Chapter 3

Stem Cell Therapy in Muscle Degeneration

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Abstract Skeletal muscle is one of the largest tissues in humans, reaching around 38% of the body weight for men and 30% for women (Janssen I, et al, J Appl Physiol 89(1):81–88, 1985/2000). Muscle tissue finely regulates essential body movements, including respiration, equilibrium maintenance and ambulation. In addition, it controls body temperature and energy balance and restores blood glucose levels. Genetic or acquired alterations in these enzymes can lead to metabolic syndromes (McArdle's syndrome (Vorgerd M, Neurotherapeutics 5(4):579–582 2008) or diabetes (DeFronzo RA, Tripathy D, Diabetes Care 32(Suppl 2):S157–S163 2009). Muscle tissue is originated during embryo development, from the fusion of mesoderm progenitors. During the neonatal and early juvenile period, even if the myofibre number remains constant, new postnatal multipotent cells, called satellite cells (SCs), can fuse and increase the number of nuclei per fibre (Biressi S, et al, Dev Biol 379(2):195–207, 2013). Adult skeletal muscle is organised in long fibres generated from the fusion of single cells into a unique syncytium able to collect thousands of nuclei and of myofibrils. Each myofibril contains multiple sarcomeres, formed by actin and myosin filaments to generate contraction and develop force. High heterogeneity is present among single fibres, including slow-contracting fatigue-resisting type I fibres (also known as slow-twitch, tonic or simply slow fibres) and fast-contracting fatigue-unresisting type II fibres (fast-twitch, phasic or fast fibres). To exert a good performance, the expression of specific contractile proteins and metabolic enzymes is necessary together with a regular connection to a single motor neuron and an efficient vascularisation. Myotendinous junctions connect myofibres from one side and bones in the skeleton on the other side, allowing transmission of contractions to other muscles. Daily wear and tear

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or excessive load and damage of the fibre integrity activate SCs that undergo fusion. The highly orchestrated process has been thoroughly studied. Nevertheless, understanding the role of local and circulating cell types in adult myogenesis still remains a matter of discussion (Relaix F, Zammit PS, *Development* 139(16):2845–2856, 2012). In this chapter after introducing the biological events occurring in acute and chronic skeletal muscle damages, cell and gene therapy approaches are considered as novel tools to preserve muscle function.

Keywords Skeletal muscle regeneration • Chronic inflammation • Adult and pluripotent stem cells • Gene editing • Clinical trials

3.1 Introduction: Muscle Damage

Muscle damage can be local and limited, so that it is easily repaired without the activation of the immune compartment. Focal myofibre damage is a reversible process involving the plasma membrane, myofibrils and myonuclei, not supported by inflammatory response activation. This kind of damage involves subcellular structures that are rapidly repaired by fusion to the plasma membrane of subsarcolemmal vesicles [6]. For instance, eccentric contraction can induce myofibrillar alterations and leads to sarcomere remodelling. The same stressing effort induces also disruption of focal plasma membrane, with involvement of dysferlin, caveolin-1 and other proteins. *Dysferlin* gene mutations generate Limb-Girdle Muscular Dystrophies 2B (LGMD2B) in humans [7].

The lesions can become severe due to traumatic reasons, such as muscle strains in sport medicine (in case of resistance training or myotoxin exposure), or are irreversible due to genetic defects, such as multiple kinds of myopathies or muscular dystrophies. In these cases, necrosis of the whole or segments of the myofibre takes place and activates the inflammatory response. Infiltration of activated macrophages and final fusion of SCs with a pre-existing damaged myofibre are events observed during a process called muscle regeneration. This regenerative process has been described to recapitulate the phases of embryonic myogenesis, starting with fibre necrosis, inflammatory system involvement, SC activation, proliferation and, finally, differentiation of SC-derived myoblasts [8].

3.1.1 Necrosis and Chemoattractant Release

Whenever a fracture or any other trauma activate the inflammatory system, a defined cascade of events takes place, in order to ensure the repair of damaged muscle tissue, the complete reconstruction of the muscle structure and, most important, the physiology.

After muscle fibre degeneration, fibre integrity disruption and permeability increase. All the cytosolic components are released in the circulation, together with other common enzymes (i.e. creatine kinase) [9] and microRNA

(i.e. miR-133a) [10], playing the role of chemoattractant for the innate leukocytes. Desmin, heat shock proteins, chromatin-associated proteins, High-Mobility Group Box 1 (HMGB1) and many other intracellular players are dispensed actively through non-canonic pathways. These have been demonstrated to be responsible for the immune system activation. Lately, studies in a mouse model lacking leukocyte HMGB1 in the haematopoietic compartment have revealed a new role for HMGB1 in injured muscle. This molecule responds to hypoxia controlling neoangiogenesis through angiotensin-2 expression [11].

Experimentally, damaged fibres are visible through the uptake of small molecules, like Evans blue or Procion-orange dyes, indicators of damage after extensive exercise or sarcolemmal permeability related to genetic diseases [12]. Membrane changes and calcium (Ca^{2+}) influx increment together with sarcoplasmic reticulum Ca^{2+} release contribute to the activation of Ca^{2+} -dependent proteases, such as calpains. These proteases, starting from the degradation of big myofibrillar and cytoskeletal proteins (i.e. titin), prepare the substrates for other proteolytic systems (such as ubiquitin proteasome and caspases) that participate to the degradation in amino acids and ultimately to muscle atrophy [13].

3.1.2 *Inflammatory Response Activation*

An increase in the local myeloperoxidase activity is correspondent to the activation of neutrophils, the first inflammatory cells, that can modify the damaged tissue to allow phagocytosis of debris [14]. Moreover, the neutrophils are responsible for the peak of cytokine secretion till 24 h after injury, contributing to the modulation of the regenerative process [15]. Thanks to the secretion of specific cytokines, i.e. Interleukin 4 (IL-4), eosinophils regulate macrophage attraction. Beyond this chemoattractant role, IL-4 has been demonstrated to promote skeletal muscle differentiation [16].

Due to the proximity to capillaries, myogenic progenitors attract monocytes from the blood through chemotactic molecules, such as Monocyte Chemoattractant Protein-1, macrophage-derived chemokine, fractalkine and Vascular Endothelial Growth Factor (VEGF) [17]. In vivo studies have shown the activation of both circulating and resident macrophages after injury [18].

SC activation and proliferation are concomitant to M1 macrophage activation in vitro and in vivo. The M2 macrophage population is more prominent during the first phases of muscle differentiation. In coculture with SCs, they can induce better differentiation with higher levels of myogenin, a marker for complete myogenic differentiation [19]. Both resolution of inflammation and myofibre repair are driven by the transition from M1 to M2 macrophages, through dephosphorylation of the Mitogen-Activated Protein Kinase (MAPK) p38 and anti-inflammatory cytokine production [20]. A decrease in fibre growth has been reported in case of ablation of anti-inflammatory IL-10 release in vivo, impairing the transition from M1 to M2 macrophages and, more recently, disturbing skeletal muscle differentiation of other muscle precursors [21].

Besides macrophages, emerging evidences stress the role of the lymphocyte subsets, such as Th17 and regulatory T cells, able to activate neutrophils in case of prolonged muscle activity or endurance exercise [22]. These two main groups of T lymphocytes can exert distinct roles in inflammatory conditions, both regulating muscle regeneration and secreting molecules able to cross-react with macrophages and contribute to the activation of the humoral immunity. In particular, activation of cell-mediated immunity can be due to CD4⁺ Th1 secretion of anti-fibrotic cytokines, such as IL-12, IL-2, Tumour Necrosis Factor-alpha (TNF α) and Interferon-gamma (IFN γ). On the other side, pro-fibrotic cytokines, secreted by CD4⁺ Th2, such as IL-4, IL-5, IL-6 and IL-13, activate humoral immunity [23] and maintain the polarisation of macrophages in vivo [24].

