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Concise Review: Skeletal Muscle as a Delivery Route for Mesenchymal Stromal Cells

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ABSTRACT

Mesenchymal stromal cells (MSCs) have demonstrated extensive capacity to modulate a catabolic microenvironment toward tissue repair. The fate, biodistribution, and dwell time of the in vivo delivered MSCs largely depend on the choice of the cell delivery route. Intramuscular (IM) delivery of MSCs is clinically safe and has been used for the effective treatment of local pathologies. Recent findings have shown that the secretome of the IM-delivered MSCs enters the circulation and provides systemic effects on distant organs. In addition, muscle tissue provides a safe residence for the delivered MSCs and an extended secretorily active dwell time compared with other delivery routes. There are, however, controversies concerning the fate of MSCs post IM-delivery and, specifically, into an injured site with proinflammatory cues. This review seeks to provide a brief overview of the fate and efficacy of IM-delivered MSCs and to identify the gaps that require further assessment for adoption of this promising route in the treatment of systemic disease. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:456–465*

SIGNIFICANCE STATEMENT

Mesenchymal stromal cells exhibit potent immune-modulatory properties and are used in the treatment of many diseases. However, the dwell time of the cells in vivo, especially when delivered intravenously, is short—a matter of a few days. This dwell time can be extended by using injection into skeletal muscle as the cell delivery route. This route has been shown to be safe and has the advantage of increased longevity of the secretory activity of the delivered cells. This article reviews the intramuscular delivery route of such cells and the potential advantage to treatment regimes.

INTRODUCTION

Mesenchymal stromal cells [1] have demonstrated extensive capacity to limit injury and promote regeneration through signaling and secretion of trophic factors [2]. Indeed, MSCs provide a putative treatment for immune-related, infectious, and degenerative diseases, without a requirement for engraftment [3]. Despite these beneficial therapeutic effects, one challenge is the short dwell time of the delivered cells in vivo [4]. However, Braid et al. [5] recently reported the extended dwell time of human MSCs (hMSCs) delivered intramuscularly (IM—5 months) in healthy athymic mice when compared with the same cells delivered intravenously (IV—3 days), and either subcutaneously or interperitoneally (3 to 4 weeks). Thus, skeletal muscle provides a putative advantage for MSC delivery.

To date, skeletal muscle has been principally used as a delivery route for local treatment of myopathic, neurodegenerative, and vascular related diseases. However, recent studies have emphasized the opportunity afforded by IM-delivery to effect systemic changes. The 3 main advantages of skeletal muscle MSC delivery are: (a) extended dwell time provided by dense muscle fibers that retain the MSCs in situ; (b) high vascular density that provides a conduit for systemic release of MSC trophic factors; and (c) an abundance of tissue that provides for multiple injection sites. Although the IM-delivery of MSCs has been shown to be clinically safe [6–12], it is important to critically evaluate the fate of MSCs postdelivery in skeletal muscle.

Although the trophic factors secreted by MSCs are often considered to have a paracrine or local effect, their release into the blood stream could effect systemic outcomes. We discuss herein the evidence for engraftment and differentiation of IM-delivered MSCs, their secreted factors both local and systemic, their dwell time, and biodistribution.

Table 1. Examples of clinical studies of intramuscular-MSC therapy

Disease	MSC source	Delivery site	Cell dose	Single/multiple	Outcome	Complications	Follow-up period	Ref.
CLI	Allogenic BMMSCS	Gastrocnemius	2×10^6 cells per kilogram	40–60 sites at proximity	Improvement in clinical scores (rest pain scores and ankle pressure)	Safety without occurrence of edema at the site of injury	24 wk	[6]
PAD + CLI	Autologous ADMSCs	Internal and external gastrocnemius andanterior compartment of the ischemic leg	1×10^8	15 sites in each muscle	Clinical signs such as leg pain, ulcer size, and pain-free walking was reported as significantly improved	No sign of edema, or necrosis at the site of injury	24 wk	[7]
ALS	Autologous BMMSC-NTF treated	Biceps and triceps	24×10^6	24 sites at proximity	Improvement in the CAMP amplitude	Slight edema at the site of transplantation. No infection. No tumor formation	24 wk	[12]

Clinical Safety of IM-MSC Delivery

Clinical trials that have adopted IM delivery of bone marrow-derived cells include both bone marrow-mononuclear cells and MSCs [13]. Clinical IM-MSC delivery has targeted both promotion of angiogenesis in patients with peripheral artery disease (PAD) and thromboangiitis obliterans (TAO)/Buerger disease, and amelioration of motor neuron loss in amyotrophic lateral sclerosis (ALS) patients. Previously, the chosen route of MSC delivery for PAD and TAO was either IV or intraarterial (IA) in anticipation that the cells would reach ischemic sites. However, IV-delivered MSCs are entrapped in the capillary beds of lungs with minimal engraftment to ischemic sites [14]. Clinical studies have validated the safety of IM-MSC delivery (Table 1). Gupta et al. IM-delivered allogeneic BMMSCs in the ischemic limbs of patients and reported improvement in clinical scores [6]. To overcome the low frequency of MSCs in BM aspirates, Bura et al. IM-delivered adipose-derived MSCs (AD-MSCs) in PAD patients with clinical limb ischemia and reported no sign of edema or necrosis at the site of injury. Clinical signs such as leg pain, ulcer size, and pain-free walking were all reported to be significantly improved—potentially due to revascularization [7].

