occurred as a response to environmental stimuli, it is important to explore how the changed environment in 3D culture conditions affects epigenetic mechanisms in relation to MSC self-renewal and fate determination. Guo et.al. [101] reported that increased clonogenicity and differentiation potency of 3D spheroids of human MSC were associated with two epigenetic mechanisms: histone modifications in promoter regions and alterations in the expression of miRNA regulated the mRNA transcripts of pluripotent genes (Oct4, Sox2, Nanog and TERT) and genes involved in differentiation (Table 2). The close correspondence of both mechanisms was defined: the histone acetylation in the promoter regions of multi-potency genes increased when the miRNAs, regulating the expression of these genes, enhances. The similar evidence was observed for the inhibition patterns of genes involved in differentiation.

The most pronounced chromatin change, provoked by cultivation environment, is the H3K9 methylation [103]. H3K9me3 accumulation on the promoter region is the major barrier in the expression of core pluripotent genes in somatic cells [104, 105]. Zhou et.al. [47] reported that 3D culturing of MSC significantly down-regulated the expression of Suv39h1, well known as H3K9 tri-methyltransferase. Those lead to reduced occupancy of H3K9me3 in the promoter region of Nanog. The reduction of the H3K9me3 level is likely induced by the release of the actin cytoskeleton stress. In support of that are the similar alterations observed in the 2D cultured cells after cytochalasin D treatment [47]. Thereby the changes in the expression of the Nanog gene under 3D culture may be realized by three epigenetic mechanisms - demethylation and acetylation of the H3K9 histone and alterations in the expression of miRNAs.

Jeon et.al. [102] pointed that full specter of histone-modifying enzymes (histone methyltransferases and demethylases) was changed in MSC cultured under 3D conditions. So, 3D MSC exhibited increased turnover of histone methylation/demethylation for more dynamic modifications of chromatins. Also, authors found the alteration in the microRNA expression profile of 3D MSC (Table 2). Many miRNAs with prominent expression changes were related to the cell fate control - towards epithelial-mesenchymal transition (EMT).

Differences in the epigenetic landscape of 3D cell models of human primary articular chondrocytes (hPACs) and BM-MSC were reported by Bomer et.al. [106]. hPACs possess a DNA methylation landscape that is almost identical (99% similarity) to autologous cartilage, in contrast to neo-cartilage obtained from BM-MSC. Differentially methylated regions (DMRs) are related to many transcription factors important for general development and morphogenesis. That provokes questions about the possibly negative effects of these vast methylation differences on cartilage regeneration and long terms consequences of implantation.

There were described many other new features of the spheroid MSC related to changes of mRNA and proteins expression [22, 107, 108]. However, the epigenetic mechanisms of these transformations, provoked by 3D culturing, are still unknown. The further exploring the impact of 3D culture conditions on the epigenetic changes in MSC, open a broad area

Table 2 Epigenetic control of gene expression in 3D spheroids of human mesenchyme stem cells (hMSC)

Epigenetic mechanism	Genes involved	Significant fold change to control	Reference
Pluripotent genes			
Increase of histone H3 acetylation in K9 in promoter regions	POU5F1	7.7	[101]
	SOX2	9.2	
	NANOG	2.3	
	TERT	21.4	
Decrease H3K9 me3 through decrease of the methyltransferase SUV39 H1 expression	NANOG	3.0	[47]
miRNA expression	*regulated pluripotent genes*		[101]
	MIR489	5.8	
	MIR370	3.0	
	MIR433	3.2	
	Differentiation genes		
	ALP	3.2	
Decrease in histone H3K9ac levels in promoter regions	OPN	0.1	
	FABP4	0.05	
	PPARr2	down	
miRNA expression	*regulated differentiation genes*		
	MIRLET7F1	0.2	
	MIR7–1	0.25	
	MIR21	0.25	
	MIR145	0.5	
	MIR24	0.5	
miRNA expression	*regulated* epithelial-mesenchymal transition (EMT):	≥2 ≤ − 2	[102]
	MIR166	89	
	MIR175		
	MIR-146b, key inducer of EMT		
Histone methyltransferases expression	KMT2B, KMT2C, KMT2E, KMT2F, and KMT2H (for H3K4 methylation)	about 2	
Histone demethylases expression	KMT1E and KMT1F (for H3K9 methylation); KMT6B (for H3K27 methylation); KMT3A (for H3K36 methylation); KMT4 (for H3K79 methylation) KDM5A and KDM5B (for H3K4 demethylation), KDM3A and KDM3C (for H3K9 demethylation), KDM6B (for H3K27 demethylation), KDM4B (for H3K36 demethylation)		

