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Different priming strategies improve distinct therapeutic capabilities of mesenchymal stromal/stem cells: Potential implications for their clinical use

Miceli V *et al*. Priming strategies improve MSC properties

Abstract

Mesenchymal stromal/stem cells (MSCs) have shown significant therapeutic potential, and have therefore been extensively investigated in preclinical studies of regenerative medicine. However, while MSCs have been shown to be safe as a cellular treatment, they have usually been therapeutically ineffective in human diseases. In fact, in many clinical trials it has been shown that MSCs have moderate or poor efficacy. This inefficacy appears to be ascribable primarily to the heterogeneity of MSCs. Recently, specific priming strategies have been used to improve the therapeutic properties of MSCs. In this review, we explore the literature on the principal priming approaches used to enhance the preclinical inefficacy of MSCs. We found that different priming strategies have been used to direct the therapeutic effects of MSCs toward specific pathological processes. Particularly, while hypoxic priming can be used primarily for the treatment of acute diseases, inflammatory cytokines can be used mainly to prime MSCs in order to treat chronic immune-related disorders. The shift in approach from regeneration to inflammation implies, in MSCs, a shift in the production of functional factors that stimulate regenerative or anti-inflammatory pathways. The opportunity to fine-tune the therapeutic properties of MSCs through different priming strategies could conceivably pave the way for optimizing their therapeutic potential.

Key Words: Mesenchymal stromal/stem cells; Mesenchymal stromal/stem cell therapeutic properties; Mesenchymal stromal/stem cell paracrine effects; Mesenchymal stromal/stem cell priming; Pro-inflammatory priming; Hypoxic priming, 3D culture priming

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Core Tip: Mesenchymal stromal/stem cells (MSCs) have demonstrated promising therapeutic results in the field of regenerative medicine. However, due to their heterogeneity, the application of MSCs in clinical trials has shown moderate or poor efficacy. Here, we review data on the principal priming approaches for enhancing the therapeutic potential of MSCs. We found that different priming strategies can modify MSC properties and, in this case some therapeutic effects on different disease models can be obtained in relation to dose and/or combination of the priming factors used. The production of priming type-specific functional factors in MSCs could pave the way toward implementing new MSC-based therapies.

INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) are multipotent adult stem cells involved in the homeostasis of tissue regeneration and, because of their therapeutic potential, have been extensively investigated in various clinical conditions[1-6]. Though MSC treatment was initially thought to promote tissue regeneration thanks to MSC multipotency of differentiation[7-9], recent evidence has revealed that the efficacy of MSC-based therapies is, at least in part, linked to the production of functional paracrine factors. These cells are able to secrete numerous products, *e.g.*, growth factors, cytokines, chemokines, and extracellular vesicles (EVs), which can regulate many pathophysiological processes, such as fibrosis, immune dysregulation, angiogenesis, and stimulation of tissue resident stem cells, in order to coordinate both tissue regeneration and functional recovery[10-12]. In injured tissue, MSC engraftment is limited because they undergo cell death, and their beneficial effects are exerted through secretion of various functional factors that not only enhance the function of resident cells, but also attract immune and progenitor cells, contributing to the coordination of tissue repair[13,14]. Therefore, considering the importance of the paracrine component in mediating MSC functions, there is growing interest in the molecular basis of MSC secretion involved in the therapeutic function of these cells.

Over the years, a large number of tissues, including placenta, adipose, umbilical cord, dental pulp, bone marrow, synovial membrane, liver and others, have been used as a source of MSCs[15-20]. It is quite clear that MSCs derived from all these

sources possess a wide variety of functional effects, which they apply physiologically to their own original tissue, regulating homeostasis and regeneration. Interestingly, these effects may be useful for therapeutic applications of MSCs[3,21]. Currently, there are 1487 clinical trials registered at clinicaltrials.gov aimed at studying MSC therapeutic efficacy in the treatment of several clinical disorders, including lung, liver, kidney, orthopedic, cardiovascular, neurodegenerative, and immune diseases. In different clinical settings, MSC-therapies have been tested, showing tolerable safety, and demonstrating therapeutic benefits, and this has led to regulatory approvals of some MSC-based therapeutic products in several countries. In 2012, Cartistem, a MSC product based on the use of umbilical cord-derived MSCs for the treatment of traumatic or degenerative osteoarthritis, was approved by Korea's Ministry of Food and Drug Safety[22]. Moreover, Remestemcel-L, based on the use of bone marrow-derived MSCs (BM-MSCs), has been investigated in a phase 3 clinical trial in patients with steroid-refractory acute graft-versus-host disease (GVHD)[23]. Recently, due to the immunomodulatory properties of Remestemcel-L, which are able to work against cytokine storm linked to several inflammatory conditions, this therapy has also been tested for the treatment of coronavirus disease 2019-associated multisystem inflammatory syndrome[24]. The increasing interest in the clinical applications of MSCs as a cellular therapy has also been evidenced by the burgeoning of several companies that sell MSC therapies to United States clinics[25]. However, this has highlighted that in some cases the propensity for economic gain has outweighed the clinical advantages, despite the lack of solid scientific evidence that supports the broad use of MSCs in treating various human disorders. Indeed, in many clinical trials it has been shown that MSCs have moderate or poor efficacy, and the results from some studies are controversial[26-31]. In particular, due to both the inconsistent criteria used for the MSC identity across studies, and MSC heterogeneity, which depends on the different MSC origin[32] and the diverse harvesting and culture strategies[33], the clinical results obtained after MSC therapy are frequently variable. This makes it very difficult to obtain reliable conclusions regarding MSC therapeutic