Other soluble mediators can impact on the myogenic process influencing both macrophages and SC proliferation, thereby their differentiation. For instance, TNF α , usually present in the injured muscle microenvironment, facilitate the phagocytosis of cell debris. Nevertheless, high and prolonged doses of TNF α impair regeneration through Nuclear Factor-kappa beta (NF- κ B) activation and MyoD inhibition, resulting in the maintenance of proliferative SCs, without stimulus to differentiate [25]. The same block in differentiation is also the result of TNF α stimulation inducing phosphorylation of Small Mothers Against Decapentaplegic family members 2 and 3 (SMAD2/3) and fusion inhibition [26]. In addition, M1 macrophages produce Activin-A that delays the conversion to M2 macrophages in vitro and consequently the release of anti-inflammatory cytokines, such as IL-10 [27]. Besides the effect of TNF α , IFN γ also seems to delay myoblast fusion and differentiation in vitro, causing both defects in proliferation and low expression of late markers of myotube differentiation [28].

Besides inflammatory cytokines, the skeletal muscle itself is able to secrete myokines with both a paracrine and endocrine effect [29]. With paracrine effectors, people mainly refer to cytokines, such as IL-15 and Leukaemia Inhibitory Factor (LIF) [30], while the term myokines is used to list molecules able to induce anti-inflammatory cytokines, such as IL-10 and IL-1 [31], usually with an endocrine range of action, such as myonectin, IL-6 and oncostatin M [32], irisin and calprotectin [33].

3.1.3 Skeletal Muscle Regeneration: Activation, Proliferation and Fusion of Muscle Precursors

After muscle degeneration, the process of regeneration both in physiological (extensive exercise) or pathological conditions (musculopathies) is a finely organised sequence of events, characterised at first by the proliferation of muscle precursors. At a morphological level, newly formed fibres are small centronucleated structures, actively synthesising proteins, thereby basophilic and positive for embryonic

Myosin Heavy Chain (MyHC) [34]. Architectural changes due to incomplete fusion of some of the SCs or the myotubes can be the origin of forked myofibres that are usually more present in old subjects or patients suffering from neuromuscular diseases, suggesting a possible alteration in regenerative ability [35]. Finally, increased scar tissue could avoid the continuity between the two sides of a damaged myofibre, leading to formation of new myotendinous tissue and to the decrease in elasticity of the entire tissue.

SCs are quiescent cells characterised by a subliminal position and by a G₀ phase. Pax7 expression is verified for SCs till the quiescent state is maintained. The disruption of Pax7 in SCs leads to cell cycle arrest and dysregulation of myogenic transcription factors [36].

SCs upon activation are extensively migrating to the site of injury stimulated by different cues. Once arrived, SCs start to express two of the myogenic regulatory factors, Myf5 and MyoD [37], with different function in adult myogenesis [4]. *Myf5*-null mice develop SCs with compromised proliferation and show myofibre hypertrophy [38]. Genetic ablation of MyoD reduces murine muscle mass and induces impairment of muscle regeneration with an accumulation of cells in the damaged area unable to complete the differentiation process. The importance of this gene has been as well demonstrated by ectopic expression of MyoD in NIH3T3 fibroblasts, as sufficient to complete the myogenic activation. Terminal differentiation starts when the myogenin expression takes place. Myogenin controls the fusion of myoblasts in both myotubes in vitro and myofibres in vivo [39]. At the end of the process of regeneration, the centronucleated myofibres grow in size (protein content) but also in number of myonuclei that, once pushed to the periphery of the fibre, will allow recovery of the morphological and physiological properties of the entire muscle.

3.2 Chronic Inflammation: When the Inflammatory Process Goes Too Far

Numerous papers are pointing at the effect that the immune system has on the differentiating environment in skeletal muscle during the onset of regenerating conditions. To this extent, physical exercise, and, in particular, long-term training, has been demonstrated to educate the immune system to survey the host microenvironment against muscle micro-injuries [40]. Eventually, this activation has a role against potential malignancies and acquired pathologic conditions. Indeed, whenever the inflammation acquires a chronic aspect, it can compromise wound healing and cause fibrosis, with the possibility to induce muscle wasting. In these cases, inflammatory cells acquire a deleterious effect with consequences on the myeloid population and on the lymphoid compartment of the immune system [41].

3.2.1 Immune Cell Involvement in Skeletal Muscle Injuries

To discriminate the role of different cells in fibrosis and other molecular hallmarks, the development of different mouse models has been essential to display deficiencies in the immune system associated to the absence in dystrophin expression (*mdx* mice, representing characteristics of Duchenne Muscular Dystrophy, DMD). *Scid/mdx* mice deficient in functional lymphocytes have shown a decrease in Transforming Growth Factor beta (TGF β) levels and fibrotic tissue in the diaphragm, allowing the study of T cells in the development of fibrosis [42]. Same results were obtained for osteopontin-deficient *mdx* mice, in which a decrease in Natural Killer (NK) cells and neutrophils has been associated to reduced infiltration and fibrosis [43]. Whether this is a peculiarity of DMD pathologic condition or the normal terminal point reached when the immune system pushes peripheral tolerance to breakdown is still a matter of discussion. The involvement of myeloid cells in the *mdx* mouse model has been demonstrated reporting macrophage activation. Moreover, since the diaphragm of *mdx* mice exhibits high levels of C-C Chemokine Receptor type 2 (CCR2) and its ligand, together with inflammatory monocytes (Ly6C^{high}) and accumulation of CD11b^{high} monocyte-derived macrophages, a new role for monocytes has been proposed. Indeed, CCR2 loss of function reduces the number of monocytes recruited from the bone marrow and restores macrophage polarisation preventing a pro-inflammatory phenotype. Similar results have been obtained when a mutated isoform of the ligand is released by implanted Mesenchymal Stem Cells (MSCs), showing a better histopathological condition for the diaphragm and increased skeletal muscle force [44].

Modulation of the inflammatory response, minimal off-targets and survival improvement have been obtained in case of injections of myeloid cells (F4/80⁺ mature macrophages) together with GFP⁺ SCs, in situ, in *mdx* or injured muscles. Conflicting results on which type of macrophages could be more suitable for transplantation make the need of further studies more and more necessary. Several studies have identified an involvement for mast cells in normal skeletal muscle repair. Of interest for this section, mast cells have been shown in the proximity of vessels in *mdx* mice and in human clinical trials, some stabilisers of these cells (oxatomide and cromolyn) have been tested for the possibility to improve muscle repair [45, 46].

3.2.2 Fibrotic Component in Skeletal Muscle Regeneration

If the injury is not solved and physiologic conditions are not restored, fibrinogen or fibrin fragments accumulate and induce the production of connective tissue, exacerbating chronic dystrophic failure. It has been described that *mdx* mice in a *DBA/2* background (*D2-mdx*) exhibit loss of skeletal muscle weight, weakness, fibrosis and fat accumulation and, after multiple injuries, poor regeneration ability. Differently, when fibrinolytic treatment in skeletal muscle avoids fibrin and myeloid

cell accumulation, in particular M2 macrophages, muscle tissue permeability is reduced in *mdx* mice. Activated M2 macrophages express high levels of TGF β and arginase 1, both important molecules able to drive fibrosis and impair muscle regeneration. Moreover, inducing M2 macrophages to express arginase 1 at progressive stages of the disease, in the skeletal muscle and heart, worsens fibrosis and contributes to lethality, while its ablation decreases the fibrotic process. The role of fibrinogen is to drive inflammation signalling through Mac-1 ($\alpha_M\beta_2$ -integrin) on the surface of myeloid cells inducing the expression of pro-inflammatory cytokines and chemokines [47]. As a matter of fact, lower levels of fibrosis in the diaphragm, decreased infiltration and low levels of pro-inflammatory cytokines were reported in mutated Mac-1 mice [48].

3.2.3 Immunomodulatory Drugs

Since fibrosis development could negatively interfere with both gene and cell therapies, reducing the integrity of the tissue for cell safe engraftment, many strategies are focusing on the treatment of fibrotic cells and molecules [49]. In particular, halofuginone (also known as HT-100), a drug promoting regeneration with decreased degree of inflammation, was reported to improve histopathology in dysferlinopathies. Interestingly, the same molecule was described as specific inhibitor of Th17 cell differentiation, suggesting an immunomodulatory effect in dystrophic muscles [50]. A phase I–II clinical trial has been started on the safety and tolerability of this drug (NCT01847573). Thereby, immunomodulator administration in DMD patients has positive effects, reducing M1 macrophages and pro-inflammatory cues [51, 52]. On this line, cyclosporin-A (CyA) treatment has been demonstrated to be beneficial when administered to *collagen VI $\alpha 1$* -null mice, both in restoring the mitochondrial compartment and recovering muscle strength. CyA increased the SC pool and stimulated the differentiation into myofibres [53]. Furthermore, immunosuppressive drugs have also shown to induce myogenesis in myopathic patients, recognising the link between immune system and muscle regeneration, not strictly reduced to Muscular Dystrophies (MDs) but extended to different myopathies. Patients with Ullrich Congenital Muscular Dystrophy (UCMD) have received a treatment of CyA able to correct mitochondrial dysfunction, increase muscle regeneration and decrease the number of apoptotic nuclei. Long-term treatment with CyA has been confirmed to ameliorate performance in the limbs [54].