List of abbreviations used in Table 2**.**

ALP: *Alkaline phosphatase.*

FABP4: *Fatty acid-binding protein 4.*

H3K9ac: Histone H3 acetylated at lysine 9.

H3K9 me3: Histone H3 trimethylated at lysine 9.

H3K4: Histone H3 lysine 4.

H3K36: Histone H3 lysine 36.

H3K79: Histone H3 lysine 79.

KDM3A: Lysine (K)-specific demethylase 3A.

KDM3C: Lysine (K)-specific demethylase 3C.

KDM4B: Lysine (K)-specific demethylase 4B.

KDM5A: Lysine (K)-specific demethylase 5A.

KDM5B: Lysine (K)-specific demethylase 5B.

KDM6B: Lysine (K)-specific demethylase 6B.

KMT1E: Histone-lysine N-methyltransferase 1E.
KMT1F: Histone-lysine N-methyltransferase 1F.
KMT2B: Histone-lysine N-methyltransferase 2B.
KMT2C: Histone-lysine N-methyltransferase 2C.
KMT2E: Histone-lysine N-methyltransferase 2E.
KMT2F: Histone-lysine N-methyltransferase 2F.
KMT2H: Histone-lysine N-methyltransferase 2H.
KMT3A: Histone-lysine N-methyltransferase 3A.
KMT4: Histone-lysine N-methyltransferase 4.
MIR: Micro RNA.
MIRLET7F1: Micro RNA let-7f-1.
NANOG: Nanog homeobox.
OPN: Osteopontin.
POU5F1: POU domain, class 5, transcription factor 1.
PPARr2: Peroxisome proliferator-activated receptor γ2.
SOX2: SRY (sex determining region Y)-Box Transcription Factor 2.
SUV39 H1: Suppressor of Variegation 3–9 Homolog 1.
TERT: Telomerase Reverse Transcriptase.

for research due to variety of factors influenced epigenetic machinery such as a tissue cell origin, an inter-individual variability in epigenetic signature and difference of the 3D architecture/or material used.

Beneficial Properties of MSC Spheroids

Enhanced Pluripotency of MSC Spheroids

The transcription factors Oct4, Sox2 and Nanog have an important role in maintaining pluripotency and self-renewal potential of embryonic stem cells in culture [109]. The expression of these factors in MSC is also associated with enhanced potential for proliferation and differentiation [110], but they diminish significantly through long-term monolayer cultivation. BM-MSC spheroids showed increased expression of pluripotency marker genes Oct4, Sox2, Nanog, compared to monolayer cells [29, 32]. 3D culture, from placenta derived MSC also displayed increased mRNA levels of Oct4, Sox2, Nanog, as well as TERT gene [15, 101], which is responsible for the self-renewal properties of cells. In vitro studies revealed higher protein and mRNA expression of Oct4, Sox2, Nanog, c-myc and Klf-4 in 3D cultures of UC-MSC than in 2D adherent cells [111, 112] whose role is associated with the maintenance of stemness properties. In comparison to monolayer cells, the AT-MSC spheroids exhibited significant increase in expression of Oct-4, Sox-2, Nanog and REX-1 at protein and gene level [14, 31, 113]. The increased expression of pluripotent markers in cells cultured in spheroids suggests enhanced stemness properties of MSC, regardless of the source from which they are obtained.