efficacy. Thus, while MSCs demonstrate a good margin of safety as cellular treatment, they have usually been therapeutically ineffective in humans[21].

These issues have underscored the urgent need to optimize the clinical use of MSCs or enhance MSC therapeutic effects. After determining the most appropriate cell source to use both in terms of invasiveness for cell isolation and cell yield, specific standardized production methods are needed to ensure MSC therapeutic abilities and, therefore, their clinical efficacy. MSCs can be considered a key regulatory component in the tissue stem cell niche and, starting with the physiological role that these cells play in regulating tissue regeneration following injury[3,4,6,34-39], specific priming strategies can be understood and adapted for MSC clinical application. In this regard, much attention has been paid to the opportunity of MSC pre-conditioning to prime the cells before their clinical use. In this case, the therapeutic properties of MSCs can be modulated by pre-treatment of cells with hypoxia, cytokines, as well as growing MSCs under three-dimensional (3D) culture. In those instances, in response to MSC priming, the phenotype of MSCs was switched toward an anti-inflammatory, pro-trophic and more regenerative potential, which results in an enhanced therapeutic function of the cells[3,40-45].

In this review, we summarize the principal priming methods aimed at improving MSC efficacy as a therapeutic product. We would also like to highlight the fact that specific priming strategies can be considered more suitable for some types of diseases, leading to new therapeutic approaches that could be used to develop more powerful and predictable MSC therapies.

THE SECRETION OF PARACRINE FACTORS MEDIATE THE THERAPEUTIC FUNCTION OF MSCS

The secretion of functional products is central to MSC-based therapy, as demonstrated in numerous studies. Indeed, individual components of MSC secretome, such as functional proteins and EVs, are involved in the regulation of various biological processes, including angiogenesis, immunoregulation, wound healing, and tissue repair/protection[14,46-49]. Among the MSC-derived functional products, exosomes (EXOs), belonging to EVs, are anuclear particles ranging from 50

to 200 nm in size that are constitutively released from the endosomal compartment of MSCs. They contain a plethora of functional protein and other molecules, including microRNAs (miRNAs), which mediate several MSC properties[15,50,51]. EXOs are key components of intercellular communication, because they are released into the intercellular space where they exert local paracrine or distal systemic effects[52]. In fact, EXOs are able to regulate numerous biological processes, including angiogenesis[53], cell proliferation[54], and the activation/inhibition of immune cells[55]. Interestingly, EXO content can be changed by various priming stimuli[3,40,55]. Recently, it has been revealed that EXO-derived miRNAs play a critical role in mediating EXO effects[56]. MiRNAs are 19-22-nucleotide-long non-coding RNAs that regulate mRNA translation, and are involved in many cellular processes[56,57]. Therefore, even if some therapeutic functions of the MSCs are mediated by cell-to-cell contact, the secretion of paracrine factors can be considered the main mechanism by which MSCs elicit functional responses in target cells[3,40,58,59]. In many *in vitro* and *in vivo* disease models, MSC-derived products have been identified as responsible for therapeutic effects[60-63]. For example, promising preclinical therapeutic effects have been obtained using MSC-derived EVs. In particular, regarding BM-MSC-derived EVs, Kim *et al*[64] found that these functional factors were able to reduce hepatic injury by modulating cytokine expression in a mouse model of fulminant hepatic failure. Preussmann[65] demonstrated that the administration of EXOs in a rat model of gentamycin-induced kidney injury, was able to improve the kidney injury score. Moreover, it has been shown that EXOs derived from umbilical cord-derived MSCs were able to accelerate wound healing in a rat skin burn model[66], and EXOs derived from BM-MSCs overexpressing hypoxia-inducible factor (HIF)-1α accelerated bone regeneration and angiogenesis in a rabbit model of steroid-induced avascular bone necrosis[67].