The IM delivery of MSCs has, more recently, been pursued as an alternative to intrathecal (IT) and/or IV transplantation in ALS patients. Petrou et al. [12] reported no significant complications, and only slight edema, associated with injection, at 24 sites in the biceps and triceps, of BMMSCS (1×10^6 cells per site)—induced in culture to express neurotrophic factors (NTF) to promote regeneration and neuroprotection. Due to the nature of ALS, direct intrathecal delivery of MSC-NTFs together with peripheral IM administration of MSC-NTF was considered to enhance the efficacy of MSC-therapy compared with the IM-MSC delivery alone [12]. However, the systemic effects of the release of NTF were not assessed. Although clinical studies have confirmed the safety of IM-MSC delivery only one, conducted in critical limb ischemia (CLI) patients receiving allogeneic placenta-derived MSCs (PLX), has reported a systemic effect—modulation of dendritic/natural killer cell interactions [15].

Preclinical Studies: IM-Delivered MSCs to Treat Local Pathologies

MSCs have been delivered IM for local treatment or to locally treat complications associated with systemic diseases (Table 2). These studies have focused predominantly on the local angiogenic and neuro-supportive effects of MSCs although the systemic sequalae of the secreted trophic factors have not been assessed. Diabetic polyneuropathy (DPN), similar to PAD, is a complication associated with diabetes. Shibata et al. [16] IM-delivered rat BMMSCs (rBMMSCs) in streptozotocin (STZ)-induced diabetic Sprague–Dawley (SD) rats. Four weeks postdelivery, the cells were observed in the gaps between the muscle fibers. In addition, a significant increase in the levels of bFGF and vascular endothelial growth factor (VEGF) were observed in the treated muscle. In a similar model in balbC mice, Kim et al. delivered mBMMSCs along the sciatic nerve and reported improvement in motor nerve conduction as early as 2 weeks, whereas no further improvement was observed after 4 weeks [17]. On the other hand, Han et al. [18] delivered allogeneic rBMMSCs in the thigh muscle of DNP-STZ induced Wistar rats near the sciatic nerve, and reported engraftment along the vasa nervosa after 4 and 8 weeks. Additionally, upregulation of angiogenic and neurotrophic genes, myelin protein, and nerve growth factor receptor gene in the transplanted muscle were all observed.

Following their initial use of neural progenitor cells [29], Suzuki et al. pursued delivery of glial cell derived growth factor (GDNF) transfected MSCs into various muscle groups [19]. In a SD-SOD1^{G93A} rat model of ALS—that develops neurodegeneration of spinal motor neurons and progressive motor deficits—^{GDNF}hBMMSCs were delivered together with daily cyclosporine (CsA). First, to ameliorate hBMMSC survival, a focal muscle injury was induced with injection of bupivacaine hydrochloride prior to cell delivery. MSC delivery into the muscle led to significant reduction in the number of denervated endplates, and abrogation of motor neuron loss. IM-transplanted MSCs were detected after 8 weeks in the muscle at the site of injection [19].

In other IM-MSC studies, human cells were xenotransplanted in animal models of CLI for preclinical and translational