Differentiation Potential of MSC Spheroids

Human MSC can differentiate along their lineage of origin when cultivated under specific conditions, such as osteoblasts, adipocytes and chondrocytes. MSC in adherent culture have low differentiation efficiency, while differentiation potential of MSC is generally enhanced in the 3D culture conditions. Both gene expression and histological observation results revealed that in 3D spheroid culture method there is a higher degree of differentiation in a shorter time.

After **osteogenic induction**, all cells from the 3D aggregate have been shown to be positive for calcium deposition when stained with Alizalin red, whereas the number of positive MSC in the monolayer was small. Spheroids from AT-MSC and BM-MSC exhibited higher mRNA expression levels of the osteogenic markers Runx2, osteocalcin and osteopontin than adherent cells [31, 113, 114]. UC-MSC 3D cultures also exhibited higher expression of Runx2, ALP, osteocalcin and osteopontin at protein and mRNA level than monolayer cells, as well as an increased mineralization and ALP activity [16, 111, 112].

Histochemical staining with Oil Red O or Nile Red of BM-MSC for adipocytes displayed almost 100% positive cells in spheroid, while positive cells in the monolayer culture were 22 ± 5% [114]. In 3D cultures lipid droplets were detected as early as 7 days after the induction of **adipogenesis**, and their number and size were growing rapidly to the end of the treatment [29]. AT-MSC spheroids also showed higher numbers of Oil Red O positive cells than cells in monolayer [31, 114]. Li et.al. [111] reported a 10-fold increase in lipid drops

deposits in spheroids from UC-MSC compared to conventional monolayer cultures. Real time-PCR analysis of adipogenic markers PPARg, C/EBPα, LPL, and aP2 showed a significantly increased expression in MSC spheroids than that in cells from the monolayer culture after adipogenic induction [16, 29, 31, 114].

Upon **chondrogenic induction**, MSC spheroids from adipose tissue and placenta displayed higher expression level of chondrogenic genes Sox9, aggrecan, and collagen type II than adhesive cell culture. Immunofluorescence analysis of both types of spheroids showed more positive cells for glycosaminoglycan and collagen type II compared to 2D culture [15].

Spheroid culture increased the differentiation capacity of MSC along the mesenchymal lineages when cultivated under specific conditions, as well as the transdifferentiating into mature cells of endodermal and ectodermal origin.

The production of cells of endodermal origin from stem cells through **transdifferentiation** would be useful both for regenerative medicine and for drug testing. After hepatogenic induction 3D clusters from AT-MSC exhibited increased protein and gene expression of hepatic markers albumin, CK-18 and CYP3A4 compared to monolayer cells revealed by immunofluorescence staining and RT-PCR analysis [28]. Spheroids from WJ-MSC displayed increased differentiation to endodermal cells, the progenitor germ layer for pancreatic and hepatic cells [115]. Guo et.al. [101] reported a more successful neurogenic differentiation in MSC spheroids from placenta compared to standard 2D culture. Most cells in the spheroids receive multi-polar neuron-like cells morphology on the seventh day after neurogenic induction. Real-time PCR and immunofluorescence analysis revealed that spheroid cells expressed higher levels of neural specific marker bIII-tubulin [101]. Enhanced neurogenic differentiation is also reported for AT-MSC spheroids, which also showed a significantly increased expression of bIII-tubulin, nestin neurofilament heavy chain and glial fibrillary acidic protein, at gene and protein level [31, 116].

In comparison to monolayer cultures spheroid formation of MSC exhibited significantly higher expression of pluripotent markers and enhanced differentiation capacity into cells of three germ layers.

