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MSC functionalization for enhanced therapeutic applications

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ABSTRACT

To date, the therapeutic efficacy of human mesenchymal stem cells (hMSCs) has been investigated in various clinical trials with moderate or in some cases inconsistent results. The still elusive reproducibility relates in part with constitutive differences in the cell preparation, translated into variable “cell potencies”. Other factors include poor cell homing and survival, and age/disease-associated host tissue impairment. It is well accepted that within *in vivo* niches MSCs exist as heterogeneous cell populations with different stemness propensities and supportive functions. Phenotype-based MSC purification of homogeneous subsets can result in cell populations with distinct biological functions. In addition, preclinical studies have shown that MSC functionalization *in vitro*, via cell priming, can boost their immunomodulatory, trophic and reparative capacities *in vivo*. Therefore, in the present review we discuss how phenotype-based MSC purification and MSC priming technologies can contribute to an improved MSC-based product for safer and more effective therapeutic applications.

IMPACT:

Culture expansion of MSC has detrimental effects on various cell characteristics and attributes (*e.g.,* phenotypic changes and senescence), which, in addition to inherent inter-donor variability negatively impact the standardization and reproducibility of their therapeutic potential. The identification of innate distinct functional MSC subpopulations, as well as the description of *ex vivo* protocols aimed at maintaining phenotypes and enhancing specific functions have the potential to overcome these limitations. The incorporation of those approaches into cell-based therapy would significantly impact the field, as more reproducible clinical outcomes may be achieved.

INTRODUCTION

Safety and efficacy of human mesenchymal stem cells (MSCs)-based therapies are being investigated in a growing number of clinical trials for various disorders including musculoskeletal, cardiovascular, autoimmune, neurodegenerative and gastrointestinal (http://clinicaltrials.gov). Initial results from many of such studies reveal that these therapies show a significant degree of variability with cases of non-reproducible clinical data. The inconsistent evidence potentially relates to intrinsic differences in the cell-based products used, including lack of standardized features in the preparations reflected in "potency" discrepancies, as well as to factors related with the therapy itself (*e.g.,* cell homing to target tissues and subsequent survival), and the host receiving the therapy (*e.g.,* age and disease-associated conditions).

Active research is being conducted to help minimize the reported variability, ultimately aiming at increasing clinical reproducibility. Efforts include thorough characterization of cell preparations including the description of constitutive variations (*i.e.,* cell heterogeneity), and methods to stimulate MSCs with chemical and physical conditions to induce and/or to modulate specific attributes of the cells (*i.e.,* cell priming). In this review, we collect and discuss available evidence of such efforts, focusing on two main aspects: the identification of specific subsets/subpopulations of MSCs from different sources (heterogeneity), and the derivation of stimulatory protocols applied to the cells a priori to modulate cellular phenotypes and homing capacity (priming).

1. MSC SUBSETS/SUBPOPULATIONS

The fact that standard MSC cultures consist of functionally heterogeneous cell subsets is well recognized in the literature, however, the reasons for such heterogeneity are poorly understood. In our opinion, such heterogeneity arises from two different factors: firstly, from an inherent heterogeneity of culture-initiating MSCs, and second, from culture-induced accumulation of senescent cells, which in later passages become predominant and therefore significantly impact on cultures' potency. As the latter phenomenon has been reviewed extensively (1, 2), this section will focus on tissue-inherent MSC heterogeneity, which is closely linked to their local niches and tissue topography.

It is widely accepted that MSC populations within the different niches and tissues are highly heterogeneous; purification of homogeneous subsets with distinct biological functions is a challenge. However, isolated MSCs are generally analyzed for their clonogenic potential by the CFU-F assay and only limited information exists about markers that discriminate between developmentally, functionally and morphologically distinct MSC subsets (3). In here, we summarize recent studies aimed to identify different MSC subsets in the bone marrow (BM) as well as adipose and synovial tissues.

1.1 Bone marrow MSCs

Location markers: CD146 and CD271.

Bone marrow contains stem and progenitor cells for both hematopoietic and non-hematopoietic lineages. Bone marrow MSCs (referred also as multipotent stromal cells and skeletal stem cells - SSCs) reside in the postnatal BM cavities and give rise to bone, cartilage, marrow-fat and hematopoiesis-supportive stroma following a specific sequence of events during postnatal development (4-6). A pioneering work by Tormin et al has clearly demonstrated that human BM MSCs were present in two different regions within the BM cavities: in the perivascular niche, where they were characterized by CD146 marker expression, and in the bone-lining location, where MSC lacked this marker (7). Both, $CD146^{+}$ and $CD146^{Neg}$ MSC subpopulations were $CD271^{+}$ and had similar clonogenic capacities and gene expression profiles characterized by the simultaneous expression of multiple-lineage transcripts. Therefore, while $CD271^{+}CD146^{Neg}$ cells are present in bone surfaces, $CD271^{+}CD146^{+}$ MSCs constitute the *bona fide* perivascular component of the BM (Figure 1). Furthermore, adherent cultures derived from these two subpopulations had similar differentiation capacities *in vitro* and *in vivo*. In a more recent study Espagnolle et al have expanded BM MSCs at the clonal level and showed the presence of $CD146^{high}$ and $CD146^{Neg/low}$ clones (8). In agreement with Tormin et al study (7), they found no differences in the tri-lineage as well as hematopoietic support potentials between these two types of clones, however clonal cultures derived from the $CD146^{high}$ subset had slightly slower growth rates and a more pronounced vascular smooth muscle phenotype, which was tested using functional cell contraction assay in a collagen gel. These findings supported Tormin et al study (7) in which BM $CD146^{+}$ cells were shown to have a topography of pericytes. The significance of CD146 expression on these perivascular MSCs remains to be investigated. In regards to CD146 expression on BM MSCs, it is interesting to mention that Maijenburg et al have also found CFU-Fs in the $CD271^{Neg}CD146^{+}$ subset of fetal BM (9). CD146 expression was shown to be dependent on calcium (10) and oxygen levels (7) and can therefore reflect the niche environment in which this specific BM MSC subset resides. Less information is so far available with respect to $CD146^{Neg}$ BM MSCs. Because significant reduction of $CD271^{+}CD146^{+}$ population was found in elderly adults, compared with children (9), it is possible that $CD146^{Neg}$ cells represent a subset of 'aged' BM MSCs, however, this is not consistent with Tormin et al data (7) that showed no differences in the clonogenicity between $CD271^{+}CD146^{+}$ and $CD271^{+}CD146^{Neg}$ subpopulations.

The perivascular localization concept of BM MSCs has been recently reinforced by Lin et al. pioneering study, showing that intra-arterially injected BM MSCs can serially engraft from the circulation into irradiated BM and proliferate *in vivo*, retaking the perivascular space around BM vessels and sinusoids (11). Recent reports

(12, 13) expanded the notion of MSC subpopulations beyond the CD146 and CD271 discrimination reported in human BM, incorporating additional markers thus far identified in mouse studies. For instance, the perivascular BM MSCs can be further divided into two main categories according to the blood vessel they associate with: the periarteriolar MSCs (Nestin^{bright}, NG2^{+}, αSMA^{+}, CD271^{+}, CD146^{+}), and the perisinusoidal MSCs (Nestin^{dim}, leptin receptor (LepR)$^{+}$, CD271^{+}, CD146^{+}). These subpopulations have distinct functions as they support hematopoiesis and probably are related with local homeostatic responses. Along those lines, Mendez-Ferrer et al. showed that Nestin^{+} MSCs contain all BM colony-forming-unit fibroblastic activity, are spatially associated with hematopoietic stem cells (HSCs) and highly express HSC maintenance and retention genes such as those encoding the cytokines chemokine (C-X-C motif) ligand 12 (CXCL12) and stem cell factor (SCF) (14, 15). These perivascular cells known as CXCL12-abundant reticular (CAR) cells express Nestin, LepR, myxovirus resistance-1 (Mx-1), the transcription factor paired related homeobox-1 (Prx-1) that characterize cells of limb bud mesoderm, and transcription factor osterix (OSX or SP7) that regulates osteoblast maturation (13). Importantly, Sachetti et al. suggested that CD146^{+} human MSCs may be the *in vitro* counterpart of CAR cells as they acquire the same phenotype in *in vivo* transplantation models reconstituting the hematopoietic environment (16). Therefore, except CD146^{+} MSCs' ability to reconstitute bone and BM stroma after orthotopic and heterotopic transplantation models, they have an important role in HSC niche maintenance. To further support this notion, Corselli et al. showed that CD146^{+}CD34^{Neg}CD45^{Neg} perivascular cells support the HSCs' stemness, through cell-to-cell contact and Notch signaling activation, and HSCs' ability to engraft to primary and secondary immunodeficient mice. In contrast, unfractionated and CD146^{Neg} MSCs induce differentiation and inhibit ex vivo HSCs' maintenance (17).

In summary, the combined literature to date indicates that within human BM stroma, the CD146^{+} subpopulation has a preferential perivascular topography and hematopoietic support function, whereas CD271 is expressed on both perivascular and bone lining MSCs; precise functions of these molecules on BM MSCs remain to be investigated.

