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Review

Cell therapy strategies and improvements for muscular dystrophy

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Understanding stem cell commitment and differentiation is a critical step towards clinical translation of cell therapies. In past few years, several cell types have been characterized and transplanted in animal models for different diseased tissues, eligible for a cell-mediated regeneration. Skeletal muscle damage is a challenge for cell- and gene-based therapeutical approaches, given the unique architecture of the tissue and the clinical relevance of acute damages or dystrophies. In this review, we will consider the regenerative potential of embryonic and somatic stem cells and the outcomes achieved on their transplantation into animal models for muscular dystrophy or acute muscle impairment.
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Muscular dystrophies are a heterogeneous group of inherited diseases, primarily characterized by severe and chronic skeletal muscle degeneration. Duchenne muscular dystrophy (DMD) is the most severe disease among similar dystrophic diseases and is caused by frame-shift deletions, duplications, or point mutations in *dystrophin* gene. Patient mobility is highly affected, usually resulting in wheelchair dependency, and death occurs due to respiratory or cardiac failure.[1,2]

New strategies for the treatment of this disease are currently being investigated and are categorized by two approaches: endogenous activation and exogenous delivery (Figure 1). The first strategy consists of re-activating endogenous cells to achieve muscle hypertrophy, counteracting the mass/force loss in inflamed fibres. To reach this goal, a growing range of small molecules or recombinant proteins has been tested, including insulin-like growth factor 1 (IGF1),[3,4] MagicF1[5] or valproic acid.[6] The second strategy, on the contrary, relies on exogenous tools (gene and/or cell therapies) to improve muscle regeneration, thus providing new, functional fibres to the dystrophic muscle. Gene therapy targets the genetic defects, attempting to overcome pathological mutations by providing the muscle with the correct form of the gene[7] or by correcting the splicing through the exon-skipping vectors[8,9] or drugs, such as PTC124.[10] Cell therapy, however, is based on stem cell-driven muscle regeneration, by systemic or local injections.

This review will focus on *in vivo* cell therapy strategies and improvements in the treatment of sarcoglycan/dystrophin complex-related dystrophies, such as Duchenne or limb-girdle muscular dystrophies.

Animal Models for Sarcoglycan/Dystrophin Complex-Related Muscular Dystrophies

Sarcoglycan/dystrophin complex-related muscular dystrophies are caused by disruption of the sarcoglycan–dystrophin complex that normally anchors the actin fibres to the sarcolemma, generally resulting in chronic muscle wastage, progressive fibrotic infiltrations and force decrease. Moreover, cardiac involvement is often described, in terms of chronic