Anti-Inflammatory Properties of MSC Spheroids

MSC cultured in spheroids have been shown to up-regulate immunoregulatory factors [22] inspiring new approaches in the research for their clinical implementation. The 3D format of culturing keeps the cell to cell interaction closer to the in vivo situation and probably unfolds their immunoregulatory potential, otherwise also seen in a monolayer culture [117–122]. Indeed, MSC-derived conditioned medium (CM) and exosomes from 3D cultures have been found to exert greater inhibitory influence on PBMC proliferation as compared to CM and exosomes deriving from monolayer MSC culture [123]. Moreover, preconditioning of MSC in 3D culture and transplantation of MSC spheroids or spheroid – derived cells have been shown as significantly more effective in suppressing inflammation in animal models of inflammatory diseases when compared to monolayer cultured MSC [22, 124].

It is believed that the cell-aggregation in 3D structures itself promotes enhanced anti-inflammatory phenotype. Several studies have shown that MSC spheroids are self-activated to produce PGE2 and TSG6 and are more effective in suppression of LPS-stimulated macrophages or PBMC as compared to adherent MSC [22, 125]. Besides, MSC spheroid-derived conditioned media have been found to affect LPS-stimulated macrophages decreasing the secretion of pro-inflammatory cytokines (TNFa, IL6, IL12), while increasing IL10 and IL1ra secretion and up-regulating the expression of the macrophage regulatory marker CD206 [84]. As for the MSC-derived PGE2 and TSG6, major roles in two separate negative feedback loops of immunomodulation in innate immune responses have been proposed in a review by Prockop et al. [126]. Both of these factors are known to be stimulated by pro-inflammatory cytokines, IL-1 and TNFa [126] and even further augmented when MSC self-organize in spheroids [5]. Interestingly, these immunomodulatory effects of MSC spheroids vanish if FBS is not present in the medium [127], meaning that besides the pro-inflammatory cytokines other factors are essential for the immunomodulatory activity of MSC [128].

On the other hand, MSC-mediated suppression of the adaptive immunity is rather requiring IFNg stimulation. Namely, when aggregation of MSC into spheroids is combined with sustained exposure to INFg, stable suppression of the activation and the proliferation of CD3/CD28 stimulated T cells, in a co-culture with the preconditioned MSC-spheroids, was achieved [127]. In addition, MSC stimulated with IFNg have been reported to secrete IDO which lead to suppression of activated T cells and stimulation of Treg in T/MSC co-culture [117, 118, 127].

MSC are also capable to secrete pro-inflammatory factors. Bartosh et al. [5] reported that, self-activation of MSC in spheroids involves up-regulation of IL-1 production and signaling. This autocrine stimulated IL-1 signaling turns out to be required for the secretion of PGE2 and TSG6 by MSC spheroids and the transition of macrophages from M1 to M2 phenotype when co-cultured with MSC [5, 129]. At the same time, in vitro priming of spheroid MSC cultures with IL-1 did not have anti-inflammatory effect on LPS-stimulated microglia cells, probably because of the concomitant stimulation of IL-6 and decrease of IL-10 secretion by the MSC [130]. Pro-inflammatory polarization of MSC has been observed also upon Toll-like receptor 4 (TLR4) priming which leads to secretion of pro-inflammatory cytokines IL-6 and IL-8 [131]. Although MSC are capable to secrete pro-inflammatory

factors this doesn't necessarily translate to pro-inflammatory effects over immune responses. However, one would always assume the possibility that the MSC-secreted pro-inflammatory cytokines could contribute to ongoing inflammation.

Although, the immunomodulatory potential of MSC is well defined and has been harnessed for the therapy of inflammatory and autoimmune diseases [132], further optimization of the MSC pre-conditioning protocols, contributing to the enhanced secretion of anti-inflammatory factors by spheroid MSC poses advantages for many therapeutic applications.

Anti-Tumor Activity of MSC Spheroids

Interaction of MSC with cancer cells in tumor microenvironment is of a crucial importance for the tumor fate. MSC are important stromal cells that contribute to tumor progression [133] and affect immune response through secretion of certain mediators. However, the complex roles of MSC in tumor development are not yet resolved because of the lack of an extracellular milieu that mimics the specific tumor microenvironment [134].