Other markers:

In recent years, a number of novel markers to identify distinct MSC subsets within the CD271^{+} fraction in human BM were proposed, including CD140b (18), MSCA-1 (19, 20), CD90 and CD106 (21), CD140a (22) and SUSD2 (20); however their expression *in situ* has not been performed therefore the precise tissue locations of these putative subsets remain unknown. Another surface marker of interest is CD56. Early studies have shown that CD56 molecule was highly-specific for bone-lining cells (23), in the same location of CD271^{+}CD146^{Neg} MSC subset in the Tormin et al study (7) (Figure 1); however CD56 has not as yet been used to isolate BM MSCs as a single marker or in combination with CD271. An interesting study from Buhring's laboratory (19) showed a distinct morphology and potential chondrogenic bias of the CD271^{+}MSCA-1^{+}CD56^{+} BM MSC subset. These findings are awaiting an independent confirmation, but were confirmed in part by Cuthbert et al who found MSCA-1 expression in the CD45^{Neg}CD271^{+} fraction of BM aspirates (24). Busser et al also found better chondrogenic potential of MSCA-1^{+} selected MSCs than unselected ones and higher chondrogenic capacity from BM MSCs

grown from the $CD271^{+}$ fraction (20). Contrary, Jezierska et al found better chondrogenesis of cultures expanded from $CD271^{Neg}$ rather than $CD271^{+}$ cells, after differentiation on gelated collagen microspheres (25).

CD271 marker is lost during culture expansion, therefore other markers were studied to discover MSC subpopulations present in both in fresh and early-passage expanded cells. When Qian et al tested CD44 as one of those markers, they found that CD44 was absent in freshly isolated BM MSC population because the cells that co-expressed CD271 and CD146 and displayed the clonogenic and multipotent functions were $CD44^{Neg}$ (26). However, CD44 marker was acquired by $CD44^{Neg}$ subset after *in vitro* expansion. This finding is unexpected because CD44 has always been considered as a "well-known" MSC surface marker in expanded-MSC studies (reviewed in Sousa et al (27)). In agreement with Qian et al study, Busser et al confirmed the higher clonogenicity of the $CD44^{Neg}$ fraction in the BM (20). These authors also found that $CD44^{Neg}$ fraction showed greater osteogenic potential than the total unselected population, but in contrast, no differences in chondrogenic potential were found between $CD44^{Neg}$ and $CD44^{+}$ cells (20). These authors also found greater osteogenic potential and clonogenic capacity in $CD34^{+}$ than $CD34^{Neg}$ cells or unselected BM MSC population. Again, $CD34^{Neg}$ and $CD34^{+}$ fractions did not show differences in the chondrogenic potential. As a result of different studies, CD34 had become another possible, but controversial marker for MSC subset selection from fresh BM aspirates (reviewed in Lin et al (28)).

Functionally selected MSC subpopulations:

Other approaches use cell division as functional parameter for identifying MSC subpopulations in early-passage MSCs (29), or clonal expansion methods (8, 30). After clonal expansion, Dickinson et al performed a comparative analysis between greatest and poorest chondrogenic clones and found that the expression of ROR2 positively correlated with chondrogenesis (30). *In situ* immunostaining corroborated the presence of $ROR2^{+}$ cells co-located with $CD146^{+}$ cells surrounding the blood vessels in the adult human BM. As mentioned, Espagnolle et al (8) performed a selection of the expanded clones based on CD146 expression, for further analysis. Other markers were used to study heterogeneity in culture-expanded MSCs, such as ganglioside-based membrane microdomains (31).

Based on this information, it is fair to conclude that the existing literature supports the notion for at least two distinct topographies of MSCs in the BM (perivascular and bone-lining), however further work is needed to assess what effect these niches may have on MSC functionality assessed after their expansion and priming *in vitro*. It is possible that standard culture conditions lead to MSC's convergence to a common phenotype (32) therefore future investigations of BM MSC heterogeneity would benefit from a direct analysis of these cells immediately after purification.

Pre-clinical studies with MSC cultures derived from selected subpopulations

Some selected BM subpopulations, mainly grown from $CD271^{+}$ cells, have been evaluated in pre-clinical studies. In one study, $CD271^{+}$ subpopulation of cultured BM MSCs when injected into infarcted murine heart, showed improved cardiac function (33). Another study reported improved repair of full thickness cartilage defects

in rats using MSCs sorted for $CD271^+$ and expanded to form cell pellets that were placed in and covered with atelocollagen sponges (34). Also, CD271-MSCs promoted significantly greater lymphoid engraftment than did plastic adherence-MSCs when co-transplanted with HSCs in immunodeficient mice (35). Other preclinical studies with BM MSC subpopulations have been recently reviewed in Mo et al (36) and Lv et al (37).

1.2 Adipose tissue MSCs (ASCs)

Location markers: CD146 and CD34

Adipose tissue is a complex tissue of mesodermal origin that contains various cell types including adipose tissue MSCs, preadipocytes and mature adipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells (ECs), monocytes/macrophages and lymphocytes (38). Obtaining MSC from adipose tissue, a waste product in several treatments, is popular due to the easy access to the tissue in large quantities. As for BM MSCs, two distinct MSC subsets have been discovered in human adipose tissue, but this time they are $CD146^+CD34^{Neg}$ pericytes and $CD146^{Neg}CD34^+$ adventitial cells (both negative for CD45) (39, 40). *In situ* immunostaining of adipose tissue obtained from abdominoplasty showed $CD146^{Neg}CD34^+$ cells localized in the outer vascular ring and $CD146^+$/α-SMA^+ cells immediately adjacent to the vascular intima (39). In one study, a third population of $CD146^+CD34^+$ was observed with a very low frequency by flow cytometry (approximately 0.5% of the nucleated stromal vascular fraction cells), presumably a transitional population between the pericytic and supra-adventitial perivascular ring cells. Further studies are necessary to confirm these markers *in situ*. The frequency of these subsets differs significantly depending on donor demographics and the way of tissue processing, but is still unclear how these variables affect the different subsets.

Other markers

Bajek et al (41) studied the differences in early-passage ASC markers from tissues obtained either from mechanical or ultrasound-assisted liposuction, and they only found statistical differences in the expression of CD166. Other authors (20) did not found differences in CD166 expression that was low in both stromal cells from abdominoplasty and lipoaspirate, although they observed that $CD271^+$ selected ASCs were highly proliferative and clonogenic only in lipoaspirate samples, being the contrary for abdominoplasty ones ($CD271^{Neg}$ fraction). Interestingly, CD271 marker was not mentioned in many characterization studies (42, 43). Importantly, Baer et al. (42) performed the first comprehensive phenotypic characterization of cultured adipose-derived stromal cells (ASCs) isolated from lipoaspirates. They found that ASCs expressed the characteristic MSC's markers CD29, CD44, CD73, CD90, CD105 and CD166 but there was high donor variability for 49 of the 242 markers tested, including CD34 and CD200. These findings can be explained by the fact that CD271, as well as other molecules present on native adipose MSC subsets can be lost in culture.

The difficulty in analyzing adipose tissue MSC literature is that adipose tissue is highly vascular and it is widely accepted that following digestion and removal of red cells, the remaining cells consist of leukocytes, stromal cells and ECs. Subsequent dissection of stromal cells and ECs is complicated by the fact that many 'MSC' specific molecules - CD73, CD105, CD146 and some authors even consider CD90 - are also expressed

on ECs (reviewed in Lin et al. (44)). Conversely, a typical endothelial progenitor molecule CD34 can be expressed on adipose MSCs (reviewed in Sidney et al. (45)), as stated above. So, these markers are not very useful alone for segregating MSCs from ECs however $CD31^{Neg}CD45^{Neg}$ combination can discriminate leukocytes and ECs from adipose MSCs as published by the ISCT together with the International Federation for Adipose Therapeutics and Science (IFATS) (43). These authors published a guidance for minimal criteria for stromal cells from adipose tissue, as previously performed for general MSC. This guide differentiates between freshly-isolated stromal cells as "uncultured stromal vascular fraction" (SVF), identified mainly as $CD45^{Neg}CD235a^{Neg}CD31^{Neg}CD34^{+}$ subset, and cultured stromal cells (ASCs), characterized as $CD45^{Neg}CD31^{Neg}CD36^{+}CD106^{Neg}$ cells. Overall, the literature agrees that $CD34^{+}$ may be present on a subset of native adipose MSCs, but it is lost in prolonged culture expansion (46) and is not useful as a sole marker as CD34 is also expressed on ECs and other cell types (44, 45). However, CD34 was considered a good marker for MSC enrichment from SVF fraction by Busser et al (20) as $CD34^{+}$ ASCs were significantly more clonogenic and proliferative than parental cells. Furthermore, Bourin et al (43) observed that both SVF and ASCs express CD73, CD90 and CD105, although CD44 expression is only present in the cultured ASCs, which seems to be similar to CD44 pattern of expression on BM MSCs (26). Also, Bourin et al (43) suggested additional positive markers with variable expression, as CD146. Despite this, one study did not found expression of CD106 and CD146 markers in culture expanded-ASCs, but passage number at which cells were analyzed was not specified (47).

Functionally selected MSC subpopulations

Different from BM, Busser et al (20) found that adipose MSCs did not express SUSD2, MSCA-1/TNAP and CD44 *in situ*, but this data that needs to be confirmed. Hardy et al (48), performed enrichment of $CD146^{+}CD34^{Neg}$ and $CD146^{Neg}CD34^{+}$ subsets from adipose MSC tissue collected by lipoaspiration, focusing on the aldehyde dehydrogenase (ALDH) staining intensity. After culturing single-cell sorted cells, transcriptional profile analysis was done and $ALDH^{bright}$ cells were considered as the most primitive cell population within both subpopulations. Considering that this study was only performed using one sample, further confirmation is necessary.