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Abbreviations: ABCG2, ATP-binding cassette, sub-family G (WHITE), member 2; ALP, alkaline phosphatase; αsg, α-sarcoglycan; bg, natural killer (NK) cell-deficient mice (beige); BM, bone marrow; BMD, Becker muscular dystrophy; BMSCs, bone marrow-derived stem cells; BMT, bone marrow transplantation; βsg, β-sarcoglycan; c-MYC, cellular myelocytomatosis oncogene; cDNA, complementary DNA; Cxcr4, chemokine (C-X-C motif) receptor 4; DMD, Duchenne muscular dystrophy; ESCs, embryonic stem cells; FACS, fluorescence-activated cell sorting; Flk1, fetal liver kinase 1; GFP, green fluorescent protein; GLP–GMP, good laboratory practice–good manufacturing practice; GRMD, Golden Retriever muscular dystrophy; Gy, Gray (unit); HCT, haemopoietic cell transplantation; hMADS, human multi-potent adipose-derived stem cells; HLA, human leukocyte antigen; Hmgb1, high mobility group box 1; IGF1, Insulin-like growth factor 1; iPS, induced pluripotent stem cells; KLF4, Kruppel-like factor 4; KO, knockout; KSN, mice strain with high natural killer activity; LGMD, limb-girdle muscular dystrophy; MABs, mesoangioblasts; Mac1, integrin alpha M; MagicF1, cMet-activating genetically improved factor1; MAPCs, multipotent adult progenitor cells; mdx, muscular dystrophy X-linked (?); μDYS, micro-dystrophin; MGF, mechano growth factor; MMP9, matrix metallopeptidase 9; mRNA, messenger ribonucleic acid; MyoD, myoblast determination protein; Myf5, myogenic factor 5; NCAM, neural cell adhesion molecule; NG2, chondroitin sulfate proteoglycan 4; NICD, Notch1 intracellular domain; NOD, non-obese diabetic; OCT4, octamer-binding transcription factor 4; Pax3, paired box gene 3; PDGFRα, platelet derived growth factor receptor alpha; PIGF, phosphatidylinositol glycan anchor biosynthesis, class F; PTC124, 3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid ($C_{15}H_9FN_2O_3$); RT-PCR, retrotranscription-based polymerase chain reaction; Sca1, stem cell antigen 1; scid, severe combined immuno-deficiency; SDF1, stromal-derived factor 1; SMPs, skeletal muscle precursors; SOX2, SRY (sex determining region Y)-box 2; SP, side population; TNF-α, tumor necrosis factor-α; VEGFR2, vascular endothelial growth factor receptor 2; Wnt3a, wingless-type MMTV integration site family, member 3A
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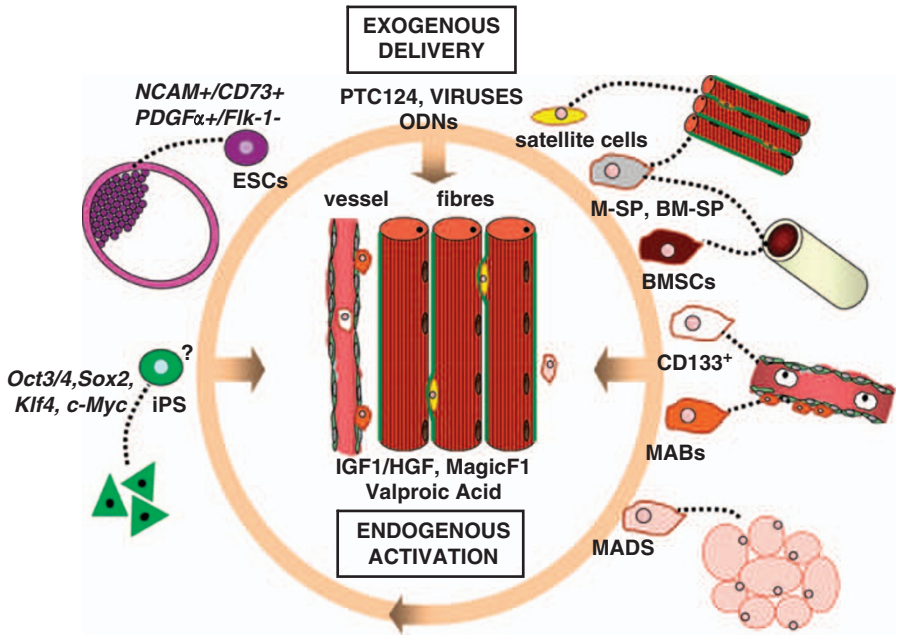

Figure 1 Combining endogenous activation and exogenous delivery to enhance muscle regeneration. Currently, two therapeutical approaches are investigated for the regeneration of the skeletal muscle: the endogeneous activation of physiological repair potential, such as targeting of satellite, circulating or perycite cells through small molecules, or the exogenous delivery of stem cells or genetic tools, such as antisense oligonucleotides (ODNs), drugs (PTC124) or viral vectors. M-SP, muscle side population; BM-SP, bone marrow side population; BMSCs, bone marrow-derived stem cells; MABs: mesoangioblasts; MADS, multipotent adipose-derived stem cells; iPS, induced pluripotent stem cells, not yet injected in animal models for muscular dystrophy; ESCs, embryonic stem cells

dilatative cardiomyopathy, scar infiltrations, aneurisms and repeated microinfarctions.

DMD is the most severe form and it is caused by frame-shift mutations or huge deletions in the *dystrophin* gene. It is one of the largest gene in the human genome and encodes a 14-kb mRNA. In this type of dystrophy, the protein is completely or partially lost. A less severe phenotype is observed in Becker dystrophy, in which mutations still affect the *dystrophin* gene, but myofibres retain a truncated and low-active isoform of the protein. Some forms of limb-girdle muscular dystrophy (LGMD2) are also caused by mutations in sarcoglycan complex proteins, for example, α- or β-*sarcoglycan* (α*sg* or β*sg*) depletion, and can result in severe pathological phenotypes.