Table 2. Intramuscular-MSC therapies to treat local pathologies

Disease model	Species model	I.S.	MSC source	Delivery site	Cell dose ($\times 10^6$)	Single/multiple	Engraftment & differentiation	Systemic effect	Biodistribution	Method of assessment	Study length	IM-dwell time	Comments & general outcome	Ref.
DPN-STZ induced	SD-rat	N/A	rBMMSCs	Thigh and soleus	1	Single, unilateral	Engraftment in the gaps between fibers, no differentiation	Not assessed	Transplanted muscle	Muscle histology, $PKH26^+$	4 wk	4 wk	↑bFGF, ↑VEGF, amelioration of diabetes complications	[16]
DPN-STZ induced	balbC mice	N/A	mBMMSCs	Along the sciatic nerve	1	Single, 4 sites, bilateral	Engraftment, did not assessed differentiation	Not assessed	Transplanted muscle	Functional loss of improvement	4 wk	<4 wk	↑NGF, ↑NT-3, improvement in sciatic motor nerve conduction	[17]
DPN-STZ induced	Wistar-rats	N/A	rBMMSCs	Thigh near sciatic nerve	5	Single, bilateral	Engraftment to sciatic nerve, no differentiation, assessed with Lectin after 4–8 weeks)	Not assessed	Transplanted muscle, along vasa nervosa	Muscle histology, DiI-MSCs	8 wk	>8 wk	Upregulation of factors involved in angiogenesis, neural function, and myelination	[18]
ALS + local IM injury (cardiotoxin-induced)	$SD–SOD^{G93A}$ rats	CycloA	hBMMSCs-GDNF	Tibialis anterior, fore limb triceps branchii, long muscles of the dorsal trunk muscle	0.12	Multiple:3 injections 1 week apart, bilateral	Engraftment and differentiation. Expression of human myosin heavy chain IIx/d(MyHC-IIx/d) gene in rat muscle	Not assessed	Transplanted sites	Histology and RT-PCR (hcDNA)	8 wk	8 wk	Amelioration of motor neuron loss locally and within the spinal cord which connected to the limb muscles with transplants	[19]
Limb ischemia	MHC mismatched ACI rats	N/A	rBMMSCs or rFM	Ischemic thigh muscle	5	Single, 5 sites, unilateral	Engraftment but not differentiation (assessed with Lectin after 1 week)	Not assessed	Transplanted site	Histology, GFP+ MSCs	3 wk	3 wk	Improvement of blood perfusion and capillary density	[20]
Limb ischemia	C57BL/6 mice	N/A	mADMSCs	Ischemic adductor muscle	1	Single, unilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	Bioluminescence imaging	4 wk	4 wk	Improvement of vascular density and perfusion rate	[21]
Limb ischemia	balbC mice NOD/SCID mice	N/A	hPLX-PAD	Thigh musculatures	1	Single, unilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	Bioluminescence imaging	3 wk	4 days 3 wk	Improvement of vascular density and blood flow, reduction of endothelial damage	[22]
Limb ischemia	balbC	N/A	hPDAC	Proximal and distant to the ischemic muscle	1	Single, 2 sites, unilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	Bioluminescence imaging	49 days	<8 days	Improvement of vascular density and blood flow	[23]
Limb ischemia	NOD/SCID-IL2Rγ−/− (NSG) mice	N/A	hBMMSCs-VEGF	Ischemic hamstring	0.5	Single, 2 sites, unilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	Bioluminescence imaging and PCR (hgDNA)	24 wk	4 wk, small quantity 4.5 m	Improvement of vascular density and blood flow	[24]
Limb ischemia	Lewis rats	N/A	rBMMSCs	Anteromedial ischemic muscle	5	Single, unilateral	Engraftment and differentiation to endothelial cells that expressed (hVIII), also differentiation to skeletal muscle fibers (expression of desmin) and adipocytes	Not assessed	Transplanted site	Histology LacZ+ MSCs, X-gal identification	3 wk	3 wk	Improvement of vascular and arteriolar density, regeneration	[25]

(Table 2 continued on next page.)

Table 2. (*continued*)

Disease model	Species model	I.S.	MSC source	Delivery site	Cell dose ($\times 10^6$)	Single/multiple	Engraftment & differentiation	Systemic effect	Biodistribution	Method of assessment	Study length	IM-dwell time	Comments & general outcome	Ref.
Limb ischemia	BalbC	N/A	mBMMSCs	Adductor muscle proximal to the site of injury	1	Single, 6 sites, unilateral	Engraftment. B-gal+ cells found distributed between muscle fibers without incorporation into the vessels, therefore no trans-differentiation	Not assessed	Transplanted site	Histology (GFP+, β-gal+)	17 days	17 days small quantity	Improvement in perfusion of ischemic tissue and collateral modeling. Attenuation of muscle atrophy and fibrosis	[26]
Limb ischemia	Lewis rats	N/A	rBMMSCs	Ischemic thigh muscle	5	Single, unilateral	Engraftment, and some transplanted MSC were positive for vWF, an endothelial marker	Not assessed	Transplanted site	Histology PKH26	3 wk	3 wk	Improvement of vascular density and blood flow	[27]
Muscle dystrophy-CTX (Latoxan)-induced	NMRI nu−/−mice	N/A	hSM-MSCs	Injured muscle	0.5	Single, unilateral	Engraftment and detection of human β2M and human dystrophin and MyHC-IIx/d in injured muscle	Not assessed	Transplanted site (60%) detected after 4 weeks	In situ hybridization for human Alu repeats and PCR (hDNA)	24 wk	4 wk, small quantity 4 wk	Transplanted MSCs differentiated and contributed to myofibrils and satellite cells but did not fuse with murine cells	[28]

Abbreviation: I.S., immunosuppressant.

