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Chapter

Mesenchymal Stem Cells for Regenerative Medicine for Duchenne Muscular Dystrophy

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Abstract

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be isolated from both foetal and adult tissues. Several groups demonstrated that transplantation of MSCs promoted the regeneration of skeletal muscle and ameliorated muscular dystrophy in animal models. Mesenchymal stem cells in skeletal muscle, also known as fibro-adipogenic progenitors (FAPs), are essential for the maintenance of skeletal muscle. Importantly, they contribute to fibrosis and fat accumulation in dystrophic muscle. Therefore, MSCs in muscle are a pharmacological target for the treatment of muscular dystrophies. In this chapter, we briefly update the knowledge on mesenchymal stem/progenitor cells and discuss their therapeutic potential as a regenerative medicine treatment of Duchenne muscular dystrophy.

Keywords: mesenchymal stem cells, induced pluripotent stem cells, induced MSCs, Duchenne muscular dystrophy, immune response, paracrine factors, cell transplantation, muscle regeneration, dystrophin, satellite cells, inflammation, skeletal muscle, fibrosis, adipocyte

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked progressive muscle wasting disorder caused by mutations in the DMD gene [1, 2], affecting 1 in 3500–5000 male births. Serum creatine kinase (CK) levels are elevated at birth, and motor milestones are delayed. Reduced motor skills between age 3 and 5 years provoke diagnostic evaluation. Quality of life for boys with DMD is further affected early in life, with the inability to keep up with peers of early school age and loss of ambulation by 12 years of age; premature death occurs at 20–30 years of age due to respiratory and cardiac complications (https://www.duchenne.com/about-duchenne; https://ghr.nlm.nih.gov/condition/duchenne-and-becker-muscular-dystrophy).

Mutations of the DMD gene cause complete (Duchenne) or partial (Becker) loss of dystrophin protein at the sarcolemma [3]. In normal muscle cells, dystrophin

forms a complex with glycoproteins at the sarcolemma, forming a critical link between the extracellular matrix (ECM) and the cytoskeleton [4]. Without the complex, the sarcolemma becomes fragile and is easily disrupted by mechanical stress [4, 5].

Except for corticosteroids, there is currently no effective treatment for DMD [7]. In this chapter, we discuss the potential of mesenchymal stem cells as a therapeutic tool for DMD patients. Many researchers prefer the term 'mesenchymal stromal cells' or 'mesenchymal progenitors' to mesenchymal stem cells because mesenchymal stem cells with self-renewal and trilineage differentiation potential are a minor subpopulation in tissue-derived primary cultures of mesenchymal cells. In this chapter, however, we uniformly refer to them as mesenchymal stem cells.

2. The pathological changes in DMD muscle

The absence of dystrophin causes loss of the dystrophin-associated protein complex (DAPC) at the sarcolemma. The sarcolemma lacking the complex becomes vulnerable to mechanical stress. In addition, signalling through dystrophin-DAPC-associated molecules such as nNOS is disturbed [4, 5]. As a result, myofibres die in large numbers by contraction-induced mechanical stress, and to regenerate injured myofibres, inflammatory cells begin to remove debris of the muscle tissue; at the same time, muscle satellite cells are activated, proliferate and fuse with damaged myofibres. In the case of DMD, however, the cycle of degeneration and regeneration of myofibres repeats throughout life. Therefore, secondary pathological changes gradually develop, including perturbation of calcium homeostasis, activation of Ca^{2+}-dependent proteases, mitochondrial dysfunction in myofibres, impaired regeneration of myofibres due to exhaustion of satellite cells, prolonged inflammation, disturbed immune response, fibrosis and fatty infiltration, with poor vascular adaptation and functional ischaemia [7]. These secondary pathological changes accelerate the disease course of DMD, resulting in severe loss of myofibres and