MSCs can also secrete a number of cytokines/chemokines that control both the innate and adaptive immune responses, resulting in immunoregulation and the induction of tolerance[68]. Indeed, it has been shown that MSCs can produce both anti- and pro-inflammatory factors which, depending on their ratio, regulate the pro- or anti-inflammatory activity of MSCs[69]. In this case, final immunoregulatory

properties may be affected by cell culture conditions that can prime/enhance MSC properties[3,70,71]. MSCs also have the ability to roll and adhere to post-capillary venules, and migrate to injured tissues, contributing to tissue repair/regeneration[72]. In this case, once MSCs reach the site of the injury, these cells put in place an active regulation by producing paracrine factors that impact tissue survival/repair, and activate tissue resident stem cells[3,73,74]. The secretion of various soluble factors has also been found to be responsible for the pro-angiogenic and anti-apoptotic effects of MSCs[75]. Though not well understood, the beneficial effects of conditioned media (CM) derived from MSCs have been clearly demonstrated by various experimental findings, supporting the concept of paracrine effects[76]. Several preclinical studies have tested the efficacy of CM in different diseases models. MSC-derived CM has been shown capable of improving cell viability and reducing inflammation in both *in vitro* and *in vivo* models of lung ischemia/reperfusion injury (IRI)[59,77]. Moreover, it has been demonstrated that BM-MSC-derived CM was able to reduce lung inflammation and edema in a mouse model of lipopolysaccharide-induced lung injury[78], and to improve renal tissue pathology in a mouse model of cisplatin-induced kidney injury[79]. Youdim *et al*[80], in a rat model of fulminant hepatic failure, found that the CM derived from BM-MSCs reduced leukocytic infiltrates and hepatocellular death. The CM derived from the same cells, in a mouse model of antigen-induced arthritis, was also able to reduce joint swelling, cartilage loss, and tumor necrosis factor (TNF)-α secretion[81]. In a rat model of lung fibrosis and hypertension, using CM derived from adipose MSCs (AdMSCs), demonstrated the ability of secretome to reduce collagen deposition and improve lung blood flow[82]. In a rabbit model of surgical bone lesion, Linero *et al* found that the CM produced from AdMSC cultures induced bone regeneration[83].

THE SECRETION OF MSC PARACRINE FACTORS CAN BE MODULATED BY VARIOUS PRIMING STRATEGIES

Given the heterogeneity of results supporting the efficacy of MSCs in the treatment of different human disorders, there is a need to improve the therapeutic properties of MSCs, and the best way might be that of preconditioning/priming. Though this

approach has been widely used in the field of immunology, has also been effectively applied to MSCs[3,84,85]. Among commonly used priming strategies, leading approaches can be attributed to three main categories: (1) MSC priming with inflammatory molecules; (2) MSC priming with hypoxia; and (3) MSC priming with 3D cultures. These priming signals activate potential MSC mediators, including surface receptors and ligands, signalling molecules that induce survival/growth, regulatory molecules such as miRNAs, and transcription factors, which can modify the MSC phenotype[86-89], with a consequent boosting of MSC therapeutic functions (Figure 1).

Priming with inflammatory molecules

Numerous studies have revealed that the immunosuppressive properties of MSCs are not intrinsically possessed, but require priming of MSCs by inflammatory factors[90-92]. Depending on the inflammatory conditions, it has been demonstrated that MSC phenotypes can be polarized into MSC type 1 (with pro-inflammatory properties) and MSC type 2 (with immunosuppressive properties)[93,94]. Several strategies have been implemented to modulate/enhance the secretion of functional molecules in MSCs. As shown in Figure 2, the treatment of MSCs with inflammatory cytokines, including interferon-gamma (IFN-γ), interleukin (IL)-1α/β, IL-25, IL-6, TNF-α, and IL-17 enhanced the immunomodulatory properties of MSCs[40,95-112]. These treatments increase the production/secretion of functional factors, including hepatocyte growth factor (HGF), transforming growth factor-β, IL-6, prostaglandin E2 (PGE2), leukemia inhibitory factor, granulocyte colony-stimulating factor, IL-10, macrophage inflammatory protein-1α, indoleamine 2,3-dioxygenase (IDO), intercellular adhesion molecule, programmed death ligand 1-2, monocyte chemoattractant protein (MCP)-1, monokine induced by IFN-γ, induced protein 10, and macrophage inflammatory protein-1β, which in turn confer more paracrine immunomodulatory properties to MSCs (Figure 2). It has been demonstrated that CM enriched with the above-described factors was able to inhibit T cell proliferation/activation, reduce the secretion of inflammatory mediators, and induce monocyte polarization towards anti-inflammatory the M2 phenotype[40,102,105-112]. It

has been shown that the treatment with inflammatory cytokines was also able to improve the immunomodulatory capabilities of EXOs, and these effects appear to be mediated by specific miRNAs, such as miR-21, miR-23a, miR-26b, miR-125b, miR-130b, miR-140, miR-146a, miR-203a, miR-223, miR-224, and miR-320a[40,109,111,113].