3.2.4 Detrimental Effects of Cytokines During Chronic Inflammation

SC proliferation is repressed in case of IFN γ secretion and M1 subtype activation [19] with increase in degradation of damaged dystrophin [55]. In case of high

concentration of IL-4, IL-10 and corticosteroid, M2 macrophages are activated and produce TGF β , accumulating in fibrotic areas. M2 macrophages have been found in the fibrotic areas of *mdx* mice diaphragm [47]. TGF β has been related to inhibition of SC proliferation, differentiation and degeneration into fibroblast-like cells able to produce collagen and increased reactive O₂ species in *mdx* mice. TGF β or TNF α and HMGB1 inflammatory cytokines can all mediate the activation of p38. In particular, α and β isoforms, once activated, promote MyoD heterodimerisation [56] and Mef2 transcriptional activation [57], while γ isoform in SCs inhibits MyoD transcriptional activity by direct phosphorylation [58]. In contrast, p38 α represses SC determination, avoiding the exit from cell cycle [59]. On this line, other inflammatory cytokines, like IL-6 and LIF, through Janus Kinase (JAK) activation and Signal Transducer and Activator of Transcription 3 (STAT3) phosphorylation, repress *MyoD* target gene favouring the expansion of Pax7⁺ cells [60].

3.2.5 Chronic Inflammation in Muscle Degenerative Processes

An inflammatory environment is not only the result of continuous damage and regeneration, happening in case of myopathies and MDs, but can be as well the final step of many other acquired syndromes (i.e. cancer cachexia). On this line, the inflammatory cues usually established during cancer are responsible for proteolytic system hyperactivation in myofibres and increased proliferation in skeletal muscle precursors. It has been reported that SCs and other precursors, both in mice and human samples, are able to proliferate but do not achieve an adequate muscle differentiation [61, 62]. New insights to limit such an increment or to unravel the stimuli that induce muscle precursors differentiation could be a useful strategy to counteract muscle atrophy. In this perspective, the activation of the transcription factor NF- κ B following pro-inflammatory cytokines sustains Pax7 expression and inhibits MyoD activation [62]. Moreover, mesenchymal progenitors expressing Platelet-Derived Growth Factor Receptor- α (PDGFR α) and Stem cell antigen-1 (Sca-1) are unable to complete their support in myogenic differentiation. Analogously, proliferation of these cells seems dependent on IL-4/IL-13 signalling and able to inhibit adipocyte differentiation [63]. In old animals, this population is unable to activate any myogenic differentiation, showing a different behaviour depending on their environment [64]. Transplantation of young mesenchymal progenitors into an old dystrophic environment has shown their plasticity, opening the possibility for novel applications in clinical trial [65].

Recently, in old animals, SC number might not critically influence muscle atrophy, although it could delay skeletal muscle regeneration and induce fibrosis [66]. Moreover, the possibility for SCs to switch from a quiescent to a senescent state in geriatric conditions has already been addressed [67]. A fine regulation of STAT3 in mouse and human SCs is important for the progression of differentiation [68]. Chronic degenerative stimuli could favour this pro-differentiative pathway leading to SC pool exhaustion in condition of sarcopenia, the natural process

of muscle mass and function decline with age. A pharmacologic inhibition of STAT3 could have a therapeutic relevance in chronic regenerative cases. As already proposed in the past, SCs during elderly could easily convert into fibrogenic fate mainly due to humoral factors that activate Wnt signalling pathway [69]. Finally, also in old patients, a regular and not extensive exercise could educate the immune system in order to repress pro-inflammatory monocytes and increase regulatory T cells [40]. Indeed, exercise remains the most physiological stimulus that can regulate both myogenesis and metabolic pathways able to counteract muscle wasting and educate the inflammatory system [70].

3.3 Stem Cell Therapies in Degenerating Muscles

Several stem cell types have been described in the skeletal muscle tissue, mainly located in the interstitium. The majority of these cells seem to be directly involved in muscle regeneration, although some of these exert supportive effects on muscle fibres. The ideal cell candidate contributing to muscle regeneration (Table 3.1) should respect the following criteria: (1) to exist in humans, (2) to have a myogenic differentiation potential, (3) to exert migration ability, (4) to express homing abilities into areas of muscle degeneration, (5) to be easily accessible and expand under in vitro conditions and (6) to be genetically editable and suitable in autologous context.

3.3.1 *Muscle-Resident Stem Cells*

3.3.1.1 Satellite Cells (SCs)

SCs, first identified in 1961 by Katz and Mauro in the muscles of frog and rat [71], represent the adult stem cell population of skeletal muscles. SCs are mononucleated cells located between the basal lamina and sarcolemma of the skeletal muscle fibres. In mature healthy muscles, SCs are in a quiescent unipotent state and enter the cell cycle as multipotent precursor cells (MPCs) to contribute to the formation of new muscle fibres in response to muscle injury, disease or during muscle physical activity (exercise or stretching) [72]. After activation, SCs proliferate extensively generating myoblasts that fuse rapidly to form multinucleated myofibres. When SCs are ablated, no other cell types can start skeletal muscle regeneration, indicating the need of resident SCs to recruit or guide any other possible contributor to muscle differentiation [73]. The localisation in specific anatomical structures and the exposure to the skeletal muscle niche signalling direct SC self-renewal. SCs undergo both symmetrical and asymmetrical cell division, mainly depending on the location of the daughter cells with respect to the myofibre. A small proportion of SCs do not undergo terminal differentiation, but repopulate the reserve pool of quiescent cells

Table 3.1 Adult stem cells involved in adult myogenesis

Cell type	Source	Anatomic localization	Molecular markers	Myogenic differentiation	Model	Systemic delivery	References
SCs (myoblasts)	Skeletal muscle	Between basal lamina and sarcolemma of muscle fibres	Pax7, MyoD, M-cadherin, CD56, desmin	Spontaneous	<i>nude</i> mice, <i>scid/mdx</i> mice, <i>scid/beige</i> mice, <i>nod/scid</i> mice, <i>Rag2</i> ^{-/-} (<i>γC</i> ^{-/-} <i>C5</i> ^{-/-} <i>/mdx</i>) mice, <i>Rag2</i> ^{-/-} <i>il2rb</i> ^{-/-} (<i>/mdx</i>) mice and GRMD dogs	No	[71, 72, 74]
					<i>scid</i> mice, <i>scid/beige</i> mice, <i>nod/scid</i> mice, <i>Rag2</i> ^{-/-} (<i>γC</i> ^{-/-} <i>C5</i> ^{-/-}) mice	Yes	[88, 89]
Pericytes	All body tissues	Perivascular, surrounding capillaries and microvessels in all tissues	ALP, CD140b, desmin, NG2, αSMA	Spontaneous			
MABs	Skeletal muscle and heart	Perivascular	ALP, CD140b, Sca-1, NG2	Embryonic: spontaneous; Adult: induced by C2C12 coculture	<i>scid/mdx</i> mice, <i>scid/beige</i> mice, <i>Rag2</i> ^{-/-} (<i>γC</i> ^{-/-} <i>C5</i> ^{-/-}) mice and GRMD dogs	Yes	[94, 96]
					<i>scid</i> mice	Not tested	[79]
MyoECs	Skeletal muscle	Interstitial	CD34, CD56, CD144	Spontaneous			
MDSCs (“MuStem cells”)	Skeletal muscle	Endomysium	Sca-1, vimentin, desmin	Spontaneous	<i>scid/mdx</i> mice and GRMD dogs	Not tested	[108, 110]
					<i>mdx</i> mice and <i>scid/mdx</i> mice	Yes	[112, 113]
PICs	Skeletal muscle	Interstitial	PW1, CD34, Sca-1	Spontaneous			
SP cells	Skeletal muscle	Interstitial	Abcg2 transporter	Induced by C2C12 coculture	<i>mdx</i> mice	Yes	[114]