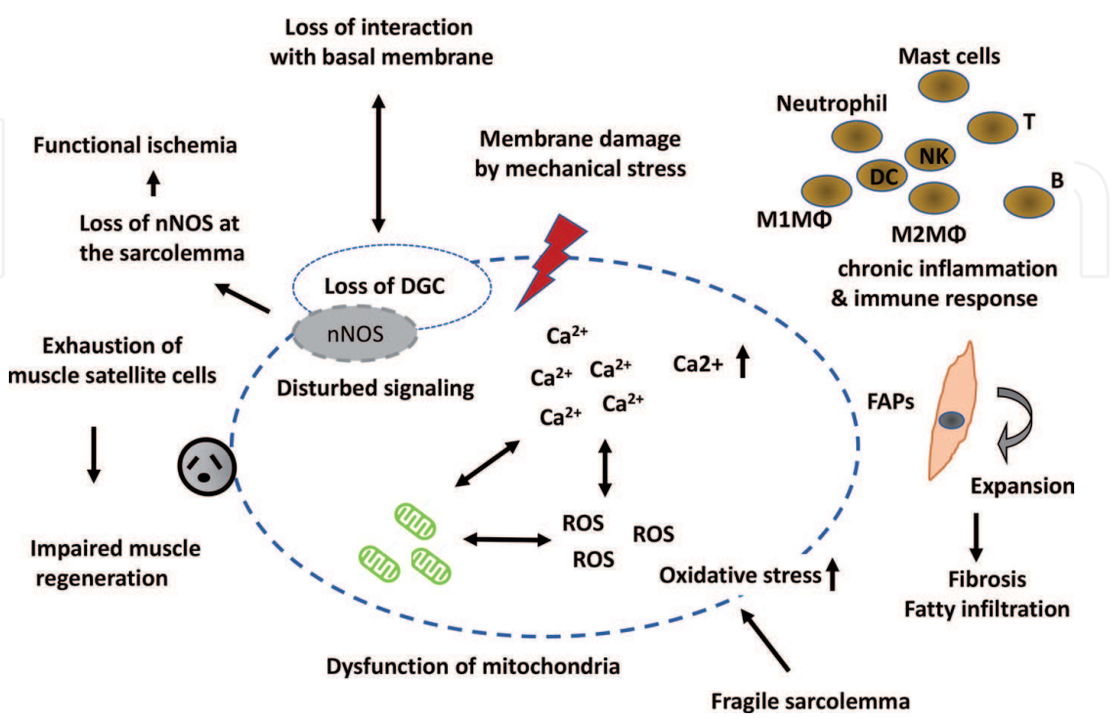

Figure 1.
Deficiency of dystrophin protein at the sarcolemma causes multiple pathological changes in DMD muscle [6, 7].

muscle atrophy. Therefore, in addition to the restoration of dystrophin protein by gene therapy or stem cell therapy, blockage of secondary pathological events is an important therapeutic strategy for DMD (**Figure 1**).

3. Muscle stem cells as a cell-based therapy for DMD

Upon injury, muscle satellite cells are activated, proliferate, and either fuse with damaged myofibres or fuse with each other to form new myofibres [8]. In DMD muscle, satellite cells compensate for muscle fibre loss in the early stages of the disease but eventually are exhausted. As a result, in DMD muscle, the myofibres are gradually replaced with fibrous and fatty connective tissue. Therefore, stem cell transplantation is expected to be a potential therapy for DMD [9].

There are different kinds of stem cells with myogenic potential in skeletal muscle. Muscle satellite cells are authentic unipotent skeletal muscle-specific stem cells [8]. Muscle-derived stem cells (MDSCs) [10] and mesangioblasts [11] were reported to be multipotent and transplantable *via* circulation; therefore, they are expected to be promising tools for cell-based therapies for DMD. Recently, muscle progenitors were induced from pluripotent stem cells as a cell source for cell-based therapy of DMD because induced pluripotent stem cells (iPSCs) can be expanded without losing pluripotency [12]. Myogenic cells induced from iPSCs are usually at a foetal stage and poorly engraft in the muscle of immunodeficient DMD model mice [13, 14].

In addition, muscles affected by muscular dystrophies are in a state of continuous inflammation and are characterised by marked and sustained infiltration of inflammatory and immune cells with fibrosis and adipose replacement. Such pathological microenvironments would not support survival, proliferation, and differentiation of the transplanted stem cells. Therefore, researchers have started to consider not only the properties of stem cells but also the microenvironment.