Priming with hypoxia

Differently from inflammatory cytokines, hypoxic treatment of MSCs seems to stimulate primarily the secretion of functional factors involved in the processes of angiogenesis and tissue proliferation/regeneration (Figure 2). Hypoxic preconditioning was able to promote angiogenic potential of MSCs *via* the activation of the HIF-1α-GRP78-Akt axis, and the overproduction of vascular endothelial-derived growth factor (VEGF) and HGF[114]. Lee and Joe[115] demonstrated that hypoxia priming induces an increase in HIF-1α expression and consequent VEGF production, improving the ability of MSCs to stimulate migration and tube formation of human umbilical vein endothelial cells (HUVECs). Moreover, Bader *et al* found that hypoxic preconditioning induces the anti-apoptotic and pro-angiogenic effects of MSCs compared with untreated cells. In particular, Bcl-xL, BAG1, and VEGF were overexpressed after hypoxic priming, enhancing HUVEC proliferation and migration[116]. Hypoxic MSCs are also able to produce numerous factors related to tissue remodelling, including matrix metallopeptidase 1 (MMP1), MMP2, and MMP9[117-119], as well as crucial factors such as IL-8 and MCP-1, involved in the chemotaxis and activation of innate immune responses[120,121]. Also with regard to EVs, hypoxic priming has been shown to have important effects. Xue *et al*[122] discovered that EXOs derived from hypoxia-treated MSCs were able to increase migration and tube formation of HUVECs through the PKA signalling pathway. Moreover, Ge *et al* demonstrated the efficacy of hypoxic MSC-derived EXOs in enhancing angiogenesis. In particular, they showed that hypoxic EXOs containing miR612 promoted, through HIF-1α activation, the production of VEGF in human brain microvascular endothelial cells, inducing proliferation, migration, and angiogenic activities of these cells[123].

Priming with 3D culture of MSCs

Various *in vitro* strategies have been applied for the production of MSCs, with improved therapeutic properties, and priming with inflammatory factors may impact the expression of HLA-DR, thus altering allogeneic therapeutic possibilities[124-126]. MSC priming through 3D culture techniques, which allows for the generation of MSC spheroids, strictly recapitulates the *in vivo* MSC niche and enhances the phenotypic profile of MSCs, increasing both trophic and immunomodulatory functionalities. MSC spheroid action is exerted by the paracrine secretion of functional factors that possess anti-inflammatory, angiogenic, anti-fibrotic, anti-apoptotic, and mitogenic properties (Figure 2)[127]. Recently, through omics approaches, such as RNA sequencing and analysis of DNA methylation, it has been demonstrated that, compared with conventional 2D culture, MSC spheroids were able to modify their transcriptome profile by overexpressing genes that can regulate proliferation/differentiation, as well as immunomodulatory and angiogenic processes[128]. Concerning immunomodulatory and regenerative effects, 3D culture of MSCs seems to have more intermediary functions than the above-mentioned priming strategies (priming with inflammatory molecules or hypoxia) (Figure 2). 3D MSC spheroids have been shown to be capable of secreting multiple functional factors. For example, it has been found that various regenerative and immunomodulatory factors, such as stromal cell-derived factor-1α, growth-related oncogene α, MCP-1/3; IL-4, IL-10; epidermal growth factor (EGF), leukemia inhibitory factor, placental growth factor-1, VEGF-A/D, HGF, insulin like growth factor 1, TNFAIP6, STC1, platelet-derived growth factor B, transforming growth factor-β, PGE2, and IDO were up-regulated in 3D MSC spheroids compared with those of the MSCs cultivated under conventional 2D conditions[43,44,59,73,128-131] (Figure 2). The paracrine effects of 3D MSC appear to be also mediated by EVs. In particular, EXOs derived from MSC 3D cultures have been shown to have higher yields and enhanced activity. Indeed, compared with 2D cultures, EXOs isolated from CM of MSC spheroids were able to inhibit T cell proliferation and stimulate angiogenesis *in vitro*[44], as well as attenuate inflammation and periodontitis *in vivo*[132], and stimulate tissue regeneration in both *in vitro* and *in vivo* models[133].

THERAPEUTIC PROPERTIES OF PRIMED MSCS IN PRECLINICAL MODELS

Principal priming strategies to treat chronic immune-related disorders

By virtue of their immunomodulatory properties, MSCs are being studied to treat numerous chronic conditions, including GVHD and inflammatory bowel disorders, in order to attenuate inflammation and induce tissue recovery (Table 1). As already mentioned, treating MSCs with inflammatory factors enhances their immunomodulatory properties, and renders these cells able to inhibit T cell proliferation/activation and induce monocytes toward an anti-inflammatory phenotype. This quality makes these cells more clinically effective when applied to chronic inflammatory-related diseases (Figure 2). Indeed, several experimental studies have demonstrated that the treatment of MSCs with inflammatory factors, such as IFN-γ, IL-1β, and IL-25, enhanced MSC therapeutic effects in *in vivo* models of chronic colitis[95,96,98]. Rafei *et al*, in a mouse *in vivo* model of autoimmune encephalomyelitis, found that treatment with allogeneic MSCs primed with IFN-γ reduced clinical signs in a dose-dependent manner. In this study the authors showed that, though the priming treatment induced the increase of CCL2 and MHCI/II expression in IFN-γ-primed MSCs, it inhibited manifestations of autoimmune encephalomyelitis while keeping their immunogenicity low[134]. The use of IFN-γ- or TNF-α-primed MSCs has also been shown to attenuate symptoms of GVHD[101,103]. In these cases, in the first study it was shown that therapeutic effects of MSCs were mediated by overproduction of IDO induced through the IFN-γ-JAK-STAT1 pathway[101]. In the second study, the therapeutic function of MSCs was activated by TNF-α, which induced overexpression of Chi3 L1 and consequent suppression of T-helper 17 cells[103]. Recently, it has been revealed that the priming of MSCs with IL-1β relieved the side effects of sepsis[109,111]. In particular, Song *et al*[109] demonstrated that IL-1β makes MSCs more effective in inducing macrophage polarization toward an anti-inflammatory M2 phenotype, and this effect was mediated, at least in part, through overproduction of EXOs containing miR146a. Similar results on M2 macrophage polarization were also obtained by Yao *et al*[111], who revealed the ability of IL-1β to stimulate the production of MSC-derived EXO containing miR21. The