FAPs	Skeletal muscle	Interstitial	CD34, CD140a, Sca-1	No myogenic differentiation, paracrine effect	<i>mdx</i> mice	Not tested	[118, 119]
MSCs	All body tissues	Bone marrow	CD73, CD90, CD105	Sporadic myogenic differentiation	<i>nude</i> mice, <i>mdx</i> mice and <i>Rag2</i> ^{-/-} (<i>γC</i> ^{-/-} <i>C5</i> ^{-/-}) mice	Yes	[120, 121]
CD133 ⁺ cells	Peripheral blood	Peripheral bloodstream	CD133, CD34, CD90, CD44, CD45, LFA-1, PSGL-1, VLA-1, L-selectin, CXCR4	Induced by C2C12 coculture	<i>scidl</i> (<i>mdx</i>) mice and <i>Rag2</i> ^{-/-} (<i>γC</i> ^{-/-} <i>C5</i> ^{-/-}) mice	Yes	[129]
CD133 ⁺ cells	Skeletal muscle	Interstitial	CD133, CD34, CXCR4	Spontaneous, but limited	<i>scidl</i> (<i>mdx</i>) mice and <i>Rag2</i> ^{-/-} (<i>γC</i> ^{-/-} <i>C5</i> ^{-/-}) mice	Yes	[129]

to mediate further rounds of muscle regeneration. The identification in different cell states (quiescence, activation or proliferation) for both murine and human SCs is based on the expression of numerous molecular markers [74].

In mice, paired box transcription factor Pax7 [75], adhesion molecule M-cadherin, sialomucin surface receptor CD34, integrin $\alpha 7\beta 1$, transmembrane Heparan sulphate proteoglycan syndecans 3 and 4 (Hspg3 and Hspg4), C-X-C chemokine Receptor type 4 (CXCR4) [76] and cytoskeleton protein desmin [77] are used as markers. In human CD56 (also known as Neural Cell Adhesion Molecule, NCAM) [78], although not SC specific, and the nuclear transcription factor Pax7 [75] are usually combined with the nuclear laminin A/C to identify SCs in their *in vivo* niche [78].

In the human adult skeletal muscle, a subpopulation of SCs that co-express vascular system markers has been identified as myoendothelial cells (MyoECs). These cells can be expanded for long-term *in vitro* and support muscle regeneration superior to myoblasts. MyoECs, located between the muscle fibres, represent approximately 0.4% of the muscle-resident stem cells, sharing myogenic (CD56⁺, CD34⁻, CD144⁺) and endothelial features (CD56⁻, CD34⁺, CD144⁺) [79]. The strong myogenic commitment and feasible isolation and expansion of SC-derived myoblasts *in vitro* pointed out these cells as the first candidates for stem cell therapy in muscular diseases. However, pioneering studies in mice models showed poor survival and migration abilities for transplanted SCs [80].

Preclinical and Clinical Studies Using SCs

SC-derived myoblasts were the first cell type used for cellular therapy, seen their rapid exhaustion in DMD patients [81]. In 1989, the group of T. Partridge demonstrated that intramuscular injected myoblasts derived from neonatal mice could restore dystrophin expression in *mdx* mice [82]. Subsequently, multiple studies confirmed these results by using murine neonatal [83] or adult [84] myoblasts as well as myoblasts from human origin [85]. In the beginning of the 1990s, these results were translated to several human clinical trials. Although no adverse effects were observed, only in a few cases myoblast-mediated dystrophin expression was documented. Overall, transplantations failed to provide sustained clinical benefits to the patients due to immunological rejection by the host, poor survival and dispersion of the injected myoblasts that significantly decrease their engraftment. Therefore, in general, SCs are cultured on soft substrates to preserve their engraftment ability. Another strategy to increase SC engraftment is to pretreat cells with growth factors, leading to partial colonisation in pilot clinical studies [86]. Nowadays, intramuscular injections of SC-derived myoblasts are still tested as potential treatment for localised forms of muscle disorders, although several multiple injections would be required (NCT02196467, Table 3.2). A recent study, investigating the possibility of the systemic delivery of myoblasts via the intraarterial route, showed that myoblasts are unable to cross the endothelial blood vessel barrier [87]. Promising results

Table 3.2 Stem cell-based clinical trials for MDs

MD type	Phase	Trial status	Number	Eligibility	Cell type	Dose	Route	Principal investigator sponsors/collaborators
DMD	I-II	Recruiting	NCT02196467	16 years and older, M	Eterologous myoblasts	30×10 ⁶ cells/cm ³ in extensor carpi radialis	i.m.	Craig Campbell and Jack Puymirat-Centre Hospitalier Universitaire de Québec, CHU de Québec, Canada
OPMD	II	Completed	NCT00773227	18-75 years, M/F	Autologous myoblasts	178×10 ⁶ cells into constrictor muscles of pharynx	i.m.	Assistance Publique – Hôpitaux de Paris, France
FSHD	I	Recruiting	NCT02208713	18-50 years, M/F	Autologous MDSCs and adipose-derived MSCs	Unknown	i.m.	Leila Arab, MD-Royan Institute, Tehran, Iran
DMD	I-II	Completed	EudraCT-2011 - 000176-33	6-14 years, M	HLA-identical allogeneic MABs	50×10 ⁶ /kg (0.5×10 ⁸ /kg), distributed in 4 increasing doses	i.a.	Giulio Cossu, Fondazione Centro San Raffaele del Monte Tabor, Milan, Italy
Refractory idiopathic inflammatory myopathy diseases	I	Recruiting	NCT00278564	16-65 years, M/F	Autologous HSCs	Unknown	i.v.	Richard Burt, MD, Northwestern University, Chicago, USA
DMD	I-II	Recruiting	NCT01834066	6-25 years, M/F	Autologous BM-MSCs	100×10 ⁶ each dose, 6 doses in 3 months	i.v.	Dr. Sachin Jamadar, C0- Investigator, Chaitanya Hospital, Pune, India
DMD	I-II	Recruiting	NCT02285673	7-20 years, M	UCMSCs	Unknown	i.v.	Ercument Ovali, Acibadem University, Istanbul, Turkey

i.m. intramuscular, *i.a.* intraarterial, *i.v.* intravenous

have been achieved in the case of Oculo Pharyngeal Muscular Dystrophy (OPMD), characterised by muscle wastage in eyelid and pharyngeal muscles. Autologous transplantation of unmodified myoblasts isolated from healthy muscles has shown beneficial effects in preclinical work and has entered clinical trial (NCT00773227, Table 3.2) for the treatment of dysphagia in OPMD. Another clinical study for Facio Scapulo Humeral muscular Dystrophy (FSHD), involving myoblast autotransplantation without immunosuppression or genetic correction, is currently ongoing (NCT02208713, Table 3.2).

Overall, SCs/myoblasts have two main disadvantages for cell therapy purposes: (1) these cells are often exhausted in dystrophic conditions (like DMD), and (2) they cannot be administered systemically but only intramuscularly [88]. Therefore, other stem cell types, distinct from SCs, have been studied and identified extensively for their myogenic potentials in animal models over the past years or in clinical trials, including vessel-associated cells, Muscle-Derived Stem Cells (MDSCs), side population (SP) and muscle interstitial cells. In a human setting, the correspondent of these stem cell types has not yet been found.