4. Muscle-resident mesenchymal stem cells (progenitors) are indispensable for muscle homeostasis

Skeletal muscle regenerates when it is injured. The regeneration process is complex but well organised, depending on the interaction among different types of cells: muscle stem/progenitor cells, muscle-resident mesenchymal progenitors and cells involved in inflammatory and innate and adaptive immune responses. Dynamic extracellular matrix (ECM) remodelling is also required for successful muscle regeneration. In the case of a minor traumatic injury, muscle regeneration is rapidly completed by the interplay of these cells. In muscular dystrophies, however, the degeneration/regeneration process is repeated for a long time, causing exhaustion of muscle satellite cells and finally resulting in severe atrophy of skeletal muscles with a loss of myofibres and extensive fibrosis and fat deposition [15].

Fibro/adipogenic progenitors (FAPs) are tissue-resident mesenchymal stem (or stromal or progenitor) cells [16, 17]. Recently, the necessity of FAPs for skeletal muscle regeneration and maintenance was demonstrated using mouse models [18]. The authors demonstrated that depletion of FAPs resulted in loss of expansion of muscle stem cells (MuSCs) and haematopoietic cells after injury and impaired skeletal muscle regeneration [18]. Furthermore, FAP-depleted mice under homeostatic conditions exhibited muscle atrophy and a loss of MuSCs, revealing that FAPs are essential for long-term homeostatic maintenance of skeletal muscle and the MuSC pool [18].

FAPs have dual functions [19, 20]. In small-scale traumatic muscle injury, they are activated, expand and promote muscle regeneration. When regeneration is completed, FAPs are cleared from the regenerated muscle. In pathological conditions, such as muscular dystrophies, they continue to proliferate and contribute to fibrosis and fatty tissue accumulation.

How is the fate of FAPs regulated? Apparently, FAPs are regulated by signals from myogenic cells and immune cells. Altered signals from these cells in dystrophic muscle change the pro-regenerative FAPs to fibrotic and adipogenic types. Recently, Hogarth et al. reported that annexin A2 accumulation in the myofibre matrix promotes adipogenic replacement of FAPs in dysferlin-deficient LGMD2B model mice. The authors also showed that an MMP-14 inhibitor, Batimastat, inhibited adipogenesis of FAP. The authors speculate that Annexin A2 and MMP-14 both prolong the inflammatory environment, therefore causing excessive expansion of FAP in diseased muscle [21]. Pharmacological inhibition of FAP expansion may be a good strategy to prevent fibro/adipogenic changes in dystrophic muscles.

The signals that regulate FAPs remain largely unclear. Interestingly, treating FAPs of young *mdx* mice with trichostatin A (TSA), a histone deacetylase inhibitor, blocked their fibrotic and adipogenic differentiation and promoted a myogenic fate [22] by changing chromatin structure [23]. TSA treatment decreased the expression of adipogenic genes and upregulated myogenic genes in FAPs [22].

5. Inflammation and immune responses in muscular dystrophies

Inflammatory and immune cells (neutrophils, eosinophils, basophils, macrophage NK cells, dendritic cells, T cells, B cells, etc.) are key regulators of muscle regeneration. In particular, macrophages orchestrate the regeneration process. In the early phase of muscle regeneration, M1 (inflammatory) macrophages remove necrotic tissues by phagocytosis and inhibit fusion of myogenic precursor cells. In the later stage, M2 (regulatory) macrophages gradually replace M1 macrophages and play anti-inflammatory and pro-regenerating roles by promoting the differentiation of myogenic cells and the neovascularization of regenerating muscle regeneration [24].

DMD muscle, which remains dystrophin-deficient, experiences continuous cycles of necrosis and regeneration of myofibres. This causes chronic inflammation and evokes T cell-mediated immune responses, which involves the coexistence of both M1 and M2 macrophages and T cells in the muscle, and it further damages myofibres and exacerbates fibrosis and adipocyte infiltration [6, 25, 26]. Therefore, pharmacological inhibition of excess inflammation and immune response is a reasonable therapeutic strategy for DMD.

6. Mesenchymal stem cells as a therapeutic tool for DMD

As a therapeutic tool for regenerative medicine, mesenchymal stem cells (MSCs) have received significant attention in the recent years due to their high growth potential, paracrine effects, immunomodulatory function and few reported adverse effects [27, 28]. Since MSCs show relatively low immunogenicity due to low expression of major histocompatibility (MHC) antigens and their immunomodulation function, they are being used even in allogeneic settings.