In general, differently from BM MSCs, most of the studies using adipose MSCs don't study chondrogenesis. There also exists controversy about MSC variability due to age, body mass index, niche location and the way of tissue processing that complicates data interpretation but may lead in the future to discovery of many more subtypes of adipose MSCs. For example, the possibility of finding different MSCs subpopulations that can support different types of blood vessels. So probably, different vascularity seen in lipoaspirates and solid fat tissues can be the reason for different types of MSCs found but more work is needed to study the relationship between these blood vessels and MSCs. Despite of all these unanswered questions, surprisingly, SVF are being used for clinical treatment of human cartilage damage (reviewed in Pak et al (49)).

Pre-clinical studies with MSC cultures derived from selected subpopulations:

In pre-clinical studies using AT MSCs, mainly $CD34^+$, $CD105^+$ and $CD90^+$ subpopulations were tested. One study compared $CD90^+$ and $CD105^+$ subpopulations of cultured MSCs seeded on hydroxyapatite (HA)-coated polylactic-co-glycolic acid (PLGA) scaffolds in calvarial defects in mice and showed better bone formation from $CD90^+$ subpopulation (50). In calvarial defects in mice, cultures grown from $CD105^{low}$ sorted cells and seeded on HA-PLGA scaffolds formed more robust bone than cultures grown from $CD105^{high}$ cells (51). Other preclinical studies with selected AT MSCs have been reviewed in Johal et al (52).

1.3 Synovial MSCs

Location markers

The synovial membrane is a specialized tissue of mesodermal origin lining the spaces of diarthrodial joints, bursae and tendon sheaths. It contains two compartments, the intima inner continuous cell compartment composed of fibroblast-like synoviocytes, and the subintima compartment composed of few macrophages and lymphocytes, fat cells, and blood vessels (53). Interestingly, the origin of synovium-derived MSCs in the synovial lining is still under investigation and can be related to infiltrated MSCs through vasculature or BM originated MSCs that connects to intra-articular space. Recently, two studies showed that single or double positive Prg4-lineage and Gdf5-lineage cells, both present in the synovium, contribute to repair of articular cartilage injuries in mice (54, 55). Despite the hypothesis of the involvement of synovium-derived MSCs in cartilage repair is beginning to gain strength (56), *in vivo* and *in vitro* studies about synovium MSCs are scarce compared with MSCs from other tissues such as adipose or BM. A comprehensive review of clinical studies utilizing intra-articular MSC therapy showed that only 7% of the studies reviewed used MSCs from synovial membrane (57). The same applies to *in vitro* studies, in which much fewer investigations were focused on synovial MSCs, mainly culture-expanded cells, compared to BM MSCs.

In a pioneering study, Karystinou et al (58) reported for the first time the presence of cell subsets with distinct characteristics within the synovial membrane. They performed cell cloning by limiting dilution and found variations in MSC proliferation and potency: all clonal populations were chondro-osteogenic and only 33% of them were also adipogenic, unlike the parental MSC population that was tripotential. Phenotype evaluation of tripotential extensively-expanded clones by flow cytometry showed positivity for markers such as CD13, CD73, CD81, CD90, CD105, CD166 and SSEA-4. However, these authors did perform neither enrichment of these tripotential MSCs nor phenotypic analysis of bi-potential clones. Besides, in this study not all of the clones isolated were culture-expandable. Taking this into account and also the fact that markers as CD271 are undetectable after extensive expansion (59-61), recent studies are being focused in early-passage expanded (passages 0 to 1) or freshly-isolated MSCs.

Hermida-Gomez et al (62) performed the first MSC topographic analysis of the synovial tissue, showing $CD271^+$ cells present in its intima lining in healthy donors but also along vascular subintima in osteoarthritic patients, a likely route for the mobilization of the MSCs to reach the cartilage damage. However, other authors associated the increase of $CD271^+$ MSCs in synovial membrane of arthritic patients with pro-inflammatory function (61). Hermida-Gomez et al (62) found that $CD271^+$ cells present in synovium had a high co-expression

of CD44 and CD90 but low levels of CD105. The low positivity of CD105 in addition to CD166, in freshly isolated synovial MSCs was confirmed by Jones et al (63) using flow cytometry. In spite of the low percentage of $CD105^{+}$ MSCs present in synovial membrane, early-passage-CD105-selected cultures had good chondrogenic capacity after spheroid formation (64), but this study did not compare results with $CD105^{Neg}$ or total MSCs because these cells were not capable to form spheroids.

More recently, Mizuno et al (65) proposed different phenotypes of MSC in the osteoarthritic synovium based on their topography: $CD55^{+}$ MSCs in the surface region, $CD271^{-}CD55^{-}$ in the stromal region and $CD271^{+}$ in the perivascular region. However, these results need to be confirmed in healthy synovium.

Other markers

Another marker used for subsets analysis and enrichment from early-passage culture-expanded synovial MSCs was CD73, which enriched for cells with higher chondrogenic potency than CD106 marker (66). Also, CD44 expression was correlated with chondrogenic capacity (63), however no CD44 enrichment was performed in this study. Combinations of markers, such as CD73 and CD39 were later used for enrichment of synovial MSCs from early and late passages synovial cultures, and $CD73^{+}CD39^{+}$ subset was found to be more chondrogenic and osteogenic than $CD73^{+}CD39^{Neg}$ subset (67). However, when these authors compared culture-expanded and freshly isolated synovial cells they found no differences in clonogenicity and chondro-osteogenic potential between $CD73^{+}CD39^{+}$ and $CD73^{+}CD39^{Neg}$ subsets confirming the necessity of performing this type of analysis using freshly isolated synovial cells. Furthermore, as commented above, markers such as CD271 are known to be downregulated following MSC culture-expansion; similarly CD34 marker was detected in directly isolated synovial cells, but declining following MSC passaging (60).

To summarize, more work in this area is required to provide a convincing argument for the use of specific markers for the isolation of any functionally relevant subsets of synovial MSCs. To the best of our knowledge, no pre-selection of synovial MSC populations was performed in pre-clinical studies.

Altogether, while MSC are being largely used in clinical trials as bulk-expanded heterogeneous preparations (reviewed in Squillaro et al (68), Kouroupis et al (69)), to our knowledge there is no clinical studies reported on a comparison between different selected subpopulations. In this area, an unanswered question yet remains: do phenotypically-same subpopulations from different tissues behave similarly upon implantation, or the tissues of origin, rather than phenotypes, are more important?

2. MSC PRIMING

Efficacy and reproducibility of MSC therapies are not only affected by the composition of the cell preparation (above), but also by the capacity of the transplanted cells to consistently reach and interact with dysregulated tissues (*i.e.,* homing and engraftment) and subsequently to predictably induce and/or modify specific host responses (*i.e.,* therapeutic effect). In other words, after administration (*e.g.,* injection, infusion,

etc.), MSCs have to migrate, home, engraft, survive, sense the local environment and reactively mount a paracrine reparative response. Previous studies have shown that systemically-infused MSCs (*e.g.,* intravenous or intra-arterial administration) are capable of migrating and homing to distant sites of active injury including BM, intestine, liver and lung (11, 70-72). However, only a small percentage of infused cells (i.e., 0.1-2.7%) actually reaches the target tissues (73-78). Once at the injured tissue, it is well accepted that MSCs exert immunomodulatory (for both innate and adaptive immunity) and trophic activities (79, 80), recently suggested to be collectively called "medicinal" signaling activities (81, 82). These local activities are performed through both direct cell-cell communication (*e.g.,* Notch receptor /Jagged-1 and PD-1/PD-L1/PD-L2 (83, 84)) and locally secreted paracrine mediators. The latter involves a list of chemokines released by MSCs including C-X-C motif chemokine 12 (CXCL12) (85), stem cell factor (SCF) (86), platelet-derived growth factor (PDGF) (87), transforming growth factor β (TGF-β) (88), vascular endothelial growth factor (VEGF) (88), tumor necrosis factor-α stimulated gene/protein 6 (TSG-6) (89), erythropoietin (EPO) (88), interleukin-6 (IL-6) (88), interleukin-10 (IL-10) (88), indoleamine 2, 3-dioxygenase (IDO) (88) and prostaglandin E2 (PGE2) (90). Some of these factors (*e.g.,* TSG-6) have been shown to also exert a remote paracrine therapeutic effect, demonstrated in myocardial infarction (91), lung injury (92), and corneal injury (93), suggesting that the induction of its secretion even without interaction with the target tissue might be sufficient for a therapeutic effect. On the other hand, host tissue conditions (*e.g.,* age and disease) also have an effect on the paracrine activity of engrafting MSCs (94-96). For instance, elevated levels of proteases such as elastase, cathepsin, and dipeptidylpeptidase (DPP) present in the aged tissue destabilize trophic factors induced by MSCs. Interestingly, the administration of a pharmacologic inhibitor of DPP prior the therapy enhanced the stability of CXCL12 and increased the engraftment and function of $CXCR4^+$ progenitor cells in an acute myocardial infarction mouse model (97). Collectively, this evidence suggests that both the "pharmacokinetic" properties and therapeutic activity of MSCs can be modulated and/or boosted a priori, through multiple *ex vivo* priming protocols. We now discuss a few techniques proposed to effectively prime MSCs before their transplantation *in vivo* (Figure 2).