Tumor microenvironments are inherently three dimensional. However, currently the most popular tumor models involve 2D cell monolayer cultures, which do not resemble the native 3D morphology and cellular heterogeneity of a tumor thus failing to recapitulate drug diffusion kinetics or patient drug scaling [135–137]. Additionally, cancer research has been limited due to the inability to accurately model tumor progression and signaling mechanisms in a controlled environment. Although animal models allow limited manipulation and study of these mechanisms, they are not necessarily predictive of results in humans.

So far, it has been found that human MSC cultured as spheroids in hanging drops exert enhanced anti-tumor properties through considerable increases in the expression of several factors, including leukemia inhibitory factor (LIF); tumor suppressor protein IL-24; TNFa -related apoptosis inducing ligand (TRAIL), a protein with selectivity for killing certain cancer cells; CXC chemokine receptor 4 (CXCR4), a receptor involved in MSC homing; and decreased expression of dickkopf 1 (DKK1), an inhibitor of Wnt signaling [22].

Moreover, several studies reported that MSC spheroids-derived conditioned medium suppressed the prostate cancer LNCaP cell growth by cell cycle arrest and up regulation of their apoptosis and necrosis [22, 138]. Similarly, Frith et.al. [29] observed decreased viability of the various prostate cancer PC3, VCAP, LNCAP, and DU145 cell lines when cultured in medium conditioned by dynamic 3D MSC. Moreover, their study showed that 3D MSC conditioned medium did not influence the viability of normal immortalized cell lines due to the up regulation of IL-24, known to specifically impair the viability of tumor cells while leaving normal cells unaffected [29].

Nevertheless, a promotion of tumor cells growth by interaction with MSC cultured under 3D conditions has been also reported in several publications. Interestingly, in a co-culture environment established in 3D bioprinted matrix, MDA-MB-231 cells significantly inhibited osteoblast and MSC growth. Conversely, tumor cells growth was distinctly promoted by the presence of osteoblasts or MSC in the co-culture environment [139]. Also, another study showed that human MSC invaded the MCF-7 spheroids affecting the motility of breast cancer cells in 3D cultures. The presence of even a few MSC altered the morphology of the spheroids and reduced MCF-7 cell–cell adhesion via degradation of E-cadherin by the sheddase ADAM10 [140]. Besides, soluble factors secreted by MSC in both 2D and 3D co-culture conditions were found to protect leukemic cells against chemotherapy cytotoxic effects providing the niche that allows leukemic cells survival and proliferation [141].

In fact, it has been reported that MSC also influence metastatic properties of tumor cells by facilitating epithelial-mesenchymal transition (EMT) and secretion of factors that increase tumor cells mobility. To follow tumor cell invasion Park et al. [142] established a novel platform of 3D spheroid-clusters consisting of either a mixture of human MSC and breast cancer cells (MDA-MB-231) or MSC only within a collagen gel matrix. Using this model, they observed higher migration activity of cancer cells through analyses of morphological changes, movements, and biochemical properties in the mixed clusters as well as by evaluation of the expression levels of invasion and metastasis markers, such as CD44, fibronectin, α-SMA, and CXCR4 [142].

Perspective of MSC Spheroid Technology

As the 3D MSC culture technologies resembles more closely in vivo cell environments, they have a perspective for broad research in biology, medicine and pharmacology as well as clinical application due to ability to provide better precision of results. MSC are the most widely used stem cell in clinical trials based on their properties of multilineage differentiation, immunomodulation, homing at site of injury and regeneration together with easy accessibility and culture expansion in vitro for a prolonged period of time [143]. In the past ten years there have been increasing amount of evidences about enhanced therapeutic efficacy of MSC cultivated in spheroids due to increased paracrine secretion, proliferation, ability to enhance the angiogenesis and stemness of MSC and providing reduction of inflammatory responses at the site of transplantation [17, 144, 145] (Fig. 2). MSC spheroids have been shown to achieve rapid promotion of osteoblastogenesis and bone formation in vitro and improved new bone regeneration in vivo [146]. Up regulation of chondrogenesis-related, anti-apoptotic and anti-inflammatory genes was observed in aggregated