6.1 Definition

To facilitate research on MSCs, the International Society of Cellular Therapy (ISCT) formulated minimal criteria for defining multipotent MSCs in 2006 [29]. First, MSCs must be plastic adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73 and CD90 and must not express CD45, CD34, CD14, CD11b, CD79alpha, CD19 and HLA-DR surface molecules. Third, MSCs must differentiate into osteoblasts, adipocytes and chondrocytes under standard in vitro differentiation protocols [29].

6.2 Preparation

Historically, MSCs were isolated from bone marrow [30–33]. Currently, MSCs are shown to exist in the perivascular niche in nearly all tissues and are prepared from a variety of tissues, such as the umbilical cord [34], placenta [35], adipose tissue [36] and dental tissues [37]. Preparation of MSCs from those tissues is less invasive than it is from BM. MSCs from different tissues have similar functions, but detailed comparative studies revealed that MSCs of different origins possess different properties [38].

6.3 Differentiation

MSCs are multipotent stem cells that undergo self-renewal and differentiate into multiple tissues of the mesenchymal lineage and into a non-mesenchymal lineage, including neurons, glia, endothelial cells, hepatocytes and β cells in the pancreas [27]. This wide range of differentiation capacities is one reason why mesenchymal stem cells are being tested in almost 1000 clinical trials in regenerative medicine for the musculoskeletal system, nervous system, myocardium, liver, skin and immune diseases (http://ClinicalTrial.gov). Importantly, the differentiation potential of MSCs varies according to their origin, method of isolation and in vitro propagation procedures [39–41].

6.4 Secretome of MSCs

MSCs secrete a variety of bioactive molecules, such as growth factors, chemokines and cytokines. These molecules regulate the survival, proliferation and differentiation of target cells, promote angiogenesis and tissue repair and modulate inflammation and innate or acquired immunity. It is widely accepted that the therapeutic effects of MSCs in preclinical and clinical trials are largely due to their paracrine function [27]. Importantly, the secretome of MSCs varies depending on the age of the donor and the niches where the cells reside [42]. Therefore, it is expected that the therapeutic effects of MSCs with different origins exert will be different.

6.5 Transplanted MSCs ameliorate dystrophic phenotypes of DMD muscle?

6.5.1 Mechanisms of amelioration of the dystrophic phenotype by MSCs

Recently, there has been considerable interest in the clinical application of MSCs for the treatment of muscle diseases. However, the myogenic potential of MSCs is controversial.

Sassoli et al. found that myoblast proliferation was greatly enhanced in coculture with bone marrow MSCs [43]. Myoblasts after coculture expressed higher levels of

Notch-1, a key determinant of myoblast activation and proliferation. Interestingly, the effects were mediated by vascular endothelial growth factor (VEGF) secreted by MSCs [43]. A VEGFR2 inhibitor, KRN633, inhibited the positive effects of MSC-CM on C2C12 cell growth and Notch-1 signalling [43]. Linard et al. showed successful regeneration of rump muscle by local transplantation of bone marrow MSCs (BM-MSCs) after severe radiation burn using a pig model [44]. The authors speculate that locally injected BM-MSCs secreted growth factors such as VEGF and promoted angiogenesis. The authors also showed that MSCs supported the maintenance of the satellite cell pool and created a good macrophage M1/M2 balance. Nakamura et al. reported that transplantation of MSCs promoted the regeneration of skeletal muscle in a rat injury model without differentiation into skeletal myofibres. The report suggests that MSCs contribute to the regeneration of skeletal muscle by paracrine mechanisms [45]. Maeda et al. reported that BM-MSCs transplanted into peritoneal cavities of dystrophin/utrophin double-knockout (dko) mice strongly suppressed dystrophic pathology and extended the lifespan of treated mice [46]. The authors speculated that CXCL12 and osteopontin from BM-MSCs improved muscle regeneration. Bouglé et al. also reported that human adipose-derived MSCs improved the muscle phenotype of DMD mice *via* the paracrine effects of MSCs [47].