assessment of the human MSC functionality in ischemia. Prather et al. [22] IM-transplanted luciferase expressing PLX, 5 hours after arterial ligation. Cells were delivered locally at the site of injury in both immunocompetent (balbC) and immunocompromised (NOD/SCID) mice. Loss of luminescence signal in the immunocompetent balbC mice was observed after 4 days, whereas in NOD/SCID mice, cells were still detected for 3 weeks. Similarly, Francki et al. IM-delivered placenta-derived adherent cells, luciferase-transduced, in the ischemia-induced hind limb muscle of BalbC mice 24 hours after injury, and reported significant improvement in the blood flow and vascular density by 35–49 days. Furthermore, at 49 days, the injured muscle showed a reduction of inflammatory infiltrate and improvement in the structure of the regenerated muscle fibers [23]. Beegle et al., in a similar CLI model, IM-transplanted hBMMSCs over-expressing VEGF in the hamstrings of immunocompromised NOD/SCID-IL2Rγ−/− (NSG) mice. Significant loss of MSCs was reported within the first 28 days; a small number of cells were detected after 4.5 months, but no cells were detected after 6 months [24]. Although these studies reported local upregulation of angiogenic growth factors, they showed model-dependent variations in the dwell time of MSCs. Even within a single model differences were seen. For example, Braid et al. [5] showed a 2-log decrease in cells over the first 4 days following IM delivery, although a secretorily active population remained at the injection site for up to 5 months.

IM-Delivered MSCs to Treat Distant and Systemic Conditions

MSCs are shown to secrete a plethora of immunomodulatory factors in response to inflammatory stimuli [30] and also to stimulate endogenous cell regeneration [31, 32]. IM-MSC delivery has demonstrated a potential to treat distant or systemic conditions where the long dwell time of secretorily-active cells would provide an advantage over the rapid disappearance of cells from the lungs following IV delivery. The systemic release of the IM-delivered MSC secretome was first demonstrated in 2001 by Bartholomew et al. who showed that human erythropoietin (hEPO) was released for up to 1 month by baboon MSCs, genetically modified to express hEPO, when IM-delivered in NOD/SCID mice [33].

Shabbir et al. IM-delivered porcine BMMSCs (pBMMSCs), two injections 2 weeks apart, into the hamstrings of cardiomyopathic TO2 hamsters. Significant ventricular function improvement (i.e., attenuated chamber dilation) and increased systolic wall thickening were reported 3 weeks after a second IM-delivery of MSCs. MSCs were also shown to reduce apoptosis and myocardial tissue injury, as well as decreased myocardial-pathological fibrosis by ∼50%. The systemic increase in the level of HGF, LIF, and GM-CSF were suggested to be the mediators of myocardial repair, which was concomitant with upregulation of HGF, IGF-II, and VEGF in the myocardium ([34]; Table 3). Similarly, Zisa et al. IM-delivered hBMMSCs in the hamstrings of TO2 hamsters and reported improved left ventricle ejection fraction (LVEF) by 30%, 4 weeks post-MSC therapy [40]: VEGF was considered to be the main factor that improved cardiac repair. Similarly, Mao et al. IM-delivered human umbilical cord Wharton's jelly MSCs (hWJMSCs) into both fore limb and hind limbs of doxorubicin-induced SD rats (a model of dilated cardiomyopathy), two injections 2 weeks apart. Improved

cardiac function with increased systemic levels of HGF, IGF-1, LIF, GM-CSF, and VEGF and cardiac tissue expression level of HGF, VEGF, and IGF-1 was observed 2 weeks after the second MSC injection [36]. Furthermore, Liu et al., using human soluble tumor necrosis factor receptor (hsTNFR) transduced hBMMSCs demonstrated a prophylactic reduction in joint inflammation in an antibody-induced/LPS-challenged murine rheumatoid arthritis (RA) model, although the naïve hBMMSCs showed no effect [37]. In another study, Braid et al. showed that a depot of IM-delivered human umbilical cord perivascular cells (HUCPVCs), genetically modified to secrete an antiviral monoclonal antibody, provided systemic protection against exposure to Venezuelan equine encephalitis virus (VEEV), with secretorily active MSCs detectable for 109 days [39]. The engineered HUCPVCs were IM-delivered in the thigh muscle of balbC$^{nu/nu}$ mice 24 hours or 10 days prior to intranasal inoculation with VEEV. No significant difference was observed between 24 hours or 10 days prophylactic protection. We have also IM-delivered hBMMSCs, mBMMSCs, or HUCPVCs in the hind limb of immunocompetent CD1 mice, and reported systemic downregulation of TNF-α and abrogation of neutrophil infiltration at an anatomically distant (contralateral) site of inflammatory injury [38].

These studies provide evidence that factors released from MSCs are the primary therapeutic mediators independent of their engraftment and differentiation at the site of injury and therefore illustrate that IM delivery could be used to treat any condition where a sustained level of circulating mediators secreted by the MSCs would be required. Nevertheless, 3 important factors that affect the efficacy of IM-MSC delivery for a systemic effect are the dwell time of the cells, the cell dose and frequency of injection.