2.1. Membrane and cytoplasmic MSC priming

MSCs express a variety of membrane adhesion molecules including intercellular adhesion molecules-1 and 2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule-1 (VCAM-1), L-selectin, CD18, CD24, CD29, CD44, and CD49a-f (98, 99). Previous studies have shown that the adhesion molecule expression profile presents an intra-population heterogeneity (71), which is not only determined by the tissue of origin (100) but also by isolation and culture procedures. For instance, Aldridge et al., showed that early and late passaged MSCs have different adhesion molecule profiles, with CD49d exhibiting variable expression within the same cell population (71). Despite the potential deleterious effect this variability may have on cell adhesion processes, MSCs' homing to target sites can be enhanced through diverse *in vitro* membrane modification techniques that incorporate natural and induced processes such as: a) the hematopoietic stem cell/leukocyte transendothelial migration process (*i.e.,* diapedesis); b) chemical or non-covalent interactions; and c) biospecific recognition. Early evidence has shown that engineering transmembrane glycoproteins on MSCs using characterized selectin ligands affects MSC migration through vasculature after systemic infusion *in vivo*, similar to leukocytes

extravasation to inflamed tissues (101). Ruster et al. identified that MSCs, like leukocytes, roll and adhere to postcapillary venules via cellular interactions with ECs engaging P-selectin as well as VCAM-1/(CD106) - VLA-4(CD49d/CD29) pathways (102, 103). As MSCs do not express P-selectin ligands such as glycoprotein ligand-1 (PSGL-1/CD162) or CD24, alternative ligands have been proposed (*e.g.,* CD44 glycoprotein which binds hyaluronic acid)(104).

Hematopoietic cell E-/L-selectin ligand (HCELL), a specific isoform of CD44 that binds strongly E-selectin (105), has been associated with MSC homing to BM in vivo (106) and thus its modification constitutes a promising approach to enhance that process. Using enzymatic conditions, Sackstein et al. (104) converted the MSC membrane native CD44 glycoprotein into HCELL allowing their efficient binding to E-selectin. The resulting sialofucosylated glycan moiety on CD44 is known as Sialyl-Lewis X (SLe^x), the active selectin-binding carbohydrate motif site for HCELL. Interestingly, intravenously infused $HCELL^+$ MSCs infiltrated mouse BM within hours of infusion (104). Enzymatic membrane modification targets only glycoproteins that already exist on the surface of MSCs, whereas further chemical alterations enable the cells to present several epitopes via covalent bioconjugation method, potentially enhancing the effect. Importantly, Sarkar et al. used several approaches to covalently attach SLe^x on MSC membrane through biotin-streptavidin binding, conferring leukocyte-like rolling characteristics to MSCs without affecting their phenotype and multilineage differentiation capacity (107, 108). These approaches resulted in increased MSC rolling on P-selectin at a physiological shear of 0.5 dyn/cm^2 *in vitro*. In an ear inflammation mouse model, SLe^x engineered MSCs exhibited a robust rolling response on inflamed endothelium (109).

Another method to improve MSC homing is via antibody conjugation approach. To localize MSCs at a target tissue, researchers have used palmitated protein G or A pre-coating of the cell membrane as a step to bind antibodies on the MSC surface. Interestingly, Dennis et al. (110) have pre-coated the membrane of MSC chondrogenic progenitors with palmitated protein G and bound to the protein G antibodies specific for cartilage matrix antigens. Effective homing of membrane modified chondrogenic progenitors was observed to cartilage injury site in rabbit osteochondral explants (110). In another study, homing rate and repairing efficacy of MSCs improved using anti-CD29 and anti-myosin light chain bispecific antibodies in a mouse myocardial fibrosis model (111). Importantly, in this study Deng et al. (111) also used highly focused ultrasound mediated stimulation of micro-bubbles, which significantly increases homing of MSCs to the mouse injured myocardium (112). Finally, another non-conventional method to enhance MSC homing is the internalization of superparamagnetic iron oxide nanoparticles with exogenously-guided magnetic targeting. This approach has been used by two separate studies showing enhanced MSC homing to mouse retina (113) and tail (114) and rabbit and swine osteochondral defects (115).

2.2. Cytokine and hormonal priming of cultured MSCs

An alternative approach to enhance homing and therapeutic capacity of MSCs is to prime the cells with specific cytokines and growth factors. Shi et al. showed that MSC priming with a cytokine cocktail containing SCF, HGF, IL-3 and IL-6 increases their CXCR4 expression (116). As a result, CXCR4 upregulation enhanced

MSC chemotaxis to CXCL12 *in vitro* and homing efficiency to BM *in vivo*. Similarly, IL-1β-primed MSCs exhibited an enhanced homing capacity to inflammatory sites in an ulcerative colitis mouse model, also via upregulation of CXCR4 (117). Mechanistically, IL-1β-primed MSCs express multiple cytokines including TNF-α, IL-6, IL-8, IL23A, IL32 and chemokines CCL5, CCL20, CXCL1, CXCL3, CXCL5, CXCL6, CXCL10, CXCL11 and CX3CL1, as well as MMPs and adhesion molecules ICAM-1 and ICAM-4 (118). Interferon-γ (IFNγ) is another molecule widely used to prime MSCs. It is a major pro-inflammatory mediator that has been shown to increase MSCs homing capacity to inflamed intestine in an ulcerative colitis mouse model, by upregulating CXCR7 and lectins LGALS3BP and LGALS9 in infused cells (119). A major effect of IFNγ relies on the induction of the expression and activity in MSCs of the enzyme indoleamine 2,3-dioxygenase (IDO), involved in the direct suppression of T cells proliferation and activation (120, 121). IFNγ priming also upregulates the production of IL- 6 in MSCs (122), a cytokine with known anti-inflammatory and reparative effects in vitro and in vivo (e.g., liver fibrosis mouse model (123)). Therefore, due to its high potency in inducing immunomodulatory factors, IFNγ has been proposed by many studies as a priming cytokine to enhance MSCs efficacy *in vivo*. Other priming methods with positive effects include exposure to complement component 1 subcomponent q (C1q), which enhances MSC homing by inducing chemotactic responses to SDF-1 gradient. Glycogen synthase kinase 3β (GSK-3β) inhibitors have also been used for MSC priming, resulting in increased MSC migration via the upregulation of β-catenin, phospho-c-Raf, ERK, phospho-β-PAK-interacting exchange factor (124), enhancing CXCR4, MMP-1 and MMP-2 expression.

Hormonal priming of MSCs has been proposed as an additional method to improve MSC survival and to induce paracrine actions post-transplantation *in vivo* in various conditions such as myocardial infarction (125) and brain injury (126). Mias et al. also showed that MSCs primed with melatonin stimulated angiogenesis and proliferation of renal proximal tube cells *in vitro* via increased HGF and FGF-2 expression, and accelerated renal recovery *in vivo* following similar mechanisms and increased cell survival after transplantation (127). Melatonin MSC priming also showed beneficial effects in focal cerebral ischemia in rats, where it not only increased MSC survival post-transplantation *in vivo,* but also reduced brain infarction and increased angiogenesis and neurogenesis (126). On the other hand, oxytocin-primed MSCs reduced cardiac fibrosis and macrophage infiltration while enhanced cardiac repair for at least 4 weeks post-MSC transplantation *in vivo* (125). In a recent study, Liu et al. (128) showed that angiotensin-II hormone MSC priming results in reduced cardiac fibrosis and infarct size, improved cardiac function, increased expression of VEGF and von Willebrand factor in the ischemic myocardium but no MSC differentiation towards cardiomyocytes *in vivo*.

2.3. Hypoxia priming of cultured MSCs

Rapid loss of implanted MSCs *in vivo* has been associated with hypoxic stress at the ischemic target tissue, which can initiate cellular apoptosis (129). It is worth mentioning that MSCs are typically cultured at a pO_2 level of 142 mmHg (~20%), while the oxygen tension in the BM niche ranges from 1% to 7%, and in ischemic tissues from 0.4% to 2.3% (130). In a pioneering study, Annabi et al. identified that hypoxic environments (*i.e.,* 1% oxygen, 5% CO_2, 94% nitrogen) induce *in vitro* MSC migration and three-dimensional capillary-like structure formation through the secretion of VEGF and MMP-1 (131). Moreover, this increased migratory behavior is

explained by Akt signaling activation and induction of cMet expression, the main receptor of HGF (132), whereas the promotion of the MSC-dependent angiogenic effect is directly associated with the activation of hypoxia-inducible factor-1α (HIF-1α)-78-kDa glucose-regulated protein (GRP78)-Akt signaling pathway (133). In general, MSC priming under hypoxia enhances the upregulation of several signaling molecules including CXCR4 (134-136), CX3CR1 (134), CXCL-12 (134, 135), VEGF (135) and vascular endothelial growth factor receptor-2 (VEGFR-2) (135), MMP-2 (136), MMP-9 (136), brain-derived neurotrophic factor (BDNF)(135), glial cell-derived neurotrophic factor (GDNF) (135), erythropoietin (EPO) (135) and its receptor EPOR (135), focal adhesion kinase (FAK) (137). The *in vivo* administration of hypoxia-primed MSCs show increased homing capacity, enhanced vascularization and restored function in various ischemic conditions, including myocardial infarction, cerebral and hind limb ischemia (132, 134-137). Mechanistically, two separate studies in mouse and rat myocardial infarction models showed that hypoxia-primed MSCs improve infarcted heart function via enhanced survival of implanted MSCs, increased angiogenesis and prevention of cardiomyocyte apoptosis through cell survival factor secretion (138, 139). These results were extended to diabetic cardiomyopathy, in which anoxia-primed MSCs improved cardiac function through not only anti-apoptotic effects but also attenuation of cardiac remodeling (140). In acute kidney injury, hypoxia-primed MSCs showed beneficial effects by improving renal function, increasing angiogenesis and reducing the levels of pro-inflammatory cytokines (141, 142).