therapeutic efficacy of MSCs primed with IFN-γ was also found in an *in vivo* model of colonic wounds. Particularly, these cells were able to enhance healing of colonic mucosal wounds in both immunocompromised and immunocompetent mice[135]. Similar results were also obtained using MSCs primed with TNF-α, which were able to accelerate wound closure and angiogenesis in an *in vivo* model of wound healing[99]. The priming with inflammatory cytokines seems to also be effective for the treatment of chronic liver diseases. Indeed, treatment with IL-6 improved the ability of MSCs to reduce liver injury[104]. The study reported that in a mouse *in vivo* model of liver fibrosis, treatment with IL-6-primed MSCs reduced both fibrosis and apoptosis, and improved liver functions[104]. Moreover, TNF-α-primed MSCs were also able to attenuate inflammation in an *in vivo* model of peritonitis[136]. In this study, the authors demonstrated that TNF-α induced the overproduction of the anti-inflammatory factor TSG-6, generating a mechanism that reduces inflammation in an *in vivo* model of zymosan-induced peritonitis[136]. Interestingly, in a similar experimental model, Bazhanov *et al* found that after intraperitoneal injection MSCs formed 3D aggregates, and stimulated the production of anti-inflammatory cytokines, such as IL-10 and PGE2[137]. In this regard, Bartosh *et al* showed that the priming of MSCs with 3D culture decreased inflammation in an *in vivo* model of peritonitis[138]. In particular, the authors suggest that MSC spheroids overexpressed TSG-6, and these cells were more effective than conventional MSCs as therapy for diseases characterized by unresolved inflammation[138].

Overall, the above-mentioned studies suggest that treatment with pro-inflammatory cytokines or the 3D culture of MSCs represents promising priming strategies for enhancing the MSC immunoregulatory phenotype, making these cells more suitable for clinical disorders related to exacerbated immune responses (Figure 2).

Main priming strategies for treating acute injury

Priming strategies for MSCs have been considered a crucial tool for enhancing their therapeutic effects, making these cells more suitable for application in the field of regenerative medicine[3,85]. However, while the priming of MSCs with pro-

inflammatory cytokines potentially represents the principal strategy modulating inflammation in chronic immune-related disorders (or, in any case, conditions in which the inflammation is exacerbated), the priming of MSCs with hypoxia is thought to represent the more appropriate priming strategy for boosting MSC effects for the stimulation of tissue function recovery after acute injury (Figure 2). This has been demonstrated in numerous study models, and on different organs (Table 1). For example, hypoxia pre-conditioning significantly improved blood flow recovery in mouse models of hindlimb ischemia. Rosovà *et al* demonstrated that hypoxic MSCs better migrate to the injured site compared with non-hypoxic MSCs, thus speeding up the restoration of blood flow. The authors demonstrated that the observed effects were likely mediated by the HGF-cMET axis[139]. It has been shown that hypoxia helps MSCs to better integrate in the damaged tissue. Han *et al* revealed that hypoxic priming enhanced survival and proliferation of transplanted MSCs, thus improving the regeneration of hindlimb ischemic tissues. After MSC treatment, the authors observed inhibition of apoptosis and promotion of neovascularization and, as they showed the increased expression of the normal cellular prion protein upon hypoxia pre-conditioning, they identified this prion as a potential target for MSC therapy[140]. In a similar manner, Lee *et al*[115] recently identified GRP78 as new potential target for the development of functional MSCs. GRP78 has been shown to be induced by hypoxia, thus increasing transplanted-MSC survival and proliferation in a mouse model of hindlimb ischemia. Moreover, the authors found that the HIF-1α-GRP78-Akt axis regulates the suppression of cell death signals, and increases angiogenic cytokine secretion, thus strongly improving tissue recovery from the damage[114]. Recently, it has been found that mild hypoxia can be induced in MSCs when they are cultured as spheroids. Various studies have clearly demonstrated that 3D culture conditions induce hypoxia in the core of the spheroid, thus stimulating the production of both growth and pro-angiogenic factors, which in turn stimulate the fast recovery of damaged tissues in mouse models of hindlimb ischemia[141,142]. Interestingly, it has also been shown that the CM derived from MSCs primed by 3D culture attenuated injury and inflammation in two IRI *in vitro* models of both lung and liver[59,131]. 3D pre-conditioning has been shown to also be effective for other