Dwell Time of IM-Delivered MSCs

The extended dwell time of transplanted MSCs in the skeletal muscle (compared with other routes of administration) enables putative extended therapeutic effects. Nevertheless, the reported dwell time of MSCs delivered to the skeletal muscle varies from 72 hours to 8 months. Two key factors profoundly affect these dwell-time variations: (a) immune-rejection and (b) the methods used for MSC detection. Although autologous MSCs are often used in clinical trials, they can show disease [41] or age-related [42] impairments. Therefore, allotransplantation provides an advantage since MSCs exhibit low immunogenicity, and are expected to evade the immune system. Although the innate immune system is known to contribute to skeletal muscle repair [43]. Davoudi et al. [44] have recently reported that neutrophils and macrophages are scarce in undamaged muscle, which may contribute to the longer dwell time of allogeneic MSCs when compared with lodgment in macrophage-rich lungs following IV delivery. MSCs in vitro exhibit low expression of MHC-I, and costimulatory molecules CD40, B7-1 (CD80), and B7-2 (CD86)—which are involved in T-cell costimulation or coactivation—and lack expression of MHC-II [45]. However, it is not clear whether MSCs maintain their low immunogenicity post-transplantation [20], especially in an inflamed site. Hemeda et al. demonstrated that MSCs exposed to IFN-γ increased MHC class I expression and also triggered the expression of MHC-II cell surface markers [46]. Ishikane et al. showed a significantly lower number of T-lymphocytes in rBMMSC-transplanted healthy muscle compared with ischemia-induced MSC-transplanted muscle [47]. Even with autologous transplantation, in vitro cell culture expansion conditions may cause phenotypic changes that facilitate innate recognition of the cells when transplanted [48], resulting in physiological clearance. The only reported preclinical IM-autologous MSC transplantation study, records a dwell time of 6 weeks at the transplanted site [49], which is significantly less than the dwell time of MSCs in immunocompromised animals. Importantly, even immune-compromised animal models differ in their reaction to xenotransplanted MSCs. *Athymic-nude* rodents do not produce mature T-cells and have high activity of macrophages, natural killer (NK) and dendritic cells (DC) [50, 51]. In contrast, *SCID* mice have impaired production of mature T-cells, and severely reduced macrophages NK and DC activity. These factors all affect the dwell time of exogenously transplanted MSCs [25, 27, 52].

The majority of the preclinical studies are conducted in small animals and MSCs are often allotransplanted. Such studies have shown 17 days to 4 weeks of in situ dwell time [21, 26, 47, 53, 54], but the length of the study also affects the reported dwell time. A somewhat extended dwell time, ranging from less than 4 to more than 8 weeks, is reported when MSCs are allo-IM transplanted in noninjured muscles in models of systemic conditions such as STZ-induced DPN [16–18]. MSCs IM-delivered in immunosuppressed (CsA)-rats exhibited a dwell time of 8 weeks when transplanted in a knock out ALS model [19]. It is important to note that CsA blocks recipient T-lymphocyte reactions [28], and compromises granulocyte migration during acute inflammation. When hMSCs are IM-transplanted in immunocompetent animals, a short dwell time of 4–8 days has been reported by Prather et al. [22], Francki et al. [23], and Hamidian Jahromi et al. [38]. Exceptions are the studies by Mao et al. [36] and Shabbir et al. [34] who reported therapeutic effects for 4 weeks that may infer survival of IM-transplanted hWJ-MSC or pBMMSCs in immunocompetent SD-rats and TO2 hamsters respectively, although more probably reflect the "hit-and-run" mechanism by which MSCs are considered to have their effects [55]. On the contrary, some of the studies that have IM-transplanted MSCs in genetically immunocompromised animal models have reported significant dwell times of 3–24 weeks in injured muscle [22, 24, 56], ∼4–16 weeks in intact skeletal muscle of animals with systemic disease [37, 39], and ∼4–32 weeks in intact healthy animals [5, 33, 57–59]. One factor that was similar in all reports was the fast decay in cell density over the first 14 days with further decline up to 28 days. For example, Liu et al. [53] transplanted mouse AD-MSCs into the hind limb adductor muscle of ischemic C57BL/6 mice 24 hours postinjury; gradual loss of the IM-transplanted MSCs was reported over 28 days. Ishikane et al. IM-delivered rBMMSCs or rat fetal membrane MSCs (rFM-MSCs) in a CLI model in MHC mismatched rats [47]. Loss of MSC engraftment was observed 3 weeks post IM-MSC delivery with a small quantity of cells still present at the site of injury. The fraction of cells remaining in the muscle for a longer period has been reported to be 10% of the transplanted cells after 8 months [57].