Even though hypoxia-derived MSC priming has a positive influence on migration and homing capacities in several conditions, there is evidence of potential unwanted effects that require further investigation and that may indicate that hypoxia-priming may be application specific. This includes a negative effect on MSC osteogenic differentiation capacity (143), and the accumulation of reactive oxygen species (ROS) that alter their transcriptional factor profile (144). In contrast, Estrada et al. showed that low oxygen levels enhance cell proliferation and genetic stability by favoring a natural metabolic state of increased glycolysis and reduced oxidative phosphorylation (145). Therefore, further studies should carefully consider the long-term effects of hypoxia primed MSCs in models of disease.

2.4. Induction of specific functional MSC phenotypes

One of the main therapeutic mechanisms of MSCs at the target site is to immunomodulate local responses (80, 146). However, depending on the molecular composition of the instructive environment at the injury site, interacting MSCs exhibit a therapeutic responsive polarization into either anti-inflammatory (MSC-2) or pro-inflammatory (MSC-1) phenotypes, tightly coupled to M2/M1 macrophage skewing (147, 148). Toll-like receptors (TLRs) are the most studied pattern recognition receptor family in MSCs, which sense pathogen-associated molecules involved in the regulation of innate immunity. Previous studies indicate that human MSCs consistently express TLR1, TLR2, TLR3, TLR4, TLR5, and TLR6, whereas TLR7, TLR8, TLR9, and TLR10 expression is dependent on the MSC origin (149-153). As elaborated below, several studies indicate that activation of specific TLRs in MSCs *in vitro* prior transplantation has a profound effect on the immunomodulatory capacities of MSCs. TLR3 and TLR4 are particularly important for MSCs' downstream effects, which can be *in vitro* activated by dsRNA mimetic polyinosinic-polycytidylic acid (*i.e.,* poly(I:C)) and lipopolysaccharide (LPS), respectively (147). Waterman et al. showed that TLR3 stimulation of MSCs supports their immunosuppressive

effects preferentially through enhanced fibronectin deposition, whereas TLR4 stimulation of MSCs provides a more pro-inflammatory signature in part through release of TGF-β and collagen deposition (147). In that respect, low-dose of poly(I:C) for 24h results in TLR3 activation and induction of VEGF, CXCL12, IL-6, IL-10, IL-11, leukemia inhibitory factor (LIF) and HGF without upregulation of inflammatory cytokines (154). Therefore, it is not surprising to observe that injection of poly(I:C)-treated MSCs in a hamster heart failure model, resulted in cardiac functional improvement with a 50% reduction in myocardial fibrosis, 40% reduction in apoptosis and 55% increase in angiogenesis (154). On the other hand, a recent study showed that both TLR3 and TLR4 activation comparably enhance MSC-mediated T regulatory cells (i.e., Tregs) induction through Notch signaling and upregulation of Delta-like 1 (DL1), critical cellular mechanisms for the immunomodulatory properties of MSCs (155). In a rat acute myocardial infarction model, Yao et al. showed that LPS-dependent priming results in increased MSC survival post-transplantation *in vivo,* coupled with reduced fibrosis of the infarcted myocardium, increased neovascularization and earlier recovery of the cardiac function (156). Transcriptional profiling of LPS-primed MSCs showed that several chemokines, cytokines and adhesion molecules were highly up-regulated including *CXCL10*, *CCL20*, *IL8*, *CXCL1*, *IL6*, *CCL2*, *IL1B*, *CXCL2*, *IL1A*, *CXCL6, ICAM1, VCAM1,* and *SELE* (157).

Different studies have indicated contrasting effects of TLR activation on MSC multi-differentiation potential. In a comparative study performed by Raicevic et al., TLR activation of MSCs with post-natal and perinatal origin resulted in differential osteogenic potential depending on MSC tissue of origin (158). TLR2 and TLR4 activation in umbilical cord blood-derived MSCs promote chondrogenesis and osteogenesis with different intensities, whereas adipogenic differentiation is not altered by such TLR activation (159). MSCs harvested from the umbilical cord showed differential responses in terms of osteogenic potency, with TLR4 activation increasing and TLR3 activation decreasing it, respectively (160). On the contrary, both TLR3 and TLR4 downstream signaling promote BM-MSCs' osteogenic potency (161), through activation of Wnt3α and Wnt5a signaling (TLR4) (162). Several groups have reported comparable increased osteogenic potency with activation of TLR2-, TLR3, TLR4- primed adipose tissue MSCs (ASCs) with unaffected adipogenesis (149, 163). A novel pattern recognition receptor, the triggering receptor expressed on myeloid cells (TREM), identified to regulate myeloid cells function *in vitro* (164), has been associated also with MSCs. Zhang et al. indicated that one of TREM family members, TREM-2, is expressed in MSCs and its knockdown reduces TLR2, TLR4, and TLR6 expression, impairing MSCs' multi-differentiation potential (165). Thus, we could speculate that TREM-2 ligands would induce TLR-specific responses in favor of cell differentiation.

2.5. Priming MSCs in 3D spheroid cultures

Adult MSCs possess a remarkable ability to coalesce and assemble in tri-dimensional (3D) structures, reminiscent of their innate aggregation as limb cell precursors in the mesenchymal condensation during early skeletogenesis. In that context, 3D organoid formation *in vitro* closely recapitulates the *in vivo* MSC niche by providing spatial cell organization with increased cell-cell interactions. As a matter of fact, MSCs cultured in 3D spheroid cultures show stable immuno-phenotypic profile, with a significant enhancement in survival (166), homing (167), stemness features (168, 169), differentiation potential (168, 169), angiogenic effect (166) and anti-

inflammatory properties(170). For example, in a mouse model of hind limb ischemia, MSC transplantation as 3D spheroids improved their survival compared with 2D expanded MSCs, by suppressing a key apoptotic signaling molecule (Bax), while activating anti-apoptotic signaling (BCL-2) (166). These positive effects can also be attributed to improved resistance to oxidative stress-induced apoptosis exerted by hypoxia-induced genes (*e.g.,* VEGF-A, HIF-1α and MnSOD), elevated by the hypoxic conditions at the spheroid core (171, 172).

Additional benefits of 3D cultures account for the established reduction in size of individual MSCs (about 0.25 to 0.5 the volume of an average 2D cultured cell), which reduce cell entrapment in the lungs when systemically infused (170). MSCs stemness features are also improved in 3D MSC spheroid cultures, evidenced by: 1) higher expansion and colony-forming activities (169); 2) enhanced differentiation capacities (168, 173, 174); and 3) changes in the epigenetic status of genes indicative of a more pluripotent nature (*NANOG, SOX2, OCT4*) (168). As expected given the presence of a stimulating variable oxygen tension within the spheroids, angiogenic properties of MSCs are positively affected by 3D spheroid priming. This trophic enhancement is produced via upregulation of key angiogenic factors including VEGF, HGF, FGF-2, angiogenin (ANG), and angiopoietin 2 (ANGPT-2) (167, 172, 175). Murphy et al. showed that MSC spheroids embedded in fibrin gel secrete up to 100-fold more VEGF compare to dissociated MSCs in fibrin gel (176). Additionally, other groups have reported an increased homing capacity of MSC spheroids through a significant upregulation in the expression of the CXCL12 chemokine receptor CXCR4 (167, 169). Enhanced anti-inflammatory effects of 3D cultures have been reported by previous studies indicating that MSC spheroid highly express TGF-β1, IL-6, TSG-6, stanniocalcin (STC-1), and PGE-2 anti-inflammatory molecules (170, 177, 178). As mentioned earlier, MSCs acquire effective anti-inflammatory properties after being primed with pro-inflammatory cytokines. Interestingly, Bartosh et al. showed that MSC aggregation into 3D spheroids activates the expression of IL-1 in an autocrine secretion manner, thus initiating an “auto-priming” effect (170). Contrarily, Redondo-Castro et al. reported that the combination of IL-1 stimulation with spheroid priming resulted in significantly increased expression of IL1-Ra, VEGF and G-CSF molecules without anti-inflammatory effects on LPS-treated microglial cells in co-cultures (179). The discrepancies of the data underline the necessity for optimization of the priming methods and culture conditions. As a recent effort, Ylostalo et al. proposed specific protocols to efficiently prime MSCs in 3-D cultures under chemically defined xeno-free conditions and how to administer the primed MSCs *in vivo* (180).

Finally, MSC-based 3D spheroids have been applied in various preclinical models including wound healing (171, 181), bone and osteochondral defects (182-184) and cardiovascular diseases (185, 186). Two separate groups applied MSC spheroids for wound healing in chemotherapy-induced oral mucositis (171) and in a model of diabetic healing impaired (leptin receptor-deficient mice) (181). In both cases MSC spheroid group provide better therapeutic efficacy compared with traditional MSC suspension group. Using a rat calvarial defect model, MSC spheroids implantation resulted in full-thickness bone formation that efficiently filled the generated bone defects (184). Intramyocardial transplantation of MSC spheroids in rat (185) and porcine (186) myocardial infarction models resulted in greater heart function improvement compared with MSC suspensions.

Collectively, MSC priming strategies aim to yield cellular products of high quality and potency by enhancing homing, survival, stemness, differentiation, anti-inflammatory and other MSC properties (Figures 2

and 3). As mentioned before, various methods exist to manipulate MSC properties that possess advantages and disadvantages mainly related to the quality of the cellular product (Table 7).