3.3.1.2 Pericytes/Mesoangioblasts (MABs)

Pericytes, the mural cells of the blood microvessels, have been discovered and described in 1871 as a contractile cell population surrounding the endothelial cells of small blood vessels. Pericytes were initially described as regulators of the blood flow, although confusion still exists about their identity, ontogeny and progeny. They differentiate towards adipocytes, bones, cartilage and muscles [89]. Several molecular markers for pericytes have been characterised, depending on their presence in different tissues [90, 91]. Notably, their expression pattern is dynamic in developmental stages, in vitro culturing and after pathological insults, so that no single pericyte-specific marker has been identified. Among the general accepted markers for murine pericytes are CD140b and Nerve/Glial antigen 2 (NG2), crucial for survival and development, alanyl membrane aminopeptidase CD13, desmin and alpha-Smooth Muscle Actin (α SMA). However, in mice, quiescent pericytes are not positive for α SMA while its expression level is upregulated in tumours and inflammatory environments. In human skeletal muscles, pericytes are highly positive for NG2, Alkaline Phosphatase (ALP), annexin V, desmin, α SMA, vimentin and CD140b and negative for M-cadherin (CD146), NCAM, cytokeratins and neurofilaments (except for nestin), endothelial markers (CD31, CD34, KDR) or haematopoietic markers (CD45) [88]. Myogenic differentiation capacity is not restricted to muscle-resident pericytes, since pericytes isolated from human adult adipose tissue or placenta can contribute to myotubes in vitro and to dystrophin-expressing fibres in vivo [92]. Recently, a new expression profile has been described for the simultaneous purification of pericytes, MSCs and blood vessel-derived stem cell subpopulations from human skeletal muscles [93].

Mesoangioblasts (MABs) are vessel-associated progenitors, located in the interstitial space between the muscle fibres. MABs have been first isolated from the dorsal aorta of E9.5 mouse embryos [94] and in adult muscles from mice [95, 96], dogs [97] and humans [88]. Embryonic MABs are positive for CD34, c-Kit and Flk-1 but negative for Oct4, Nkx2.5 and Myf5 [94]. MABs of embryonic origin can differentiate to multiple derivatives of the mesodermal lineages in vitro and in vivo [98]. In mice and dogs, adult MABs are isolated from adult skeletal muscles based on their expression for ALP, Sca-1, integral membrane NG2, CD140a and CD140b (also known as PDGFR α and β isoforms, respectively) [88, 95, 99]. Adult MABs have a multipotent differentiation potential towards myo-, osteo-, chondro- and adipogenic lineages [100]. Human MABs are well studied both in vitro and in vivo. They express the adhesion molecule CD146, CD140b and NG2 but do not express haematopoietic or SC markers, including CD45, CD34, CD56, CD144 and Pax7 [88]. The human counterpart for the murine Sca-1 marker is still missing, although members of the human Ly6 proteins have been suggested as correspondent to Sca-1 with homologous functions. Intriguingly, a comparison among human MABs, MSCs and pericytes has identified some similar markers (CD10, CD13, CD44, CD73, CD90), opening new questions regarding their origin [101]. Human MABs easily proliferate in vitro and spontaneously differentiate into MyHC-positive myotubes. Moreover, they express immunomediated cytokines and receptors, indicating immunomodulatory properties by inhibiting the T-cell proliferation in vitro [102]. The ability to regulate immune responses by MABs opens new clinical applications.

Preclinical and Clinical Studies Using MABs

MABs are used in systemic cell therapy for MD in experimental models by intraarterial injection of either wild-type or genetically modified MABs. Injected MABs cross the blood vessel barrier and migrate to the dystrophic muscle, where, subsequently fused to muscle fibres, they reconstitute dystrophin expression. Moreover, MABs localise with the SCs and ameliorate morphologically and functionally the dystrophic phenotype [95, 97, 103–105]. MABs, in vitro transduced with a lentiviral vector carrying human mini- or micro-*dystrophin* after intraarterial injection in *scid/mdx* mice or Golden Retriever Muscular Dystrophy (GRMD) dogs, are able to migrate and contribute to the host skeletal muscle regeneration [88, 97]. Based on the outcome of these mice and dog studies, in March 2011, a phase I–II clinical trial has been started, in which four consecutive escalating intraarterial infusions of HLA-matched donor-derived MABs were given (EudraCT no. 2011-000176-33, Table 3.2). Safety and feasibility of the study have been proven. Two months after the last infusion, the first muscle biopsy was performed revealing low levels of donor DNA in the majority of the patients and low amount of donor-derived dystrophin just in one patient. Unfortunately, no functional improvements were observed [106].

3.3.1.3 Muscle-Derived Stem Cells (MDSCs): Skeletal Muscle Aldehyde Dehydrogenase-Positive Cells (ALDH⁺) and “MuStem Cells”

Another muscle-resident stem cell population, known as MDSCs, has been identified from mouse skeletal muscle and seems to be different from late-stage myogenic populations, like SCs and myoblasts, by their multipotent differentiation potential. Besides their myogenic capacities, they can differentiate *in vitro* and *in vivo* towards adipo-, osteo-, chondrogenic, haematopoietic, endothelial, smooth muscle, cardiac muscle and neural lineages [107]. Moreover, MDSCs secrete high levels of VEGF, promoting vascularisation and consequently facilitating tissue regeneration. MDSCs have a high expression of Sca-1, low levels of the fibroblast marker vimentin and low levels of desmin. These cells are negative for CD45, M-cadherin. Moreover, the low expression of the Major Histocompatibility Complex (MHC)-1 is a promising feature towards more successful stem cell-based therapy applications [108]. Intramuscular delivery of MDSCs, 4 days after induced injury, showed enhanced angiogenesis and reduced scar tissue formation. Interestingly, 1 week after injection, high levels of VEGF and high expression levels of antioxidant, glutathione (GSH) and superoxide dismutase were detected, increasing their survival after administration [109]. Aldehyde dehydrogenase 1A1 (ALDH) is a ubiquitously detoxifying enzyme involved in the metabolism of aldehydes and retinoic acid. ALDH⁺ cells are found in the bone marrow, umbilical cord and peripheral blood, although a cell population has been characterised with a high ALDH activity in human skeletal muscles [110]. These cells represent a small amount of the total mononucleated cells (2–4%). MDSCs have a higher survival ability compared to SCs and myoblasts after transplantation, probably due to their resistance to oxidative stress and high proliferation activity *in vivo*.

Preclinical and Clinical Studies Using ALDH⁺ MDSCs and “MuStem Cells”

Two distinct subpopulations of ALDH⁺ cells with different phenotypic and functional properties can be purified from skeletal muscle. The ALDH⁺ CD34⁺ cells have a mesenchymal profile, while ALDH⁺ CD34[−] cells rapidly upregulate CD56 *in vitro* and give rise to multinucleated myotubes. *In vivo*, ALDH⁺ CD34[−] cells injected in damaged muscles of immunodeficient mice contribute to muscle formation and migrate into the SC position. Interestingly, these CD34[−] cells display high proliferation rates after *in vivo* administration into the muscle of immunodeficient mice [110].

In 2011, MDSCs isolated from GRMD dogs, called MuStem cells, were isolated based on their delayed adhesion properties. These cells have high proliferation rates and seem to be committed to the myogenic lineage. MuStem cells have been shown to contribute to muscle regeneration in the GRMD dog, allowing dystrophin expression and relocation into the SC niche. A partial remodelling of the skeletal muscle tissue of GRMD dogs has been observed after intraarterial injection of

MuStem cells [111]. These data may indicate a potential therapeutic application for MuStem cells, although further studies have to be done to investigate their myogenic potential in humans.

3.3.1.4 PW1-Expressing Interstitial Cells (PICs)

PW1-expressing interstitial cells (PICs; also known as Paternally expressed gene 3, *Peg3*) are characterised by the localisation in the interstitium among muscle fibres. These cells are not embryonically related to SCs, since they do not derive from Pax3⁺ myogenic progenitor cells. PICs have a Pax7⁻/Sca-1⁺ and CD34⁺ marker phenotype [112]. Recently, a high expression of PW1 has been shown in MABs of mice, dogs and humans, and the expression levels correlate with their myogenic and migratory capacities. Silencing *PW1* significantly inhibits their myogenic differentiation capacity and their ability to cross the vessel wall, so consequently, their engraftment into damaged muscle tissue [113]. Therefore, PW1 may be used in the future as a biomarker to identify optimal donor populations for cell therapy in pathologic conditions.

3.3.1.5 Muscle Side Population (SP) Cells

SP cells are a rare subpopulation source of precursors associated to skeletal muscles. SP cells are characterised by the complete exclusion of the DNA-binding dye Hoechst 33342, due to their abundant expression of the *Abcg2* transporter. They show heterogeneity inside their population. 80% of the total SP cells are positive for the vascular endothelial marker CD31, while 2–10% are blood derived and show positiveness for the immune marker CD45. However, a small fraction has been characterised as highly positive for *Abcg2*, CD31 and CD45, especially during muscle damage and the early phase of regeneration. Recently, another SP cell subgroup has been identified, representing 5% of the total population, which are negative for both CD31 and CD45, and may express Pax7, Sca-1 and syndecan 4 [114, 115]. In coculture with myoblasts, SP cells are able to fuse in vitro to form myotubes. Interestingly, in vivo engraftment experiments show high myogenic differentiation capacity in regenerating muscles after acute tissue damage [116]. Moreover, several studies have shown their ability to restore dystrophin levels when injected intravenously into *mdx* mice [117].