Suzuki et al. have reported a short MSC dwell time when transplanted into intact muscle. A focal injury in the skeletal muscle, prior to transplantation, extended the MSCs dwell-time [19]. The short dwell time of MSCs in intact muscle does not corroborate the findings of Shibata et al. [16], Kim et al.

Table 3. Intramuscular-MSC therapies to treat distant and systemic diseases

Disease model	Species model	I.S.	MSC source	Delivery site	Cell dose ($\times 10^6$)	Single/multiple	Engraftment & differentiation	Systemic effect	Bio-distribution	Method of assessment of biodistribution/ engraftment/ differentiation	Study length	IM-dwell time	Comments & general outcome	Ref.
Dilated cardiomyopathy	TO2 hamster	N/A	pBMMSCs	Intact hamstrings	0.25, 1, 4	Multiple, 2 injections, 2 weeks apart, bilateral	Not assessed at the IM transplantation site	↑LIF and GM-CSF, ↓cTnI	Not assessed	Functional effect	4 wk	4 wk	Mobilization of progenitor cells to the heart. Significant cardiac output improvement and higher MSC dose resulted in higher functional improvement	[34]
Dilated cardiomyopathy	Bio-TO2 hamster	N/A	hBMMSCspBMMSCs	Intact hamstrings	2–4	Single, bilateral	Not assessed at the IM transplantation site	VEGF	Not assessed	Functional effect	4 wk	4 wk	Improved cardiac output. hBMMSCs express less VEGF compared with pBMMSCs	[35]
Dilated cardiomyopathy-doxorubicin-induced	SD-rats	N/A	hUC-WJMSCs	Fore- and hind limb skeletal muscles	0.25, 1	Multiple, 2 sites, 2 injections, and 2 weeks apart, bilateral	Not assessed at the IM transplantation site	↓BNP and cTnI, ↑LIF, HGF, GM-CSF, and VEGF	Not assessed	Functional effect	4 wk	4 wk	Improved cardiac output, MSC-dose independent	[36]
RA- antibody/ LPS induced	BalbC/SCID mice	N/A	hBMMSCshBMMSC-hsTNFR	Thigh quadriceps	2	Single, bilateral	Not assessed at the IM transplantation site	hsTNFR	Not assessed	Functional effect	27 days	27 days	Prophylactic concept, only hsTNFR transduced hBMMSCs protected joints from inflammation, 20 days of hsTNFR detection in circulation	[37]
Focal paw inflammation, induced by γ-carrageenan	CD1 mice	N/A	HUCPVCs, hBMMSCsmBMMSCs	Contralateral to the injured limb, quadriceps muscle	1.3	Single, unilateral	Engraftment, did not assess differentiation	Reduced TNF-α, increased TSG-6 (indirect measurement)	Transplanted site, no distribution to organs	Histology, RT-PCR (hgDNA)	48 hours	48 hours	Reduction of inflammation in the paw, reduction of neutrophil infiltration, HUCPVCs and hBMMSCs performed better than mBMMSCs, HUCPVCs showed earliest response	[38]
Systemic virus infection-induced with VEEV	balbC$^{nu/nu}$ mice	N/A	HUCPVCs-anti-VEEV	Thigh skeletal muscle	2.5	Single, bilateral	Engraftment, did not assess differentiation	Anti-VEEV IgG	Transplanted site	Secreted IgG, bioluminescence imaging	123 days	Bioluminescence, systemic IgG secretion: 109 days	Prophylactic concept, protection against exposure to a VEEV	[39]

Abbreviation: I.S., immunosuppressant.

[17], and Han et al. [18] where cells were injected in the intact muscle of DPN-STZ induced animal models and other studies that have injected MSCs in healthy mice ([5, 33, 49, 57–59]; Table 4). Interestingly, Laurila et al. reported detection of MSCs near the needle injury site [60] and Braid et al. [5] reported accumulation of MSCs around the site of needle injury which indeed was more pronounced when the density of IM-delivered MSCs declined over time. Although the discussed work does not support the notion of extended dwell time of MSCs in an injured site, it is understood that needle injury itself is a small focal injury created in every IM-delivery model.

Cell Dose and Frequency of Injections

To date, MSC dosing, both in clinical trials and animal studies has been chosen rather arbitrarily. For IV infusion in humans, $1–2 \times 10^6$ cells per kilogram body weight is commonly used. As expected for local delivery, lower cell numbers are reported; examples of which are from 1×10^6–10^8 for injection into OA knee joints [61], and 6×10^6 cells delivered into the intervertebral disc for the treatment of lower back pain caused by degenerative disc disease [62]. Interestingly, the latter clinical trial showed no therapeutic advantage of using the higher dose, although the clinical study was based, in part, on a sheep study employing both a low 0.5×10^6, and high 4×10^6 ovine BMMSCs in which the higher dose was more effective [63]. In an ex vivo pig lung dose escalation study using HUCPVCs, Mordant et al. [64] found a medium dose (5×10^7 cells) to be more effective than either a lower or higher dose.