3. Clinical studies using functionalized MSCs

Up-to-date no clinical trials have been executed to evaluate the efficacy of MSC subpopulations. On the other hand, three clinical trials are currently registered in www.clinicaltrials.gov using primed MSC. Despite the fact that various priming methods have been developed to enhance MSC properties *in vitro*, some with preclinical validation, the current clinical studies center on hypoxia/ischemia as the priming method to enhance MSC trophic properties. The phase 3 'STARTING-2' study (NCT01716481) evaluates the efficacy of intravenously infused autologous BM-derived MSCs pre-conditioned with autologous "ischemic serum" to treat stroke patients. A phase 1/2 study (NCT01849159) investigates the effectiveness of intravenously infused allogenic BM-derived MSCs primed *in vitro* under 1% hypoxia to regenerate the lungs of patients suffering from pulmonary emphysema. Finally, the 'TPAABPIHD' phase 1/2 study (NCT02504437) investigates hypoxia-primed autologous BM-derived MSCs for their effectiveness to treat patients with ischemic heart diseases. Consequently, additional clinical research is needed to assess the safety and efficacy of "functional enhancing" strategies to MSC before administration, including cell selection and priming protocols.

4. Conclusions

It has become increasingly clear that current MSC culture-expansion methods, although proven to be clinically safe, do not guarantee the preservation of specific native MSC characteristics and attributes, thus yielding cell-based products of variable quality, and more importantly, potency. This in addition to inherent differences secondary to various factors including: origin of the cells (i.e., autologous or allogeneic), donor age and underlying pathological condition(s), and the recipient's implantation site microenvironment. The resulting compromised standardization may account for the observed inconsistent therapeutic outcomes. Therefore, obtaining more homogeneous MSC preparations (e.g., through selection and/or induction by priming) in which the critical features are preserved may help circumvent the lack of reproducibility while enhancing their therapeutic effects (Figure 3). Thus, based on pre-clinical data, MSC subpopulation(s) selection and priming protocols may offer therapeutic advantages compared with the use of bulk/heterogeneous preparations in a number of clinical indications.

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FIGURE LEGENDS:

Figure 1: MSC topography within bone marrow. All BM MSCs are $CD271^{+}$, whereas CD146 and CD56 help discriminate their differential presence within two distinct anatomical regions of the BM: 1) the perivascular niche ($CD146^{+}$) where they interact with blood vessels (arterioles and sinusoids), and 2) the bone-lining niche ($CD146^{Neg}$ $CD56^{+}$) where they interact with cells of the osteoblastic lineage (quiescent bone lining cells and active osteoblasts in places of active remodeling).

Figure 2: MSC priming strategies include membrane modification, cytokine and hypoxia priming, Toll-like receptor priming, and 3D spheroid priming

Figure 3: MSC functionalization by priming in 2D and 3D cultures results in boosted homing, immunomodulatory and trophic effects *in vivo*.

Table 1. Membrane and cytoplasmic MSC priming

PRIMING METHOD	REFERENCE	TYPE OF CELLS	TYPE OF PRIMING	PRECLINICAL MODEL	OUTCOME IN VIVO
Membrane priming	Xia L et al., 2004 (197)	Human umbilical cord blood CD34+ cells	Enzymatic functionalization with selectin-binding motifs	Non-obese diabetic/severe combined immune deficiency (NOD/SCID) mouse model	Systemically administered functionalized-$CD34^+$ cells showed increased bone marrow homing
Membrane priming	Sackstein R et al., 2008 (106)	Human BM MSCs	Enzymatic convertion of native CD44 into haematopoietic cell E-/L-selectin ligand (HCELL)	Non-obese diabetic/severe combined immune deficiency (NOD/SCID) mouse model	Intravenously infused HCELL (+) MSCs infiltrated marrow within hours of infusion
Cytoplasmic priming	Kobayashi T et al., 2008 (117)	Human BM MSCs	MSCs labeled with internalized superparamagnetic iron oxide nanoparticles (SPIONs)	Rabbit and Swine osteochondral defect models	Local knee administration of SPIONs-labelled MSCs showed increased homing at the osteochondral defect sites
Membrane priming	Ghanem A et al., 2009 (114)	Human BM MSCs	Combination of BM MSCs with ultrasound mediated stimulation of micro-bubbles	Acute myocardial ischemia and reperfusion rat model	MSCs migrate across the myocardial endothelium whereas micro-bubbles stimulation improves MSC myocardial engraftment and allows locoregional targeting of post-ischemic myocardium
Membrane priming	Sarkar D et al., 2011 (111)	Human BM MSCs	Conjugation of the sialyl $Lewis^x$ (sLe^x) moiety to MSCs' surface through biotin-streptavidin	Ear inflammation BALB/C mouse model	Systemically administered SLe^x engineered MSCs exhibited a robust rolling response on inflamed endothelium
Membrane priming	Deng W et al., 2011 (113)	Mouse BM MSCs	Combination of BM MSCs with anti-CD29/anti-myosin light chain bispecific antibodies and ultrasound mediated stimulation of micro-bubbles	Isoproterenol-induced myocardial fibrosis mouse model	The homing rate to injured myocardium and repairing efficacy of systemically administered MSCs improved
Cytoplasmic priming	Yanai A et al., 2012 (115)	Rat BM MSCs	MSCs labeled with internalized superparamagnetic iron oxide nanoparticles (SPIONs)	Retinal degeneration rat model	Increased retina homing of SPIONs-labelled MSCs after both systemic and intravitreal infusions under magnetic field
Cytoplasmic priming	Landázuri N et al., 2013 (116)	Human BM MSCs	MSCs labeled with internalized superparamagnetic iron oxide nanoparticles (SPIONs)	Mouse model	Systemically administered SPIONs-labelled MSCs showed increased tail homing under magnetic field

Table 2. Cytokine MSC priming

PRIMING METHOD	REFERENCE	TYPE OF CELLS	TYPE OF PRIMING	PRECLINICAL MODEL	OUTCOME IN VIVO
Cytokine priming	Shi M et al., 2007 (118)	Human fetal BM MSCs	MSC priming with SCF, HGF, IL-3 and IL-6 cocktail	Sublethally irradiated non-obese diabetic/severe combined immune deficiency (NOD/SCID) mouse model	Short-term (24 hours) MSC priming with the cytokine cocktail resulted in up-regulation of CXCR4 and increased MSC homing to the BM post-intravenous infusion
Cytokine priming	Hahn JY et al., 2008 (198)	Rat BM MSCs	MSC priming with FGF-2, IGF-1 and BMP-2 cocktail	Myocardial infarction rat model	Local transplantation of cytokine primed MSCs resulted in enhanced gap junction formation, smaller infarct size and better cardiac function than untreated MSC group
Cytokine priming	Fan H et al., 2012 (119)	Human Umbilical cord MSCs	IL-1β-primed MSCs	Dextran sulfate sodium (DSS)-induced ulcerative colitis mouse model	Intravenously infused IL-1β-primed MSCs modulated the balance of immune cells in the spleen and the mesenteric lymph nodes (MLNs) and possessed an enhanced homing capacity to inflammatory gut site via upregulation of CXCR4
Cytokine priming	Duijvestein M et al., 2011 (121)	Human BM MSCs	Interferon-γ (IFN-γ)-primed MSCs	Dextran sodium sulfate (DSS)- and trinitrobenzene sulfonate (TNBS)-induced ulcerative colitis mouse model	Intraperitoneally infused IFNγ-primed MSCs show upregulated CXCR7, LGALS3BP, LGALS9 expression and increased homing to the inflamed intestines
Cytokine priming	Nasir GA et al., 2013 (125)	Mouse BM MSCs	Hepatic microenvironment priming with IL-6 intraperitoneal injections combined with MSC transplantation	Carbon tetrachloride (CCl_4) fibrotic liver mouse model	*In vivo* hepatic microenvironment priming with IL-6 resulted in increased MSCs homing to fibrotic liver and reduced fibrosis and apoptosis post-local liver MSC transplantation
Cytokine priming	Cheng W et al., 2017 (199)	Rat BM MSCs	IL-25-primed MSCs	Dextran sodium sulfate (DSS)- induced ulcerative colitis rat model	Intravenously infused IL-25-primed MSCs exert improved therapeutic effects on the intestinal inflammation which may be related to the inhibition of Th17 immune response and induction of T regulatory cell phenotype
Cytokine priming	Aktas E et al., 2017 (200)	Rat BM MSCs	TNF-a primed MSCs	Achilles tendon segmental defect rat model	- TNF-a-primed MSCs reduced IL-12 production and the number of M1 macrophages, whereas increased the percent of M2 macrophages, and synthesis of the anti-inflammatory factor IL-4 - Concentration of type I procollagen in the healing tissue and failure stress of the tendon were increased 4 weeks post-injury
Cytokine priming	Guess AJ et al., 2017 (201)	Human and Mouse BM MSCs	Interferon-γ (IFN-γ)-primed MSCs	Total body irradiated mouse model	Intravenously infused IFNγ-primed MSCs supported hematopoietic reconstitution without evidence of organ toxicity or tumor formation
Cytokine priming	Kim DS et al., 2018 (202)	Human BM MSCs	Interferon-γ (IFN-γ)-primed MSCs	Graft-versus-host disease in an immune deficiency (NOD/SCID) mouse model	Intravenously infused IFNγ-primed MSCs effectively decreased clinical symptoms and immune cell infiltration into the skin and small intestine of GVHD mice, improving their survival rate