In addition to soluble factors, recent studies demonstrated that MSCs secrete a large number of exosomes for intercellular communication [48, 49]. These exosomes are now expected to be a therapeutic tool for many diseases [50, 51]. Nakamura et al. reported that exosomes from MSCs contained miRNAs that promoted muscle regeneration and reduced the fibrotic area [45]. Bier et al. reported that intramuscular transplantation of PL-MSCs in *mdx* mice decreased the serum CK level, reduced fibrosis in the diaphragm and cardiac muscles and inhibited inflammation, partly *via* exosomal miR-29c [49]. Thus, MSC exosomes or MSC cytokines may provide a cell-free therapeutic strategy as an alternative to transplanting MSCs.

On the other hand, Saito et al. reported that BM-MSCs and periosteum MSCs differentiated into myofibres and restored dystrophin expression in *mdx* mice, although the efficiency was low (3%) [52]. Liu et al. showed that FLK-1^{+} adipose-derived MSCs restored dystrophin expression in *mdx* mice [53]. Feng et al. reported that intravenously delivered BM-MSCs increased dystrophin expression in *mdx* mice [54]. Vieira et al. reported that intravenously injected human adipose-derived MSCs successfully reached the muscle of golden retriever muscular dystrophy (GRMD) dogs and that they expressed human dystrophin [55]. Furthermore, Park et al. reported that human tonsil-derived MSCs (T-MSCs) differentiated into myogenic cells in vitro, and transplantation promoted the recovery of muscle function, as demonstrated by gait assessment (footprint analysis); furthermore, such treatment restored the shape of skeletal muscle in mice with a partial myectomy of the gastrocnemius muscle [56]. These reports suggest that MSCs directly contribute to the regeneration of myofibres and restore dystrophin expression.

7. MSCs regulate inflammation and the immune response in muscular dystrophies

In response to damage signals, perivascular MSCs are activated and recruit inflammatory and immune cells and promote inflammation. At a later stage, MSCs begin to suppress inflammation and the immune response. On the other hand, MSCs in circulation are reported to selectively home towards damaged tissue [57]. Once homed, the inflammatory environment stimulates MSCs to produce a large amount of bioactive molecules or to directly interact with inflammatory and immune cells to regulate inflammation and the immune response.

The therapeutic effects of MSCs in preclinical or clinical trials are thought to be partly the result of modulation of innate and adaptive immunity [27], especially through monocyte/macrophage modulation [28]. Inflammation and immune response are part of the pathology of DMD muscle. Therefore, the immunomodulatory functions of MSCs might be useful for the treatment of DMD.

MSCs are supposed to modulate inflammation and the immune response by (a) suppressing the maturation and function of dendritic cells [58–60], (b) promoting macrophage differentiation towards an M2-like phenotype with high tissue remodelling potential and anti-inflammatory activity [61], (c) inhibiting Th17 generation and function [62, 63], (d) inhibiting Th1 cell generation [64], (e) suppressing NK [65, 66] and T cytotoxic cell function [66], (f) stimulating the generation of Th2 cells [67] and (g) inducing Treg cells [64, 66, 68].

Pinheiro et al. investigated the effects of adipose-derived mesenchymal stem cell (AD-MSC) transplantation on dystrophin-deficient mice. Local injection of AD-MSCs improved histological phenotypes and muscle function [69]. AD-MSCs decreased the muscle content of TNF-α, IL-6, TGF-β1 and oxidative stress but increased the levels of VEGF, IL-10 and IL-4 [69]. MSC-derived IL-4 and IL-10 are reported to convert M1 (pro-inflammatory) macrophages to the M2 (anti-inflammatory) type and promote satellite cell differentiation [70]. These results suggest that transplanted AD-MSCs ameliorated the dystrophic phenotype partly by modulating inflammation.

7.1 Suppression of the immune response by MSCs potentiates gene therapy and cell-based therapy

In a clinical trial of gene therapy using a dystrophin transgene, T cells specific to epitopes of pre-existing dystrophin in revertant fibres were detected, suggesting the existence of autoreactive T-cell immunity against dystrophin before treatment [71]. Currently, exon skipping therapy to restore the reading frame of the DMD gene, and readthrough therapy of premature stop codons (e.g. aminoglycosides or ataluren), is being tested in patients with DMD. The treated patients start to produce dystrophin, which provides new epitopes to them. Suppression of undesirable immune responses against newly produced dystrophin might improve the efficiency of gene therapy.