For IM delivery of MSCs, little information is currently available and is contradictory. For example, although Petrou et al. [12] undertook a dose escalation study in patients with ALS (see above), no differential effects of the 3 dosing cohorts of combined IT and IM-delivered autologous BMMSCs were reported. In preclinical studies, Suzuki et al. [19] delivered 0.12×10^6 gene-modified human neonatal BMMSCs (see above) either unilaterally or bilaterally into 3 muscle groups (tibialis anterior, triceps brachii, and dorsal trunk musculature) of rats at 24 hours, 1 week, and 2 weeks after local muscle injury. Although the number of surviving cells was reported to increase with multiple injections, no other differences were attributed to the multiple dosing. On the contrary, Kang et al. [65] delivered high and low doses of hBMMSCs in ischemic limbs of Balb/c mice and reported no dose–effect relationship but enhanced results were obtained with higher frequency of MSC injection. Similarly, Mao et al. injected hUCMSCs twice into both fore and hind limb musculature of DCM rats (see above), 2 weeks apart, but reported no differences in outcome with low and high dose (0.25 or 1×10^6 cells) although the second treatment did result in significant increase in left ventricular ejection fraction [36]. On the other hand, Shabbir et al. reported that the highest injection dose used, of 0.25, 1, and 4×10^6 pBMMSCs into bilateral hamstrings, resulted in the most effective cardiac function improvement in the recipient hamsters [34].

As all MSCs populations are heterogeneous, but to varying extents, the therapeutically optimum cell dose for a particular delivery route can be expected to vary with MSC tissue source and the therapeutic target condition in addition to variations in the dosing regimen which, for IM administration, can include the number of IM sites chosen, their anatomical location and frequency (for multiple deliveries). Furthermore, gene-modified cells could be expected to be used at different dosing regimens than unmodified populations. Several authors have shown that neonatal MSCs are more potent than those derived from adult tissues including higher MSC frequency, growth rate, life span, and superior immunomodulatory properties [35, 66–72].

Differentiation of IM-Delivered MSCs

Environmental cues can drive the phenotype of transplanted MSCs. IM-MSC delivery has also been used to treat other local pathologies in local muscle injuries. De Bari et al. [56], assessed myogenic differentiation of human synovial membrane (hSM)-MSCs-*LacZ*$^+$, delivered either IV or IM, to treat Latoxan-induced muscle injury in NMRI nu$^{-/-}$ mice. After 4 weeks, cells expressing human myosin heavy chain type IIx/d (MyHC-IIx/d)—a terminal differentiation marker—were found in both injured and noninjured tibialis anterior (TA) muscles. In addition, human β2-microglobulin (β2M) was detected between the basal lamina and muscle fibers at the injured site, but without fusion with the latter. Similar results were obtained when hSM-MSCs were IM-transplanted in the TA muscle of Dystrophin-deficient mdx mice (C57BL/10ScSn DMD^{mdx}/J) immunosuppressed with Tacrolimus (FK506). After 4 weeks, human dystrophin and MyHC-IIx/d were detected in the injected muscle implicating differentiation and contribution of hSM-MSCs to regeneration of myofibers but without fusion [56]. Similar results were demonstrated by Suzuki et al. as the hBMMSCs transplanted in focally-induced skeletal muscle expressed β-actin and hMyHC-IIx/d suggesting myogenic differentiation [19]. Furthermore, 3 weeks post-IM-transplantation of rBMMSCs, Iwase et al. reported detection of double-positive PKH26/von Willebrand (vWF) cells [26]. Similarly, in a CLI Lewis rat model, Al-Khaldi et al. demonstrated that rBMMSCs transplanted in the ischemic limb of rats express factor VIII, α-SMA actin and desmin, markers of endothelial, smooth muscle and skeletal muscle cells respectively and concluded that the transplanted cells spontaneously regenerated the various components of muscular tissues [21]. Ishikane et al. assessed fusion of MSCs with blood vessel endothelial cells after 1 week of MSC transplantation in the ischemic limb and did not observe GFP+/Lectin double-positive cells [47] which was similar to the reported results of Han et al. observed after 4 and 8 weeks [18]. Studies that did not use specific markers reported that MSCs reside in the gaps between the fibers without differentiation [16, 54]. The collective opinion is that myogenic environmental cues affect the phenotype of exogenously transplanted MSCs, and that this may happen earlier in an injured site.