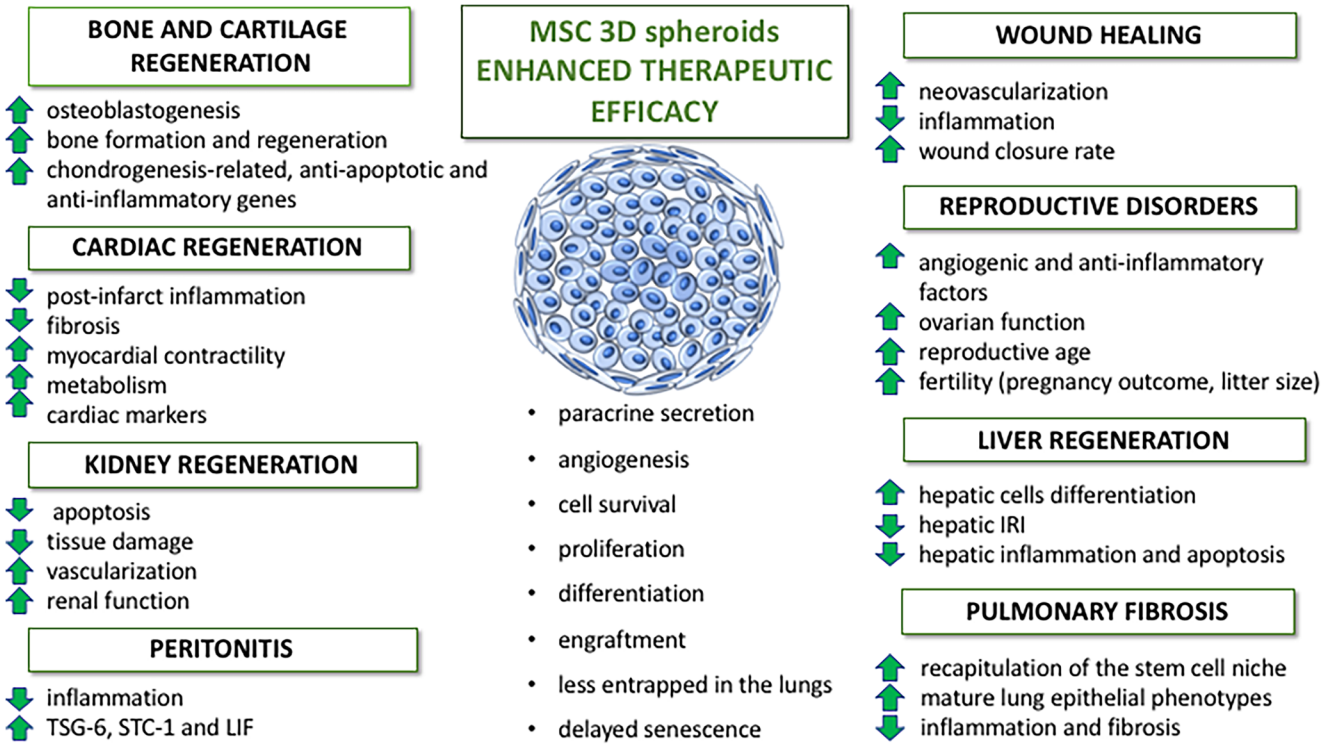

Fig. 2 Enhanced therapeutic potential of 3D spheroid MSC. Cultured in 3D spheroids MSC showed up-regulated secretion of paracrine factors that influence angiogenesis and regulate the inflammation. Stemness and differentiation capacity, as well as cell survival and engraftment after infusion were significantly unproved compared to traditional adhesive 2D cell cultures. The spheroids are less entrapped in lungs and capillaries and are found to a greater extent in all other tissues and organs. Higher proliferation, survival rate and delayed senescence were reported for 3D cultured MSC. Therapeutic approaches implementing spheroid cultured MSC in various diseases were suggested. Regeneration of injured tissues and organs, reduction of inflammation, apoptosis and fibrosis, neovascularization and improved function were documented in a variety of disorders