Table 3. Hormonal MSC priming

PRIMING METHOD	REFERENCE	TYPE OF CELLS	TYPE OF PRIMING	PRECLINICAL MODEL	OUTCOME IN VIVO
Hormonal priming	Mias C et al., 2009 (129)	Rat BM MSCs	Melatonin priming	Acute kidney injury rat model	Intra-parenchymal injection of melatonin primed MSCs resulted in increased MSC survival, angiogenesis, proliferation of renal cells and accelerated renal recovery
Hormonal priming	Kim YS et al., 2012 (127)	Human UC MSCs	Oxytocin priming	Myocardial infarction rat model	Intramyocardial injection of oxytocin primed MSCs reduced cardiac fibrosis and macrophage infiltration and enhanced cardiac repair for at least 4 weeks post-MSC transplantation *in vivo*
Hormonal priming	Tang Y et al., 2014 (203)	Rat BM MSCs	Melatonin priming	Focal cerebral ischemia rat model	Melatonin primed MSCs were stereotactically injected into the striatum of the ipsilateral hemisphere. Melatonin MSC priming increased MSC survival *in vivo*, whereas melatonin primed MSCs reduced brain infarction, increased angiogenesis and neurogenesis.
Hormonal priming	Liu C et al., 2015 (131)	Rat BM MSCs	Angiotensin-II priming	Myocardial infarction rat model	Angiotensin-II hormone MSC priming results in reduced cardiac fibrosis and infarct size, improved cardiac function, increased expression of VEGF and von Willebrand factor in the ischemic myocardium but no MSC differentiation towards cardiomyocytes after intra-myocardial injection *in vivo*

Table 4. Hypoxia MSC priming

PRIMING METHOD	REFERENCE	TYPE OF CELLS	TYPE OF PRIMING	PRECLINICAL MODEL	OUTCOME IN VIVO
Anoxia priming	Uemura R et al., 2006 (142)	Mouse BM MSCs	Hypoxic gas mixture MSC priming	Myocardial infarction mouse model	Anoxia primed MSCs improved infarcted heart function via enhanced survival of implanted MSCs and prevent left ventricular remodeling of ischemic heart through MSC paracrine signaling
Hypoxia priming	Hung SC et al., 2007 (137)	Human BM MSCs	Hypoxic gas mixture MSC priming	Xenotypic grafting early chick embryo model	Hypoxia primed MSCs showed increased homing capacity into early chick embryos and generated a variety of cell types in host tissues
Hypoxia priming	Rosová I et al., 2008 (135)	Human BM MSCs	Hypoxic gas mixture MSC priming	Non obese diabetic/severe combined immune deficiency (NOD/SCID) hind limb ischemia mouse model	Intra-arterial injected hypoxia primed MSCs increased locally muscle revascularization
Hypoxia priming	Hu X et al., 2008 (143)	Mouse BM MSCs	Hypoxic gas mixture MSC priming	Myocardial infarction mouse model	Hypoxia primed MSCs improve infarcted heart function via enhanced survival of implanted MSCs and increased angiogenesis
Anoxia priming	Li JH et al., 2008 (144)	Rat BM MSCs	Hypoxic gas mixture MSC priming	Diabetic cardiomyopathy rat model	Anoxia primed MSCs improved cardiac function through anti-apoptotic effects and attenuation of cardiac remodeling
Hypoxia priming	Hu X et al., 2011 (140)	Mouse BM MSCs	Hypoxic gas mixture MSC priming	Myocardial infarction rat model	Intravenously injected hypoxia primed MSCs showed increased homing capacity to injured myocardium
Hypoxia priming	Wei L et al., 2012 (138)	Rat BM MSCs	Hypoxic gas mixture MSC priming	Transient cerebral ischemia rat model	Intravenously injected hypoxia primed MSCs showed increased homing to the brain and resulted in enhanced angiogenesis and neurogenesis
Hypoxia priming	Wei N et al., 2013 (139)	Rat BM MSCs	Hypoxic gas mixture MSC priming	Focal cortex ischemic stroke mouse model	Intranasal administration of hypoxia primed MSCs resulted in increased homing and neuroprotective effects to the ischemic brain region
Hypoxia priming	Zhang W et al., 2014 (145)	Human AT MSCs	Hypoxic gas mixture MSC priming	Acute renal ischemia/reperfusion injury rat model	Hypoxia primed MSCs attenuate renal injury through enhanced angiogenic and antioxidative capacities
Hypoxia priming	Overath JM et al., 2016 (146)	Mouse AT MSCs	Hypoxic gas mixture MSC priming	Cisplatin-induced acute kidney injury mouse model	Hypoxia primed MSCs results in improved renal function and reduced levels of pro-inflammatory cytokines
Hypoxia priming	Liu YY et al., 2017 (204)	Rat BM MSCs	Hypoxic gas mixture MSC priming	Ischemia-reperfusion lung injury rat model	Hypoxia primed MSCs migrate into extravascular lung tissue and attenuate I/R lung injury through anti-oxidant, anti-inflammatory and anti-apoptotic mechanisms
Hypoxia priming	Kim YH et al., 2018 (205)	Human UCB MSCs	Hypoxic gas mixture MSC priming	Graft-versus-host disease in an immune deficiency (NOD/SCID) mouse model	Intravenously injected hypoxia primed MSCs resulted in significant improved survival, less weight loss, and reduced histopathologic injuries in GVHD target organs

Table 5. TLRs MSC priming

PRIMING METHOD	REFERENCE	TYPE OF CELLS	TYPE OF PRIMING	PRECLINICAL MODEL	OUTCOME IN VIVO
TLRs priming	Yao Y et al., 2009 (160)	Mouse BM MSCs	Lipopolysaccharide priming	Myocardial infarction rat model	LPS MSC priming results in increased MSC survival post-transplantation *in vivo* and as a result reduced fibrosis of infarcted myocardium, neovascularization and recovery of the cardiac function
TLRs priming	Mastri M et al., 2012 (158)	Porcine BM MSCs	Polyinosinic-polycytidylic acid priming	Cardiomyopathy hamster model	Intramuscular injection of poly(I:C) primed MSCs resulted in cardiac functional improvement with a 50% reduction in myocardial fibrosis, 40% reduction in apoptosis and 55% increase in angiogenesis
TLRs priming	Waterman RS et al., 2012 (206)	Human BM MSCs	Lipopolysaccharide priming	Epithelial ovarian cancer mouse model	Intraperitoneal injection of LPS primed MSCs (MSC1 phenotype) attenuated tumor growth and metastasis
TLRs priming	Fuenzalida et al., 2016 (207)	Human UC MSCs	Polyinosinic-polycytidylic acid priming and Lipopolysaccharide priming	Dextran sodium sulfate (DSS)- induced ulcerative colitis mouse model	- Intraperitoneal injection of poly(I:C) primed MSCs ameliorated the clinical and histopathological severity of the disease - Intraperitoneal injection of LPS primed MSCs significantly increased clinical signs of disease, colon shortening and histological disease index
TLRs priming	Qui Y et al., 2017 (208)	Human UC MSCs	Polyinosinic-polycytidylic acid priming	Trinitrobenzene sulfonic acid-induced colitis mouse model	- Intraperitoneal injection of poly(I:C) primed MSCs decreased a wide range of inflammatory cytokines and increased systemic interleukin-10 (IL-10) levels in colonic tissues - Poly(I:C) primed MSCs also impaired T-helper type 1/17 (Th1/17) cell expansion and enhanced the suppressive effects of regulatory T cells

Table 6. 3D spheroid MSC priming

PRIMING METHOD	REFERENCE	TYPE OF CELLS	TYPE OF PRIMING	PRECLINICAL MODEL	OUTCOME IN VIVO
3D spheroid priming	Wang CC et al., 2009 (189)	Rat BM MSCs	Spheroid MSC culturing	Acute myocardial infarction rat model	Intramyocardial injection of MSC spheroids showed superior heart function than MSC suspensions by increasing vascular density
3D spheroid priming	Bartosh TJ et al., 2010 (174)	Human BM MSCs	Spheroid MSC culturing	Peritonitis mouse model and NOD/SCID mouse model	- Intraperitoneal injection of MSC spheroids was more effective than MSCs from adherent monolayer cultures in suppressing inflammatory responses Spheroid MSCs - Intravenously injected MSC spheroids show better trafficking through the lungs and homing to spleen, liver, kidney, and heart due to their smaller volume from adherent cultures
3D spheroid priming	Amos PJ et al., 2009 (185)	Human AT MSCs	Spheroid MSC culturing	Full-thickness diabetic mouse wound model	Transplantation of MSC spheroids to the diabetic wounds resulted in significant increase in the rate of wound closure compared to wounds treated with an equal number of MSCs delivered in suspension
3D spheroid priming	Ma D et al., 2011 (186)	Rabbit BM MSCs	Spheroid MSC culturing	Ectopic bone formation nude mouse model and Orthotopic bone formation mandible rabbit model	- Subcutaneously injected MSC spheroids showed significantly larger and denser ectopic bone at the injection sites than the MSCs suspension group - MSC spheroids' orthotopic injection locally at the mandibular fracture gap results in increased bone healing
3D spheroid priming	Bhang SH et al., 2012 (170)	Human umbilical cord blood MSCs	Spheroid MSC culturing	Hindlimb ischemia mouse model	Intramuscularly injected MSC spheroids showed better survival compared to 2D expanded MSCs by suppressing apoptotic signaling (Bax molecule) and activating anti-apoptotic signaling (BCL-2 molecule)
3D spheroid priming	Cheng NC et al., 2012 (173)	Human AT MSCs	Spheroid MSC culturing	Hindlimb ischemia mouse model	Intramuscularly injected MSC spheroids showed higher cellular retention
3D spheroid priming	Zhang Q et al., 2012 (175)	Human gingiva MSCs	Spheroid MSC culturing	Chemotherapy-induced oral mucositis mouse model	Intraperitoneal injected MSC spheroids possessed better therapeutic efficacy than their adherent cells in reversing body weight loss and promoting the regeneration of disrupted epithelial lining of the mucositic tongues
3D spheroid priming	Suzuki S et al., 2012 (187)	- Human synovial MSCs - Rabbit synovial MSCs	Spheroid MSC culturing	Full-thickness osteochondral defect rabbit model	- Transplantation of MSC spheroids at osteochondral defect sites resulted in their prompt adherence by surface tension, without any loss. - MSC spheroid transplantation at relatively low density achieved successful cartilage regeneration
3D spheroid priming	Suenaga H et al., 2015 (188)	Human BM MSCs	Spheroid MSC culturing	Calvarial bone defect rat model	Locally transplanted MSC spheroids resulted in significant bone remodeling
3D spheroid priming	Xu Y et al., 2016 (209)	Human AT MSCs	Spheroid MSC culturing	Ischemia/reperfusion-induced acute kidney injury rat model	Injection into the kidney cortex of MSC spheroids protected the kidney against apoptosis, reduced tissue damage, promoted vascularization and ameliorated renal function
3D spheroid priming	Lee JH et al., 2016 (210)	Human AT MSCs	Spheroid MSC culturing	Hindlimb ischemia mouse model	Intramuscularly injected MSC spheroids showed better proliferation than their adherent cells