3.3.1.6 Fibro-/Adipogenic Progenitors (FAPs)

In 2010, a new muscle stem cell population located in the interstitium has been discovered. Fibro-/Adipogenic Progenitors (FAPs) are non-myogenic, mesenchymal progenitors that differentiate towards both adipo- and fibrogenic cells, indicating a

potential contribution to adipose and fibrotic deposition in diseased muscles [118, 119]. These cells were isolated as $CD34^+/Sca-1^+/CD31^-/CD45^-/Lin^-/integrin\ \alpha7^-$ [118] or $Sca-1^+/CD140a^+/integrin\ \alpha7^-$ [119]. They exert beneficial paracrine effects on the muscle fibres. FAPs were used in preclinical models of MDs with histone deacetylase inhibitors (HDACi), able to promote in vitro and in vivo myogenic differentiation. Transplantation of FAPs in more advanced dystrophic muscles enhanced the regeneration by interacting with the muscle-resident SCs. Several studies have shown that FAPs from dystrophic muscles of *mdx* mice retain a bipotency in their phenotype and functionality, either by supporting regeneration at early stages of disease progression or by differentiating in fat and fibroblasts [64, 65]. The human FAP counterpart has been isolated as CD140a-positive cells in healthy and diseased muscles, although further studies are needed to investigate the molecular and functional characterisation to develop novel pharmacological treatments or to understand their ability as a cell therapy source for muscular diseases in human settings.

3.3.2 *Non-resident Stem Cells for Skeletal Muscle Regeneration*

3.3.2.1 Mesenchymal Stem Cells (MSCs)

MSCs are non-haematopoietic multipotent stem cells that can be found in several tissues, including bone marrow, umbilical cord, blood, placenta, liver, adipose tissue, muscle and synovial membrane. The bone marrow is the principal source [120, 121]. MSCs can renew and differentiate into multiple mesenchymal lineages, including adipo-, chondro- and osteocytes. MSCs are characterised by the expression of several markers (CD73, CD90 and CD105), although not specific for MSCs, and by the lack of haematopoietic markers (CD45, CD34 and CD14 or CD11b, CD19 and HLA-DR) [122].

Preclinical and Clinical Studies Using MSCs

The clinical relevance of MSCs is their modulating properties of immunological responses, which could be important for immunotolerance induction and to prevent rejection of allogenic transplantation. Moreover, they can secrete trophic factors and chemokines, which alters the local environment to facilitate and regulate endogenous tissue homeostasis [123]. Unfortunately, studies using MSCs isolated from human tissue, like adipocytes [124] or synovial membrane [125], have shown a weak beneficial effect in muscle regeneration. However, their accessibility and abundance from different tissues and their immunosuppressive and trophic characteristics make

MSCs an interesting cell candidate to be further investigated [126]. In 1998, the first report was published, showing the transplantation of genetically modified bone marrow cells into the injured muscle of immunodeficient mice [127]. Nowadays, two clinical trials (Table 3.2) started for autologous transplantation of bone marrow-derived stem cells (BM-MSCs; NCT01834066) and for Umbilical Cord-derived Mesenchymal Stem Cells (UCMSCs; NCT02285673) involving DMD patients. In addition, genetically modified MSCs for their Notch expression were injected intramuscularly in *mdx* mice and showed a better rescue of dystrophin expression [128]. Further studies are necessary to determine the immunomodulatory properties of MSCs and their interactions with the inflamed environment into damaged tissue.

3.3.2.2 CD133⁺ Cells

Another cell population with myogenic potential has been identified expressing CD133 [129–131]. CD133⁺ cells are a small subpopulation of the mononucleated cells present in the peripheral blood [129], expressing the stem cell marker CD133. Some cells have also been identified as positive for CD133 in human skeletal muscle [131]. The function of CD133 is still unclear. However, it could be a useful marker to purify haematopoietic and endothelial progenitors. A drawback of these cells is CD133 loss in vitro after expansion, hampering cell tracking after expansion or in vivo administration. CD133⁺ cells cocultured with murine myoblasts have the capacity to form MyHC-expressing myotubes [129].

Preclinical and Clinical Studies Using CD133⁺ Cells

Intraarterial or intramuscular injections of human peripheral blood-derived CD133⁺ cells in *scid/mdx* mice produce dystrophin, by fusion with the host fibres, and colonise the SC niche, expressing typical SC markers [129]. Moreover, local injections in rat injured muscle accelerate regeneration, suggesting the ability of these cells to produce VEGF and promote vasculogenesis, by differentiating to both endothelial and myogenic lineages. In addition, the regenerative capacity of CD133⁺ cells isolated from human skeletal muscle has been studied. After intramuscular delivery in immunodeficient mice, CD133⁺ cells showed a higher regeneration capacity compared to *bona fide* human SC-derived myoblasts. A study performed in immunodeficient and dystrophic mice opens the horizon for the use of (blood- and muscle-derived) CD133⁺ cells as possible candidates for (autologous) stem cell therapy applications for DMD patients [132]. Moreover, genetic modifications together with their ability to cross the blood vessel barrier allow systemic delivery and point out CD133⁺ as suitable cell population for cellular therapy.

3.4 Human Embryonic Stem Cells (ESCs) and Induced Pluripotent Stem Cells (iPSCs): Future Challenges for Stem Cell Therapies

Human Embryonic Stem Cells (ESCs) [133] and induced Pluripotent Stem Cells (iPSCs) [134] hold great potential for regenerative medicine. These PSCs are able both to repair damaged muscles and regenerate healthy tissues (Table 3.3). However, in vitro PSC-derived muscle precursor differentiation, obtained by well-defined medium conditions or genetic modification, is still challenging and needs further improvements in order to reproduce the in vivo environment. To favour expansion and differentiation, tissue engineering methods could be necessary to mimic the muscle progenitor stem cell niche and to accelerate myocyte maturation. Tissue engineering in combination with gene and cell therapy approaches opens new perspectives towards a successful development of therapeutic treatments in several MDs [135].

3.4.1 Embryonic Stem Cells (ESCs)

ESCs have been isolated from the inner cell mass of the murine [136] and human [133] blastocyst. The therapeutic applications of ESCs have been strongly debated in the scientific community, mainly because of ethical concerns and immune rejection issues. Moreover, their use needs to be carefully evaluated, since ESCs can lead to teratoma formation [137]. Few studies have described the use of human ESCs in muscle transplantation. One study documented the transplantation of mesenchymal precursors derived from human ESCs in cardiotoxin-injured tibialis anterior (TA) muscles of *scid/beige* mice. Although only a small cell proportion could be tracked in the muscle, a possible future role for ESCs in muscle cell transplantation could be suggested, since no tumorigenic effect was observed [138]. Several groups have documented the differentiation of myogenic progenitors from ESCs. Darabi et al. used inducible Pax3 and Pax7 overexpressing murine ESCs, injected intramuscularly or systemically administered in *mdx* mice, resulting in engraftment with dystrophin-expressing myofibres and improvements in the muscle function [139]. Similar outcomes were observed in mice affected with FSHD [140]. Zheng et al. showed that human ESCs exposed to serum in the presence of EGF directed differentiation towards myogenic precursors. Subsequent addition of the DNA-demethylating agent 5-azacytidine increases even more their myogenic potential. in vitro terminal differentiation and fusion into myotubes was not observed. However, transplantation of human ESC-derived cells into injured TA reached terminal differentiation, as well as localisation in the SC compartment [141]. Barberi et al. differentiated human ESCs to obtain mesenchymal precursors for myogenic progenitor cells. After transplantation in the hindlimb muscle of immunodeficient mice, long-term survival and myofibre commitment were reported

Table 3.3 PSC derivatives to improve adult myogenesis

Cell type	Source	Pluripotency markers	Myogenic differentiation	Acquired molecular markers	Model	Systemic delivery	References
ESC derivatives	Blastocyst inner cell mass	Oct3/4, Nanog, Sox2, Lin28, SSEA4, TRA-1-60	Induced by culture conditions or gene transfer	CD56, KDR, CD73, CD90, CD140a, M-cadherin, $\alpha 7$ integrin, SM/C2.6	<i>mdx</i> mice, <i>scid/beige</i> mice and <i>Rag2^{-/-} (γC^{-/-} C5^{-/-})</i> mice	No	[142]
iPSC derivatives	Reprogrammed somatic cells	Oct3/4, Nanog, Sox2, Lin28, SSEA4, TRA-1-60	Induced by culture conditions or gene transfer	CD56, KDR, CD73, CD90, CD140a, M-cadherin, $\alpha 7$ integrin, SM/C2.6	<i>mdx</i> mice, <i>scid/beige</i> mice and <i>Rag2^{-/-} (γC^{-/-} C5^{-/-})</i> mice	No	[143, 144, 146, 147, 148]

[138]. Nowadays, several preliminary studies have been performed with PSC-derived myogenic cells, showing their capacity to form muscles *in vivo*. However, their long-term engraftment into adult muscle and their functional contribution still have to be elucidated, as well as their depleted muscle stem cell pool repopulation.