Several animal models have been developed to study muscular dystrophies, particularly for DMD and LGMD2. The most widely used model for dystrophy is the *mdx* mouse that carries an X-linked mutation in the *dystrophin* gene, thus mimicking, at least in principle, the DMD genotype in humans. In *mdx* mice, the effects of degeneration are less severe, mainly due to the presence of relatively high numbers of revertant fibres (1–3%)[11] and an upregulation of *utrophin*. Utrophin is a smaller analogue of the dystrophin and may account for the partial compensatory effect on muscle wastage.[12,13]

Recently, it has been demonstrated that *mdx* satellite cells undergo telomere erosion, which may also contribute to the inability of these cells to continuously repair DMD muscle. It is possible that muscle stem cells or myogenic progenitors that maintain telomerase activity until late passages, may contribute in part to the muscular regeneration, that provides *mdx* mice with a normal life span.[14]

Feasible models of LGMD2 are α-*sarcoglycan*- and β-*sarcoglycan*-knockout mice. These mice are very close to the human phenotype of LGMD2D and LGMD2E, respectively, as they show chronic skeletal muscle degeneration and, in the case of β*sg-KO* mice, dilatative cardiomyopathy.[15,16] In α-*sarcoglycan-KO* mice, α*sg* gene is disrupted through a *neomycin* cassette insertion between exons 1 and 9, through homologous recombination of the flanking regions. Similarly, in the β-*sarcoglycan*-KO mice, the region encompassing exons 3–6 of β*sg* gene is disrupted. LGMD mice are considered a better animal model than *mdx* mice because of their lack of revertant fibres, which often render *mdx* mice-related results controversial.[17]

Canine models of DMD are being also extensively studied.[18,19] The larger fibres in canine muscles mimic the human dystrophy effects better than that of the mouse. At present, there are two major colonies of dystrophic dogs all over the world, bearing the same mutation in different genetic backgrounds; a colony of Golden Retrievers and one of Beagle dogs. These animals are both derived through cross-breeding of a naturally born, affected founder because transgenic creation would be unethical in these animal models. The mutation lies in intron 6 of *dystrophin* gene and results in aberrant splicing that causes a premature transcription stop codon. Dystrophic dogs, from both varieties, show extremely affected motility, posing, salivation, severe chronic scar infiltrations and skeletal muscle degeneration. In these animals, revertant fibres are also almost undetectable, thus

providing a good model to analyze regeneration effects of cell and genetic therapies.

Cell Models for *In Vivo* Skeletal Muscle Regeneration

To date, the only clinical treatments for muscular dystrophy are steroid administration and non-invasive intermittent positive pressure ventilation. These treatments result in amelioration of symptoms and improved quality of life.[20] Despite the benefits of slightly longer lifespan, alleviation of pain and surgical management of scoliosis, this therapy shows many side effects, such as weight increase, and has no real beneficial effects on skeletal muscle architecture and force.[21] Thus, cell therapy represents a theoretical valuable alternative. The main goal of cell therapy is to directly regenerate wasted, adult muscle fibres through systemic or targeted injection of stem cells, which function to block muscle loss and restore, at least partially, the normal muscular activity. It is currently a difficult task to conjugate high efficiencies in cell motility, homing, engraftment and differentiation into the complex environment of a severely inflammed and degenerated muscle. Several cell models have been tested *in vivo*, with diverse results (Table 1). Three main classes of stem cells with a myogenic differentiation potential have been considered for cell therapy protocols in preclinical studies for the treatment of muscular dystrophy: (i) embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSs); (ii) bone marrow-derived stem cells (BMSCs) and circulating progenitors; and (iii) local myogenic-committed progenitors.

ESCs and iPS cells. Embryonic stem cells (ESCs) are generally considered the most promising natural source of pluripotent cells for cell therapy, but few attempts have been reported in muscle regeneration and efficiencies are still quite low. Importantly, ethical issues around their procurement and use, such as blastocyst disgregation and oocyte requirement, have raised a lot of concerns and limited the use of human ESCs (hESCs) for research purposes in several countries.