Biodistribution of MSCs after IM-Delivery

The biodistribution of MSCs is important for both safety and survival of MSCs. It is important to assess whether MSCs distribute to unwanted organs postdelivery, cause microembolism, or disappear which could shorten the duration of therapeutic effect. Although it has been shown by many that MSCs can migrate toward the site of injury, this was not demonstrated with the IM-delivery route, except if the injury site was local as shown by Han et al. [18], who demonstrated a close spatial relationship between IM-delivered BMMSCs and vasa nervora. They considered it likely that the observed increase in angiogenesis

Table 4. Intramuscular-MSC studies in healthy animals

Disease model	Species model	I.S.	MSC source	Delivery site	Cell dose ($\times 10^6$)	Single/ multiple	Engraftment & differentiation	Systemic effect	Bio-distribution	Method of assessment of biodistribution/ engraftment/ differentiation	Study length	IM-dwell time	Comments & general outcome	Ref.
Healthy	balbC$^{nu/nu}$ mice	N/A	HUCPVCs, hBMMSCs	Thigh skeletal muscle	1	Single, unilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	Histology, bioluminescence	5 m	5 m	IM: 5 m dwell time and no distribution to organs	[5]
Healthy	NOD/SCID mice	N/A	bBMMSCs-hBMMSCs-hEPO	Quadriceps muscle	1	Single, bilateral	Engraftment, did not assess differentiation	Increased human EPO and hematocrit level	Not assessed	Secreted hEPO	4 wk	4 wk	Nonhuman primate MSCs can also be engineered to deliver biological products	[33]
Healthy	balbC$^{nu/nu}$ mice	N/A	hADMSCs	Thigh muscles	1	Single, bilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site and liver	Histology, bioluminescence imaging, genomic hybridization (hgDNA)	8 m	8 m	Majority of transplanted cells disappeared within 1 wk, 10% remained in the muscle through 8 m	[55]
Healthy	Nu/nu Foxn1 mice	N/A	hBMMSCs	Hind limb skeletal muscles	1.5	Single, bilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	MRI	26 days	26 days	Significant reduction in signal between 3 to 12 days	[56]
Healthy	Merino-cross sheep	N/A	sBMMSCs	Gastrocnemius muscle	2	Single, unilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	Histology	6 wk	6 wk	CM-DiI labeled sBMMSC into skeletal muscle showed dye retention for 6 wk	[47]
Healthy	NOD/SCID mice	N/A	PLX-PAD	Thigh muscle	1	Single, 2 sites, unilateral	Engraftment, did not assess differentiation	Not assessed	Transplanted site	Histology, RT-qPCR (hgDNA)	3 m	3 m	80% of cells lost within 1 m. Neither single nor multiple caused toxicity	[57]

Abbreviations: r, rat; m, mouse; h, human; p, porcine; s, sheep; b, baboon; d, day; wk, week; m, month; LIF, leukemia inhibitory factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; cTn1, cardiac troponin 1; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; NT-3, neurotrophin-3; GFP, green fluorescent protein; gDNA, genomic DNA; cDNA, complementary DNA; RT-qPCR, reverse transcription polymerase chain reaction; I.S., immunosuppressant.

was due to both secreted cytokines and physical interaction but provided no evidence for direct cell–cell contact as an effector mechanism. MSCs transplanted in the skeletal muscle are shown to reside locally and secrete trophic factors that enter the systemic circulation. Upon loss of the IM-delivered MSCs from skeletal muscle, either a small (1.5%) portion was found in the liver [57], or none was observed in any organs other than the muscle site [5, 22–24, 38, 58, 60]. Furthermore, it has been shown that if the needle accidently punctures a major blood vessel, then the IM-delivered MSCs rapidly enter the circulation and transfer into distal organs. This could cause a problem more specifically in small animals that is, mice that exhibit small size muscles.

Concluding Remarks

The studies reviewed collectively support the notion of broadening the applicability of IM-delivery route from a local therapy to the treatment of systemic disease. Multiple studies have shown that IM-delivered MSCs safely reside in situ for an extended dwell time and are secretorily active. Current assessment of the fate of MSCs post IM-delivery is largely limited to conditions where MSCs are transplanted in an injured site consisting of a significant amount of inflammation. This is a concern, since local injury environmental cues are shown to both impair MSC viability and functionality while driving phenotypic change and lineage differentiation.

This raises many questions, of which the following are examples: *What degree of inflammation primes MSCs without affecting their viability and engraftment? What is the degree of inflammation in which MSCs can survive and still exert an immunomodulatory response?* and W*hat is the timeframe for a change in MSC phenotype*? Answers to these questions are vital in determining the dose of a particular MSC population, and the frequency of their IM-delivery to optimize therapeutic performance.

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Author Contributions

S.H.J.: literature search, drafted the manuscript; J.E.D.: manuscript revisions; S.H.J., J.E.D.: approved the final version.

Disclosure of Potential Conflicts of Interest

J.E.D. is the founding president and officer of Tissue Regeneration Therapeutics, Inc. (TRT), Toronto. The other author indicated no potential conflicts of interest.

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