3.4.2 Induced Pluripotent Stem Cells (iPSCs)

Human iPSCs can be generated from adult somatic cells by introducing a defined set of transcription factors to reprogram them towards an embryonic-like pluripotent state [134, 142–145]. Human iPSCs have been derived from fibroblasts [146] and Peripheral Blood Mononuclear Cells (PBMCs) of DMD and Becher Muscular Dystrophy (BMD) patients [147]. Myogenic lineage induction from murine ESCs and iPSCs has been described [140, 148], although terminal differentiation of human ESCs, as well as human iPSCs, is still facing some difficulties. Murine and human iPSCs have been provided to counteract muscle wasting in MDs [116]. So far, *in vitro* and *in vivo* studies have shown that myogenic precursors derived from iPSCs could form chimeric myotubes in coculture with C2C12 myoblasts. In addition, iPSC-derived myogenic precursors transplanted in dystrophic muscles contribute to an improvement in the contractile properties. The iPSC technology has been used to allow the generation of genetically corrected human DMD iPSCs by using a human artificial chromosome able to carry the full-length dystrophin gene. This strategy is an alternative to induce truncated forms of *dystrophin* (quasi-, mini-, micro-*dystrophin* genes) in mouse and human iPSCs [149]. Recently, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9-modified DMD human fibroblasts reprogrammed to iPSCs have been tested for skeletal and cardiac muscle differentiation abilities both *in vitro* and in *mdx* mice [150].

3.5 Ex Vivo Cell Therapies

In case of genetic diseases, it becomes more and more possible to envision an autologous source of muscle progenitors extracted from the patient, genetically corrected *in vitro* and consequently transplanted into the same donor. Autologous strategies would be preferable to avoid immune rejection or immunosuppressive treatment to restore the expression of the mutated proteins. Still, further studies need to be developed, to increase both efficacy and safety of genetic modification and to avoid immune reaction due to the expression of transgene products. Since most of the studies have been addressing DMD, truncated versions of synthetic *dystrophin* have been tested for their efficiency, since just the actin-binding and the cysteine-rich domains are essential for function, while many other region of the gene are dispensable [151]. Instead of native *dystrophin* 14 kb cDNA, transcript of 4–5 kb can be better included into viral vectors [152]. Therefore, lentiviral vectors have

been used to infect micro-*dystrophin* gene in SP cells [153] or SM/C2.6-positive cells [154] transplanted into *mdx* mice. In addition, genetically corrected canine [97] and human MABs [88] have been transplanted into experimental dystrophic mice and dogs.

Viral vector choice has to be carefully considered, especially with respect to vector insertional mutagenesis. Activating vector insertions near proto-oncogenes might induce T-cell leukaemia, 3–5 years after gene therapy, therefore requiring careful monitoring against oncogenic development [155]. In the last years, the Adeno-Associated Viruses (AAVs) have been pointed out as the first choice viral vectors for gene and cell therapy in MDs, due to their low immunogenicity and ability to exist in the transduced cells as an episome [156].

3.5.1 Adeno-Associated Virus (AAV)

AAV has already been used in many clinical trials since its broad tropism and infectivity, long-term expression and site-specific integration (NCT01344798; Table 3.4) [157]. Non-selective infection and organ-restricted transduction have pushed researchers to look for better vectors, based on different serotypes able to transduce more efficiently muscle tissue or to offer the best compromise between skeletal muscle expression levels and ability to escape human immune response [158]. A mini-*dystrophin* gene (rAAV2.5-CMV-mini-*dystrophin* gene vector) has been tested for safety (phase I) in the treatment of progressive muscle weakness due to DMD (NCT00428935) [159]. Analogously, a double-blind randomised study (NCT00494195) reported the administration of intramuscular injection of rAAV.tMCK.haSG gene vector in LGMD2D patients as being safe. In addition, AAV8 has been used to repress chronic inflammation and muscle degeneration specifically into *mdx* skeletal muscle, overexpressing an endogenous inhibitor of NF- κ B signalling pathway under the truncated Muscle Creatine Kinase (tMCK) promoter. Short hairpin RNA carrier AAV9 has been successfully developed to target the major subunit of NF- κ B (NF- κ B/p65) and ameliorate muscle pathologic phenotype in *mdx* mice [160]. Finally, AAV9 mini-*dystrophin* gene delivery combined to the addition of octalysine (8K)-NF- κ B essential modulator (NEMO)-Binding Domain (8K-NBD) peptide promoted a higher mini-*dystrophin* expression in skeletal muscle compared to AAV9 alone [161].

3.5.2 Exon Skipping

Since DMD is mainly caused by frameshift mutations in *dystrophin*, exon-skipping strategies have been developed. Indeed, skipping exons carrying an out-of-frame DMD mutation result in a final in-frame transcript, able to be translated in a

Table 3.4 Gene therapy or small molecule-based clinical trials for MDs

MD type	Phase	Trial status	Number	Eligibility	Strategy	Viral vector	Molecule	Principal investigator sponsors/collaborators
DMD	I	Completed	NCT00428935	5–15 years, M	Mini- <i>dystrophin</i>	rAAV2.5-CMV	N/A	Jerry R. Mendell, Nationwide Children's Hospital, Columbus, Ohio, USA
LGMD2D	I	Completed	NCT00494195	5 years and older, M/F	<i>Alpha-sarcoglycan</i>	rAAV1.tMCK	N/A	Jerry R. Mendell, Nationwide Children's Hospital, Columbus, Ohio, USA
LGMD2C	I	Completed	NCT01344798	15 years and older, M/F	<i>Gamma-sarcoglycan</i>	AAV1	N/A	Frederic Revah, CEO, Genethon, Evry Cedex, France
LGMD2B	I	Recruiting	NCT02710500	18 years and older, M/F	<i>Dysferlin</i>	rAAVrh74.MHCK7	N/A	Jerry R. Mendell, Nationwide Children's Hospital, Columbus, Ohio, USA
DMD	II	Completed	NCT01462292	5 years and older, M	Exon skipping (exon 51)	N/A	Drisapersen (GSK-2402968)	GlaxoSmithKline, Brentford Middlesex, UK
DMD	III	Recruiting	NCT01803412	5 years and older, M	Exon skipping (exon 51)	N/A	Drisapersen	BioMarin Pharmaceutical, San Rafael, CA, USA

DMD	II	Completed	NCT01396239	7–13 years, M	Exon skipping (exon 51)	N/A	Eteplirsen (AVI-4658)	Sarepta Therapeutics, Cambridge, MA, US; Jerry R. Mendell, Nationwide Children’s Hospital, Columbus, Ohio, USA
DMD	II	Ongoing	NCT01918384	4 years and older, M	Reading through	N/A	Arbekacin sulphate (NPC-14)	PTC Therapeutics, South Plainfield, NJ, USA; Yasuhiro Takeshima, Kobe University, Japan
DMD and BMD	II–III	Completed	NCT00592553	5 years and older, M	Reading through	N/A	Ataluren (PTC124)	PTC Therapeutics, South Plainfield, NJ, USA; Leone Atkinson, MD, PhD

N/A not applicable

truncated but still functional protein product [162], thereby leading to a less severe phenotype (similar to BMD). Similar genetic modification has been successfully used as well to correct other mutations such as exon 32 in *dysferlin* [163]. Morpholino oligomers have been directed against exon 51 and tested in proof-of-concept studies and clinical trials [164]. The same exon skipping has been extended to correct different cell types like CD133⁺ myogenic progenitors from DMD patients transplanted afterwards into *scid/mdx* mice. The corrected cells were able to recover histologically and functionally dystrophin presence [132]. Lately, the greatest percentage of *mdx* muscle fibres rescued in dystrophin has been calculated by immunostaining around the 38%, underlying the importance of undefined factors in the variable success of exon skipping method [165].