Transplantation of myogenic cells also evokes innate and acquired immune responses against transplanted cells in the recipient. Therefore, immunosuppression by MSCs is expected to improve the engraftment of transplanted cells and the therapeutic effects of cell therapy. In addition, MSCs support the survival, proliferation, migration and differentiation of myogenic cells by secreting trophic factors.

8. Mesenchymal stem cells induced from pluripotent stem cells (iPSCs)

8.1 MSC-like cells induced from human pluripotent stem cells (iMSCs) have properties that are different from tissue MSCs

Although BM-MSCs are well studied and widely tested in regenerative medicine, the collection procedure for bone marrow is invasive and painful. In addition, adult BM-MSCs cannot be expanded in culture beyond 10 passages [72]. To obtain MSCs with higher proliferative potential, other sources of MSCs are gaining attention, such as the umbilical cord and the placenta. MSCs from these sources proliferate better than BM-MSCs but still show limited proliferative activity [38].

hiPSCs can be expanded in vitro without loss of pluripotency and are therefore an ideal source for deriving mesenchymal stem cells of high quality in a large quantity [73–75]. In addition, unlike human ES cells, iPSCs are not accompanied by ethical concerns. To date, many protocols have been reported for the deviation of mesenchymal stem cells from human ES cells/iPS cells [73–77], although the difference in properties among iMSCs induced by different protocols remains to be determined [73, 74, 77]. For clinical use, iMSCs would be generated from well-characterised, pathogen-free, banked iPSCs with known HLA types or from patient-specific iPSCs.

8.2 Are MSCs induced from human pluripotent stem cells (iMSCs) ideal for clinical use?

MSCs induced from human iPS cells are generally characterised as reprogrammed, rejuvenated MSCs with high proliferative activity [78]. A previous study reported that MSCs from human iPSCs could be expanded for approximately 40 passages (120 population doublings) without obvious loss of plasticity or onset of replicative senescence [79]. In addition, iMSCs have been shown to exhibit potent immune-modulatory function and therapeutic properties (**Table 1**) [80]. Spitzhorn et al. reported that iMSCs did not form tumours after transplantation into the liver [81], but to exclude residual undifferentiated iPS cells, purification of MSCs by FACS using MSC markers and careful evaluation of the risk of tumour formation would be required for each preparation.

	BM-MSCs	**Induced MSCs from ES/iPS cells**
Preparation	Autologous or allogeneic, invasive	Many protocols for deviation scale-up production [73–77]
Proliferation	Limited expansion	Proliferate faster, greater proliferation capacity
Senescence	Faster	Slower
Quality	Inconsistent, heterogeneous depend on donor age [82] and health condition, and culture condition	Controllable? Closer to foetal MSCs less mature than tissue-derived MSCs
Differentiation	Trilineage (adipocytes, osteocytes and chondrocytes); hardly differentiate into skeletal muscle	Higher osteogenic differentiation [74]; poor differentiation into adipogenic cells [83]
Stemness	Lost with expansion	Kept for long culture
Safety	No tumour formation; pathogens from the donors	Genomic instability during the expansion of iPSCs; tumorigenic potential by residual hiPSCs
Paracrine effects	Inhibit apoptosis, promote proliferation and differentiation of the cells, promote tissue regeneration; different secretome [87]	
Immunomodulation function	Regulate inflammation, innate and acquired immunity	Stronger than BM-MSCs? [74, 80]
Suitable for cell therapy?	Being tested in preclinical and clinical trials without serious side effects	Unlimited source of MSCs; autologous MSCs are available
Genome editing	Difficult	Possible

Table 1.
Comparison of properties of human iMSCs with human BM-MSCs.

8.3 iMSCs for muscle disease

The therapeutic potential of iMSCs has been tested in bone regeneration [80, 84], intestinal healing [85], myocardial disorders [86, 87], limb ischaemia [79] and autoimmune disease [88, 89]. In these studies, iMSCs showed therapeutic effects that were comparable or superior to those of tissue MSCs. In the muscular dystrophy field, there are only a small number of reports so far. Jeong et al. reported that iMSCs transplanted into the tibialis anterior of *mdx* mice decreased oxidative damage, as evidenced by a reduction in nitrotyrosine levels, and achieved normal dystrophin expression levels [90]. Since direct differentiation of MSCs into myogenic cells is generally limited, the observed effects of iMSCs might be due to the secretion of bioactive molecules that exert immunomodulatory effects and provide trophic support to myogenic cells.