MSCs, which provided successful cartilage regeneration after transplantation into a knee joint [147]. Enhanced therapeutic properties of MSC spheroids in cardiac and neural disorders was reviewed by Petrenko et.al. [55]. MSC-specific growth factors and cytokines can modulate processes such as post-infarct inflammation, fibrosis, myocardial contractility and metabolism. Higher cardio myogenic potential of MSC spheroids resulted in the reparation of the myocardial infarction in animal models [138, 148]. Lee et al. reported significant preservation of myocardial contractility and prevention of LV dilatation when spheroids were injected into the myocardium at 3 days after MI in rats [144]. Increased expression of cardiac markers in vitro in MSC spheroids is also a potential benefit for myocardial infarction treatment [149].

Also, there are accumulated data related to the greater therapeutic potential of MSC spheroids in kidney and liver injury and regeneration [150–152]. MSC spheroids were more effective in protecting the kidney against apoptosis, reducing tissue damage, promoting vascularization, and ameliorating renal function compared with 2D cultured cells. The spheroid MSC were more effective in suppressing inflammatory responses in a mouse model for peritonitis [22] due to unregulated expression of anti-inflammatory genes TSG-6, STC-1 and LIF in 3D. Idiopathic pulmonary fibrosis in mice treated with self-aggregated spheroids demonstrated recapitulation of the stem cell niche and acquired mature lung epithelial phenotypes that decreases inflammation and fibrosis [153]. 3D MSC attenuated hepatic ischemia-reperfusion injury in rats by inhibiting hepatic inflammation and apoptosis [151].

Wound healing in presence of MSC spheroids exhibited significant increases in wound closure rate compared with wounds treated with an equal number of MSC suspension [113]. MSC spheroids contribute by modulating the inflammatory milieu and improved neovascularization [18, 154, 155]. Re-epithelization and capillary network formation were studied to show the effect of MSC spheroids. Spheroid MSC treated wounds at day 14 were completely regenerated, with a more mature vascular system, based on the presence of several organized capillaries, and already showing cell appendages such as glands and hair follicles [18].

Reproductive disorders also were better effected by spheroid MSC than by cells in monolayer. Rat model of Asherman's syndrome (AS) treated with spheroid MSC showed pregnancy outcome and litter size higher than in control group treated with 2D MSC [156]. The authors suggest that improved therapeutic effect is realized via enhanced secretion of angiogenic and anti-inflammatory factors. Intrauterine administration of spheroid MSC improved

fertility better that intravenous injection [156]. MSC spheroids effectively restored ovarian function and expand the reproductive age of woman, reducing medical costs due to age-related disease and morbidity [157]. Spheroid-cultured MSC significantly increase the number of ovarian follicles and the expression levels of folliculogenesis-related genes in a short period compared to 2D-MSC.

The data suggest that spheroid MSC may be more effective than adherent MSC in therapies for diseases characterized by sterile tissue injury and unresolved inflammation and for some cancers that are sensitive to anti-inflammatory agents [22]. Elevated expression of CXCR4 in MSC spheroids predicted enhanced migration toward tumors after infusion, and up-regulated caspase3/7 activation lead to more effectively killing of the cancer cells [158].

Cultured as spheroids MSC obtained features that favored 3D to monolayer cultures in regenerative medicine applications. Improved cell survival, higher efficiency of engraftment and enhanced proliferation of spheroid MSC transplanted have been reported in various in vivo models. Additionally, larger numbers of the cells trafficked through the lung after i.v. infusion and were recovered in spleen, liver, kidney, and heart [22] since they become less entrapped in the lungs and the capillaries. Other advantages described for MSC spheroids are enhanced production of growth factors and cytokines including mitogens, anti-inflammatory and angiogenic activities, augmented differentiation and stemness potential, and delayed replicative senescence. These promising features of MSC spheroids give solid base for the development of the suitable approaches for functional recovery of damaged tissues in future.