Table 7. Advantages and disadvantages of different MSC priming methods

PRIMING METHOD	ADVANTAGES	DISADVANTAGES
Cytokine and hormonal	• Increased survival, homing capacity and migratory behavior • Enhanced anti-inflammatory MSC phenotype	• Use of pro-inflammatory priming conditions *in vitro* that may affect the MHC class I and class II levels pre-implantation in *vivo*
Hypoxia	• Increased homing capacity, migratory behavior and vascularization	• Accumulation of reactive oxygen species • Putative negative effect on MSC differentiation capacity
Immunomodulatory/functional	• Increased MSC polarization into anti-inflammatory (MSC-2) phenotype • Enhanced production of immunoregulatory molecules • Increased survival, homing and migratory capacities	• Toll-like receptors (TLRs) activation influence MSC differentiation capacities in an MSC tissue of origin-dependent manner
3D spheroid cultures	• Increased stability of MSC immuno-phenotypic profile • Enhanced survival, homing, stemness features, differentiation potential, angiogenic effect and anti-inflammatory properties	• Size variability for effective *in vivo* implantation • Depending on spheroid size, limited diffusion of nutrients and oxygen in 3D spheroid core • Depending on spheroid size, necrotic spheroid core

FIGURE 1 KOUROUPIS ET AL

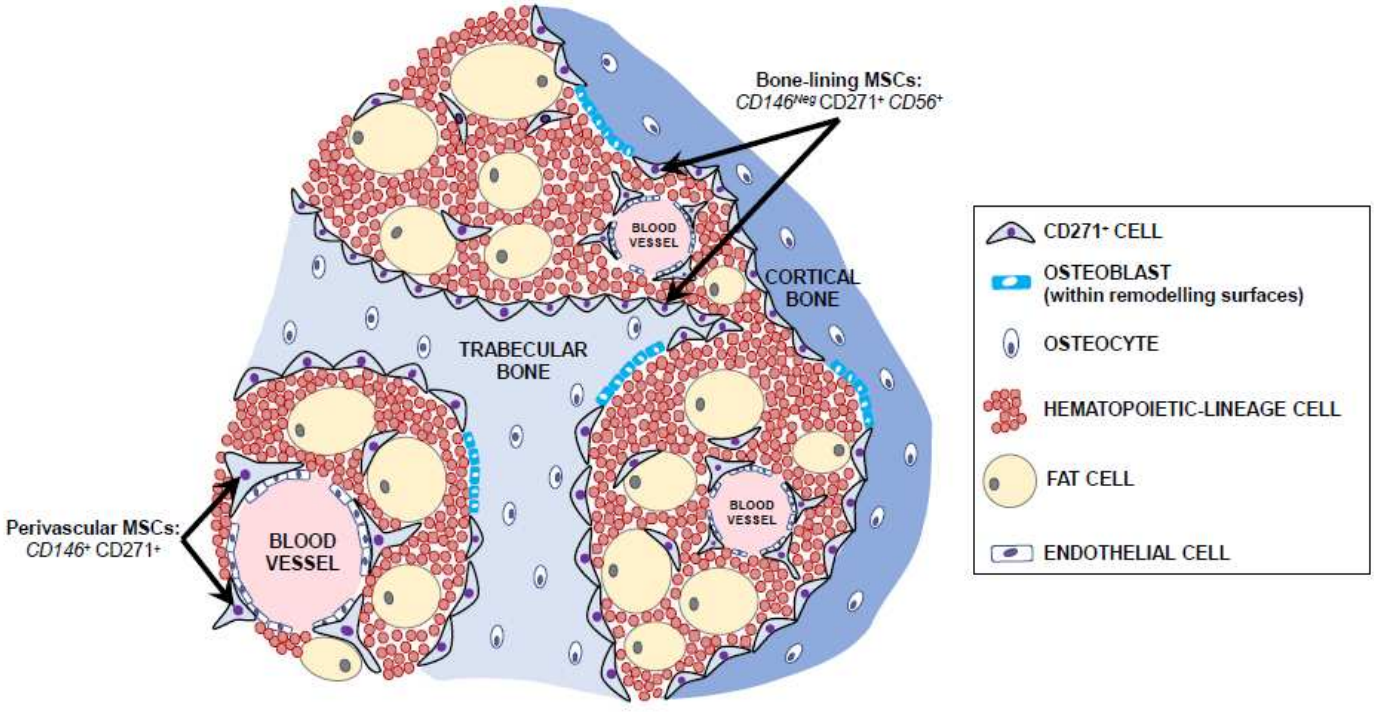

FIGURE 2

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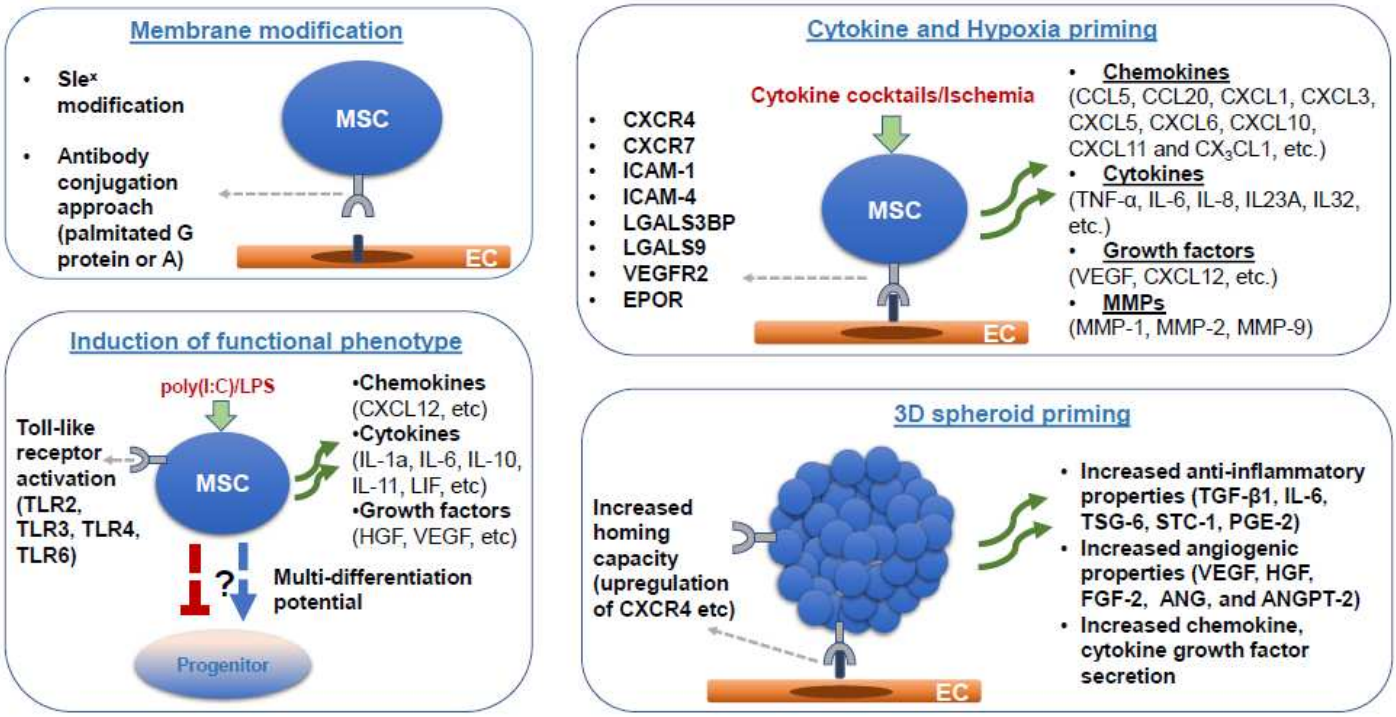

FIGURE 3

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