type of diseases, such as acute kidney injury (AKI). Xu *et al* found that 3D pre-conditioned MSCs, when transplanted in mice with AKI, are more viable than the 2D cultured cells, and exhibit higher paracrine secretions, as evidenced by the increased levels of VEGF and TSG-6[143]. Furthermore, the authors show that the paracrine secretion, which also includes basic fibroblast growth factor, insulin like growth factor, and EGF, significantly improved renal function and reduced tissue apoptosis, thus speeding up the regeneration of renal tissues upon injury[143]. Recently, the secretome of 3D MSCs transplanted for the treatment of AKI was furtherly investigated. For example, Cao *et al* found that the paracrine effect on AKI was mediated not only by soluble factors, such as anti-inflammatory cytokines, but also by EXOs, whose production is increased after 3D pre-conditioning[144]. Furthermore, by using a cisplatin-inducing AKI model in mice, the authors showed that the increased number of EXOs upon 3D culture enhanced the renoprotective and anti-inflammatory efficacy of MSCs[144]. Treatment of AKI with MSC therapy has been implemented in recent years by defining new protocols of MSC pre-conditioning. Along with 3D culturing, hypoxia priming has been used for the treatment of IRI-inducing AKI in animal models, and Zhang *et al* demonstrated that hypoxia priming enhanced angiogenic and antioxidative MSCs properties in a rat model of renal IRI. In addition, in the same model, the authors found that transplanted MSCs attenuated renal apoptosis by reducing cleaved caspase3 activation[145]. Notably, hypoxia also enhanced MSC therapeutic potential in a cisplatin-induced mouse model of AKI. Overath *et al*[146] found that hypoxic conditions increased the efficacy of transplanted MSCs in attenuating renal damage upon injury both by reducing creatinine and N-GAL serum levels, and decreasing pro-inflammatory cytokine release. MSC hypoxia pre-conditioning has also been found to be strongly effective for the treatment of IRI in the lung. For example, MSC infusion in lung perfusates demonstrated that hypoxic MSCs quickly migrate from the pulmonary artery to the lung tissue, where they attenuate parenchymal damage by reducing oxidative stress, inflammation, and apoptosis, and by stimulating cell proliferation and survival[147]. In a similar manner, MSC hypoxia has been found to have important effects also for radiation-induced lung injury (RILI). A mouse model of RILI was recently

established by exposing the lungs of mice to irradiation, thus generating tissue damage. Upon irradiation, the authors demonstrated that hypoxic MSCs reside for longer in the injured tissue compared with normoxic MSCs. In addition, Li *et al*[148] showed that hypoxia-primed MSCs enhanced cell viability and proliferation, as well as anti-oxidative and anti-apoptotic capabilities in lung parenchymal cells. Finally, the authors highlighted the role of HIF-1 in modulating resistance to lung hypoxic stress induced by RILI, thus promoting tissue repair and regeneration upon injury.

The use of MSCs as cellular therapy has also been shown to be effective for the treatment of acute myocardial injury in several preclinical models (Table 1). Also in this case, to ameliorate the therapeutic effects of MSCs various priming strategies have been evaluated. In particular in myocardial infarction (MI), it has been widely believed that tissue injury is related to ischemia and the hypoxic environment. Therefore, the *in vitro* hypoxic condition was tested to improve MSC therapeutic effects in MI animal models[149]. In a mouse model of MI, it was found that intramyocardial injection of hypoxia-preconditioned MSCs reduces infarct size, influences heart remodelling by modulating vasculogenesis, and improves heart functions, promoting cell survival[150,151]. Of note, expression analysis in hypoxic MSCs has revealed an increase in expression of pro-survival and pro-angiogenic factors, including HIF-1α, ANGPT1, VEGF, Flk-1, Bcl-2, Bcl-xL, and these proteins can act in a paracrine manner on MI, inducing functional recovery[150]. It has also been observed that hypoxic MSCs influence the expression of specific miRNAs that can be secreted through EVs. In particular, Feng *et al* demonstrated that after hypoxic treatment of MSCs an increase of miR22 was observed in EXOs, and this miRNA was considered responsible for targeting Mecp2, with beneficial effects on survival of cardiomyocytes exposed to ischemia[152]. Similarly, EVs derived from hypoxic MSCs overexpressing miR26 were able to reduce the damage from ischemia/reperfusion in a rat model[153]. In the same way, in an MI mouse model the intracardial injection of hypoxic-preconditioned MSC-derived EXOs was able to positively regulate cardiomyocyte proliferation and survival, and this effect was ascribable to the overexpression of miR125b[154]. In addition to the use of hypoxia priming, the use of 3D culture has also been shown to be effective in the

improvement of MSC therapeutic effects on the treatment of acute myocardial injury. You *et al*, in an acute MI rat model, found that treatment with 3D-primed MSCs resulted in a retention of MSCs at the epicardium, where MSCs exerted cardiac protection/repair, and functional recovery[155]. Moreover, in the same animal model, Wang *et al*[156] revealed that 3D MSCs were able to stimulate vascular density and improve cardiac function after MI.