However, continuing the research on epigenetics, genomics, and proteomics of MSC spheroids is very important for elucidation of differences in therapeutic capacities of MSC derived from different individuals and tissues [159], which will allow the precise treatment of specific disorders.

Although the therapeutic applications of MSC cultured in spheroid enlarge, there are many unclear aspects related to the behavior of these cells after transplantation. In this case, the promising perspective of spheroids technology can be their transformation in factories producing micro-vesicles (MVs) containing numerous therapeutic factors such as cytokines, micro-RNAs and others. Indeed, MSC-derived MVs are considered as ideal medicinal candidates and are preferred over MSC infusion therapy due to improved safety [160].

Except therapeutic potential, the development of 3D MSC spheroid technology can help to make drugs discovery and screening of drug properties easier and less expansive. From technological perspective promising for pharmacology have been considered the multi-cellular spheres created by co-suspending several cell types, including MSC, embedding of formed spheres into scaffolds mimicking extra cellular matrix and organoid cultures [161]. In addition, combining drug discovery with patient-derived 3D cell culture and molecular profiling data, allows screening of a personalized panel of drug candidates and can improve outcome and reduce side effects of therapy [162]. However, despite rapidly accumulating scientific data on spheroids in pharmacology much work remains to be done to develop an ideal system that accurately reproduces in vivo physiological as well as diseases conditions. The data presented above clearly point that the research on MSCs spheroid properties still needs further improvement and enlargement before being subjected to wide clinical applications.

Conclusion

In contrast to the scaffold-based 3D cell cultures, MSC spheroids can be obtained by using various low cost, easy to handle and reproducible techniques that allow large-scale production of cellular aggregates usable for live cell testing and therapeutic application. Reproducing many features exhibited by in vivo human tissues, MSC spheroids represent optimal cell structures suitable for various application in pharmacology and medicine. Having this in mind, understanding the mechanisms by which physical and biochemical cues specific for 3D MSC spheroid microenvironment impact MSC behaviors may contribute to the development of new and more effective therapeutic application strategies. Therefore, in this review, we aimed to collect data on how specific features of 3D microenvironment, including oxygen level, metabolic alterations, cell-ECM and cell-cell interactions influence MSC survival, pluripotency, differentiation and immunomodulatory potential highlighting in this way advantages and perspectives of MSC spheroids use in regenerative medicine.

All presented data clearly provide evidence that the physiology and behavior of cells in 3D system are different from the cells cultured in 2D environment. On one hand, 3D MSCs have more benefits such as higher differentiation activities, up-regulation of paracrine secretion and enhanced therapeutic potential [41, 55]. However, on other hand, the drastic epigenetic changes in MSC under 3D environment, particularly, miRNA profile related with cell fate control towards epithelial-mesenchymal transition (EMT), can negatively impact their therapeutic potential. Indeed, in vitro co-culture of these spheroids with tumor cells provoked invasion of tumor cells, while in vivo application promoted the metastasis development [106] indicating that 3D MSC spheroids could serve as activated niche for cancer stem cells.

Therefore, further detailed experimental and mathematical analyses are required in order to better understand molecular characteristics of MSC spheroids in temporal and spatial manner, as well as to identify the key regulatory factors responsible for observed changes of MSC behavior in spheroids. In addition, much effort is still needed to ensure reproducibility, high throughput analysis capacity, compatibility of readout

techniques, and better automation, to establish standardized and validated 3D cell models. Driving innovations for 3D technologies to improve the quality, consistency and predictive capacity of MSC spheroid cultures have been considered as current and future challenge within the field of stem cells research and regenerative medicine.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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