Importantly, however, Liu et al. recently reported that transplantation of BM-MSCs from C57BL/6 mice aggravated inflammation, oxidative stress and fibrosis and impaired regeneration of contusion-injured C57/Bl6 muscle [91]. Although the mechanisms are not clear, the microenvironment in contusion-damaged muscle might induce the transformation of MSCs into the fibrotic phenotype. Caution might be warranted in the clinical application of MSCs to highly fibrotic muscle.

9. Conclusions

MSCs are multifunctional cells. MSCs secrete trophic factors that help regenerate myofibres. In addition, MSCs suppress inflammation and the immune response in dystrophic mice to protect muscle. MSCs are also expected to support the engraftment of transplanted myogenic cells in recipient muscle. Fortunately, recent technology gives us an option to derive MSC-like cells from pluripotent stem cells. Thus, MSCs are a promising next-generation tool for cell-based therapy of DMD (**Figure 2**).

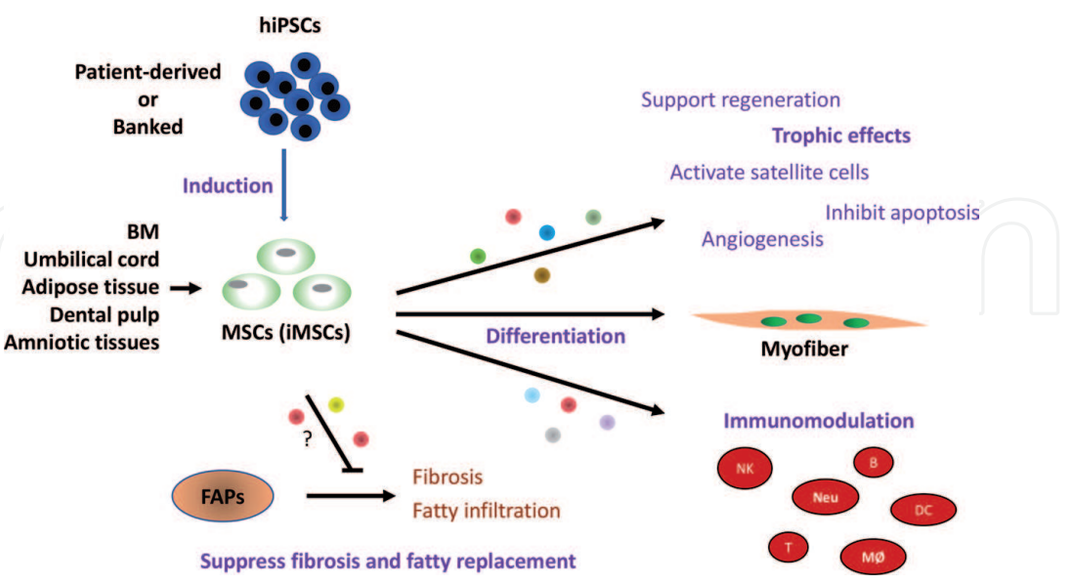

Figure 2.
Mesenchymal stem cells ameliorate the dystrophic phenotype of DMD muscle. Mesenchymal stem-like cells can be derived from human iPSCs (iMSCs). MSCs, which arrive in the muscle either through direction transplantation or via *circulation, secrete a variety of bioactive molecules that promote angiogenesis and support the proliferation and differentiation of satellite cells, thereby promoting muscle regeneration. MSCs also suppress excess inflammatory and immune responses. Whether transplanted MSCs can directly modulate the phenotype of FAPs (resident MSCs) to inhibit fibrosis and fatty replacement remains to be determined. Abbreviations: DC, dendritic cells; NK, natural killer cells; Neu, neutrophil; Mø, macrophage; T, T lymphocytes; B, B lymphocyte.*

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Conflict of interest

The authors declare no conflicts of interest.

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