It is worth noticing that two advanced clinical trials have been carried out describing two drugs promoting exon 51 skipping. Drisapersen is a 2′O-Methyl-Phosphorothioate oligonucleotide (2′OMePS) that has already been tried successfully in a phase II clinical trial on DMD patients (NCT01462292, Table 3.4). In a phase III randomised, double-blind, placebo-controlled trial, it did not show statistical difference between treated patients and placebo (NCT01803412, Table 3.4). Eteplirsen is a more promising phosphorodiamidate morpholino oligomer (PMO) that gives a gradual increase in dystrophin fibres (from already 23% at a first time point till 52% at lower or 43% at higher concentration, after 48 weeks of treatment). The presence of functional dystrophin in the sarcolemma has been confirmed both by detection of sarcoglycans and neuronal Nitric Oxide Synthase (nNOS) and resulted in ambulation benefit compared to placebo patients (NCT01396239, Table 3.4). Eteplirsen-treated patients undergoing a 6 min walking test (MWT) have reported a lower decline in ambulation compared to untreated matched healthy controls [166].

3.5.3 Reading Through

Two other clinical trials (phase II randomised, placebo-controlled, double-blind studies) have been started in patients with nonsense mutations in *dystrophin*, comparing the efficacy and the safety of two different drugs with a similar effect: arbekacin sulphate (NPC-14; NCT01918384, Table 3.4) and ataluren (NCT00592553, Table 3.4). These drugs are targeting the translation machinery and inducing the reading through premature stop codons. The study with ataluren has already been concluded reporting at lower doses an improvement in the 6 MWT at week 48, while at higher doses it did not differ from placebo effect. Moreover, time function test of skeletal muscle, from patients treated with lower doses, showed amelioration in both trends for climbing and descending four stairs and running/walking 10 m. These positive results have promoted the drug for a phase III clinical trial, in which the ability to slow the disease progression in DMD population will be hopefully reconfirmed [167].

3.5.4 *Small Nuclear RNA (snRNA) Sequences*

Small nuclear RNA (snRNA) sequences that favour sublocalisation for the RNA and ameliorate the inclusion of antisense oligonucleotides into the spliceosome are the next-generation molecules, able to be packaged in adenoviral vectors, allowing persistent repair of the DNA without continuous administration. Therapies using this approach have been developed and applied to *dystrophin* exon 51 skipping [168]. Recently, a study was performed in 18 juvenile GRMD dogs intravenously infused with different volumes of rAAV8-U7snRNA-E6/E8 [169]. rAAV8-U7snRNA-E6/E8 is an antisense nucleotide able to avoid the point mutation in the acceptor splice site of intron 6 of the canine *dystrophin* gene, causing skipping of exon 7, disruption of the reading frame and premature termination of translation. The injection was performed into one forelimb, aiming to model human treatment, to define the therapeutic dose, to set up the injection protocols and to assess both gene therapy and delivery safety. A dose-dependent exon skipping has been reported for the dogs, opening the possibility for a phase I–II clinical trial, since the highest dose resulted even in 80% of dystrophin-expressing fibres, without acute or delayed adverse effects [169].

3.5.5 *Engineered Nucleases*

Improvements in different kinds of nucleases have opened the possibility to replace in phase small frameshift and point mutations [170]. In the last years, genome editing at exon 51 by mean of engineered nucleases, able to correct the disrupted reading frame like Transcription Activator-Like Effector Nucleases (TALENs), has been demonstrated to efficiently correct and restore the expression of a functional dystrophin protein in vitro, in immortalised myoblasts [171]. A newer approach to correct specific genome sequences consists of RNA-guided nucleases and type II CRISPR/Cas9 system [172]. Single-guide RNA molecules drive Cas9 nuclease to a targeted locus in the proximity of protospacer adjacent motif and generate a double-strand break that is then repaired by either nonhomologous end-joining (with the possibility of insertion/deletion mutations) or by homology-directed repair. To favour this last foreseen repair mechanism and remove the defect, an exogenous template can be provided, in order to generate a perfect mutation of the desired locus [173].

In particular, as a proof of concept, this technique has been used to correct mutations in the germ line of *mdx* mice with a nonsense mutation in exon 23 of the *dystrophin* gene. The study has reported a high percentage (47–60%) of dystrophin-positive fibres associated to 17% of gene correction, and, in older animals, almost no negative dystrophin fibres have been observed selectively in skeletal muscle, meaning a progressive rescue of dystrophin protein, as well in the SC compartment [174]. Alternative strategies have been foreseen to correct

postnatal tissues, since germ line editing is not affordable in human settings and homology-directed repair does not occur in postmitotic cells [175]. AAV9-delivery of gene-editing components has been administered in different ways to postnatal *mdx* mice, including intraperitoneal at postnatal day 1 (P1), intramuscular at P12, and retro-orbital at P18. Dystrophin protein expression has been restored with different degrees, increasing from 3 to 12 weeks after injection, in cardiac and skeletal muscle, the latter proved by grip strength test [176]. These recent results are encouraging and in the range of therapeutic benefit, since it has been estimated that low-level expression of dystrophin (4–15%) could be enough to protect from myo-injury due to eccentric contraction and could effectively ameliorate cardiomyopathy [177]. Still, gene-editing techniques need improvements to avoid off-target effects if an eventual translation to human settings is foreseen.

3.6 Conclusion

So far many efforts have been made to increase our understanding of molecular cues of MDs, since the use of anti-inflammatory drugs administered alone, or in combination with other molecules, showed poor beneficial effects in treated patients. In the last two decades, stem cell therapies for MDs are gaining momentum. In particular, the rising on the knowledge about the biology of multipotent and PSCs allowed to identify novel muscle progenitor cells besides SCs. Those novel identified myogenic progenitors have been tested in several animal models, including mice and dogs. Promising results have been obtained in decreasing the inflammatory burden, improving dystrophin expression and tissue architecture, with a beneficial effect on muscle function. AAVs have been approached in preclinical and clinical studies to mediate the delivery of micro-dystrophin or mini-utrophin, while exon skipping strategies have been proposed to correct the mutation of the endogenous *dystrophin* and rescue protein expression. Unfortunately, so far, both gene and cell therapy strategies produced poor results in terms of dystrophin restoration and muscle function amelioration. Nevertheless, intraarterially transplanted MABs from donor cells have been proved to be feasible and relatively safe, recovering dystrophin expression, at least in one muscle biopsy from treated patients. An intriguing perspective is also to consider the non-genetic cues to improve the myogenic fates of local or transplanted progenitors, maybe in combination with miRNAs and gene-editing (CRISPR and TALEN) technologies (Fig. 3.1). However, several practical issues need to be urgently solved to improve stem cell efficacy in clinic. Low cell survival, cell motility after transplantation and immune rejection are still the main problems for cell-based therapies of MDs. In conclusion, more preclinical studies are expected on myogenic derivatives from PSCs and possibly in combination with exosomes, miRNAs and gene-editing technologies to improve the efficacy of stem cell treatment in MDs.

Fig. 3.1 Present and future of stem cell therapies in MDs. Schematic representation of skeletal muscle tissue focusing on the resident and circulating cells with myogenic potential (*right panel*). On the *left* side, somatic cells reprogrammed to a pluripotent state (iPSCs) and differentiated to myogenic precursors show early markers of muscle progenitors and represent the future challenge. Marker expression profile makes iPSC-derived muscle progenitors suitable for isolation and subsequent transplantation. Genetic modifications are necessary for autologous cell therapies and eventually to improve myogenic commitment and safety of iPSC-derived muscle progenitors

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