Over the last decade, MSCs have also been intensively studied for their potential use in the treatment of neurological acute injury, including cerebral ischemia, traumatic brain injury, and spinal cord damage. For example, in an *in vivo* model of cerebral ischemia, it has been shown that hypoxic-preconditioned MSCs enhanced angiogenesis and neurogenesis after ischemia[157]. In an *in vivo* model of traumatic brain injury, Chang *et al*[158] demonstrated that the priming of MSCs with hypoxia improved their therapeutic function, and resulted in an amelioration of neurogenesis, and motor and cognitive functions. Moreover, in a rat model of spinal cord injury, hypoxic MSCs were also able to increase axonal preservation and decrease apoptosis[159].

Principal priming strategies for stimulating tissue regeneration

MSCs are involved in tissue homeostasis, which is necessary for physiologically coordinating regeneration/repair of tissue, also after injury[3,6,36]. Thus, the use of MSCs in regenerative therapies is garnering great interest due to their potentially numerous clinical applications.

In the complex process of cutaneous wound healing, a central role is played by fibroblasts, which contribute, through the interaction with surrounding cells, to the production of ECM, glycoproteins, adhesive molecules, and various growth factors[160]. Recent evidence suggests that CM produced by primed MSCs from different sources, such as bone marrow[120], adipose tissue[160], amnion fluid[161], and placenta[162] enhanced the migration and proliferation of fibroblasts *in vitro*, and accelerated wound healing in *in vivo* models (Table 1). In all these cases, hypoxia treatment represented the chosen priming strategy for driving MSCs in increasing secretion of various angiogenic factors, cytokines, and chemokines. Therefore, the

priming of MSCs with hypoxia might well represent the main approach to improving the therapeutic effects of MSCs to be applied in the stimulation of tissue regeneration (Figure 2). This idea has also been supported by other studies (Table 1). Indeed, in both hepatectomized mouse and rat models, it has been demonstrated that hypoxic MSCs produce crucial functional molecules, including HGF and VEGF, which were considered responsible for the induction of liver regeneration[163,164]. Kuo *et al* showed that systemic infusion of MSCs restored liver function and promoted liver regeneration in rodents[165]. In this regard, in a rat massive hepatectomy model, Jun *et al* found that hypoxia-conditioned MSCs secreted significantly more VEGF than normoxia-conditioned cells, and the infusion of primed MSCs promoted proliferation of hepatocytes and liver regeneration[164]. Several studies have focused on the signalling pathways up-regulated by MSC during liver regeneration. Sang Chul *et al*, using a partially hepatectomized mouse model, found that treatment with hypoxic MSC-derived CM increased the viability of hepatotoxic hepatocytes, and enhanced liver regeneration through JAK/STAT3 signalling[163]. These data were also confirmed by Sang Kuon *et al*, who confirmed the activation of JAK/STAT3 signalling induced by MSC CM during mouse liver generation[166]. Hypoxic MSCs that secrete high level of VEGF were also able to regenerate pulp-like tissues and vasculature similar to the native pulp in a rat model of pulp repair[167]. HGF and VEGF produced by hypoxic MSCs were considered by Chang *et al*[158] to be responsible for improvement of neuronal proliferation. Moreover, Zhilai *et al*[159] demonstrated that both HGF and VEGF produced by hypoxia-primed MSCs facilitated axonal survival in a rat model of spinal cord injury. Han *et al*, in a murine hindlimb ischemia model, found that the expression levels of EGF, VEGF, fibroblast growth factor, and HGF were significantly higher in ischemic tissue treated with hypoxic MSCs, where an improvement of neovascularization was observed[140]. The efficacy of hypoxic MSCs was also tested in reducing scar formation and inducing wound healing in various *in vivo* models[120,162,168].

Despite the fact that the principal MSC priming strategy used for both *in vitro* and *in vivo* regeneration experiments was hypoxia treatment, 3D culture of MSCs as priming strategy has also been investigated in tissue regeneration (Figure 2). In fact,

MSC spheroids have also shown therapeutic abilities with regard to both bone and cartilage regeneration. In particular, it has been found that treatment with MSC spheroids was effective in inducing disc repair in an *in vivo* model of disc degeneration, bone regeneration in an *in vivo* model of bilateral calvarial defects, and cartilage regeneration in an *in vivo* model of osteochondral defects[169-171].

CONCLUSION

The therapeutic effects of MSCs have been demonstrated in both *in vitro* and *in vivo* studies. Nevertheless, due to their heterogeneity related mainly to tissue source, which can impact MSC functional properties[85,172], the application of MSCs in clinical trials has shown moderate or poor efficacy. MSCs are considered key regulators of tissue repair and, in this case, different stimuli are crucial in modulating the functional properties of these cells. In fact, it is believed that inflammation and low oxygen levels are essential signals for triggering MSC activity in a suitable manner. Moreover, it has recently been shown that different priming approaches can eliminate the functional heterogeneity of MSCs[173]. Therefore, specific priming strategies have been implemented to improve the regenerative and immunomodulatory properties of MSCs. In this review, we have explored data regarding the principal priming approaches used to enhance the therapeutic potential of MSCs. The above-mentioned data underscore that several factors play a role in the ability to modify MSC properties. Moreover, some therapeutic effects, on different disease models, can be obtained in relation to dose and/or combination of the priming factors used.

Several diseases have in common tissue injury and repair processes, in which inflammation plays a central role in coordinating different pathways that regulate tissue regeneration and functional recovery. Indeed, after acute injury, a low level inflammation (acute inflammation) occurring after specific triggers, is crucial in stimulating wound healing and tissue repair, facilitating the resolution of inflammation and restoring tissue structure/function (inflammation drives regeneration). On the other hand, in the case of abnormal damage repair, chronic unregulated inflammation can lead to pathological processes, including hormonal

metabolic changes, which culminate in the onset of specific diseases, including cancer and fibrosis[174,175]. Therefore, the regulation of both acute and chronic inflammation is essential for a proper restorative response and, in this scenario, MSCs can have a crucial physiopathological role. In fact, it has been shown that when MSCs coordinate damaged tissue for repair, they undergo local stimuli such as inflammatory cytokines, and hypoxia, which in turn boost and direct the reaction of MSCs to orchestrate tissue regeneration[85,176]. In Figure 3, we depict a hypothetical model that occurs during physiopathologic tissue injury and repair. In this model, MSCs are activated differently by various microenvironment stimuli to manage tissue functional recovery. One of the first factors that arises after tissue injury is the establishment of a hypoxic and weakly inflammatory microenvironment, which in turn activates local cells to protect/regenerate tissues[3,177]. Hypoxia rapidly up-regulates the level of intercellular adhesion molecule-1 in local-inflamed endothelium, promoting MSC migration to injured tissues[178,179]. Moreover, a mild inflammation may stimulate MSCs to release chemokines for attracting immune cells and amplifying immune responses[180]. Once MSCs reach the site of injury, the paracrine properties of MSCs to release chemotactic and angiogenic factors is significantly amplified under hypoxic conditions[181]. In this case, naïve MSC are activated to recruit neutrophils and stimulate the formation of new blood vessels. Neutrophil action is followed by monocyte/macrophage activity that ensures sustained release of pro-inflammatory cytokines and potentiation of the fibroproliferative response[182,183]. If these processes are not adequately regulated, a state of chronic inflammation occurs. Thus, cytokines such as IFN-γ, TNF-α, and IL-1 accumulate in the injured tissues, and the inflammatory environment becomes central in affecting the regulatory role of MSCs that exhibit immunosuppressive capacities[184]. The MSC phenotype is switched into a lower regenerative potential and a higher anti-inflammatory phenotype (Figure 3). Thus, high amounts of pro-inflammatory cytokine confer a dramatic immunomodulatory ability to MSCs[40,91,124,125,185,186] which, in turn, act as a homeostatic regulator to control the inflammatory response. Overall, this scenario describes what occurs when MSCs are exposed to low levels of both oxygen and inflammation, and their phenotype is

potentially inclined to low immunomodulation and high stimulation of tissue regeneration. Otherwise, high levels of inflammation can imprint a MSC phenotype inclined toward high immunomodulation and weak stimulation of tissue regeneration (Figure 3). In this regard, Vigo *et al*[87] found that IFN-γ can orchestrate MSCs functions in a dose-manner, and this is reflected in the opportunity to modulate MSC properties before their use in clinical practice. In addition, considering the heterogeneous immune regulatory functions of MSCs due to intrinsic characteristics of individual clones, the priming of MSCs with pro-inflammatory factors can equally amplify immune therapeutic properties of MSCs, and eliminate the variances among different MSC clones[173].

Priming with inflammatory signals polarizes MSCs toward an anti-inflammatory and pro-trophic phenotype allowing, on the one hand, the regulation of inflammatory responses, and on the other the final remodelling and recovery of damaged tissue. Likewise, different priming strategies can be used to direct the therapeutic effects of naïve MSCs toward specific pathological processes. As also highlighted by the studies we have noted in this review, while hypoxic priming of MSCs could be used mainly to treat acute disease, to principally stimulate angiogenesis and tissue regeneration, inflammatory cytokines could be used mainly to prime MSCs for treating chronic immune-related disorders. The change of perspective from regeneration to inflammation implies in the MSCs the shift in the production of functional factors that stimulate regenerative or anti-inflammatory pathways (Figure 2). Interestingly, the 3D culture of MSCs as priming strategy appears to be an intermediate functional priming between the two mentioned above. The production of priming type-specific functional factors in MSCs could well pave the way for optimizing their therapeutic potential, aimed at a greater effectiveness as an advanced therapy medicinal product.

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