The critical step in using ESCs resides in cell conditioning before engraftment, a necessary step that increases the differentiation rate towards myogenesis and avoids teratoma formation. A heterogeneous suspension, derived from a co-culture of male embryoid bodies and female freshly isolated dystrophic satellite cells, has been demonstrated to produce few chimeric, dystrophin-positive myofibres in injured muscles of female *mdx* mice.[22] Promising results have been obtained with hESCs, cultivated to enrich in mesenchymal precursors. The $CD73^+$ $NCAM^+$ sub-fraction was able to differentiate into myotubes *in vitro* and, remarkably, to regenerate up to 7% of injured skeletal muscle in immunodeficient mice.[23] Recently, another strategy has been developed using paraxial mesoderm progenitors that are isolated from differentiating ESCs. The $PDGFR\alpha^+/Flk1^-$ fraction of Pax3-induced, embryoid body-derived cells shows activation of myogenic transcription factors *in vitro* and good differentiation in $dystrophin^+$ fibres on transplantation into both cardiotoxin-injured and dystrophic muscle. Injected mice also show an amelioration of the contractility force. In this case, intra-arterial administration of cells resulted in higher engraftment than intravenous injection.[24] In another study, paraxial mesoderm progenitors were isolated as $PDGFR\alpha^+/VEGFR2^+$ cells, which also have been successfully tested in cardiotoxin-injured quadriceps of KSN nude mice.[25]

A new source of pluripotent cells comes from the reprogramming of adult murine or human somatic cells, by means of pluripotency transcription factor expression. Human iPS (induced pluripotent stem, hiPS) cells are reprogrammed from differentiated, adult cells, such as fibroblasts, to an ES-like status, by retroviral-mediated transduction of OCT4, SOX2, KLF4 and, even if dispensable, c-MYC.[26–28] As hiPS cells are created by reprogramming adult cells into a flexible, embryonic-like state, they have been claimed as alternative pluripotent cells to overcome the ethical issues regarding the use of ESCs. However, some reservations exist regarding the *in vivo* safety of hiPS cells, which must be addressed in the future. Recently, a wide range of disease-specific hiPS cells has been generated from patients with various Mendelian or complex diseases, including Duchenne and Becker muscular dystrophy.[29] These cells could represent a greater advancement in terms of plasticity and life span, in comparison with other cell lines tested to date. Moreover, these DMD- or BMD-specific hiPS cells show the same genetic background of the donor, thus representing a good model for *in vitro* drug testing and, if genetically corrected, a suitable cell line for extensive skeletal muscle repair.

Bone marrow-derived and circulating progenitors. Mesenchymal stem cells have been tested in acute and chronic muscle wastage, but results are still controversial. After bone marrow transplantation (BMT) in dystrophic mice, BMSCs are able to migrate and contribute to the formation of new $Myf5^+$ fibres through repeated rounds of inflammation and regeneration, which is typical of the *mdx* mouse muscles.[30] In humans, the clinical case of a young DMD patient (deletion of the exon 45 in the *dystrophin* gene) has been reported, which 12 years after BMT, showed donor nuclei fused to 0.5% of dystrophic myofibres.[31] Real-time RT-PCR analysis detected a small amount of a truncated, in-frame isoform of *dystrophin*, lacking exons 44 and 45, and trace amounts of the wild-type gene (0.0005%), although a direct correlation between the BMT-derived nuclei and the *dystrophin* isoform expression was missing. In addition, human mesenchymal stem cells isolated from synovial membrane of adult donors, on intramuscular delivery into tibialis anterior of *mdx* mice, efficiently produce new, functional myofibres, without any sign of fusion. These cells also contribute to the long-term satellite cell population and restore mechano growth factor (MGF) expression in treated muscles.[32] The myogenic potential of these cells seems to be strongly related to the microenvironment surrounding the delivered cells because when injected systemically in dystrophic mice, they are observed in almost all tissues.

Given the broad availability of the source, human multipotent adipose-derived stem cells (hMADS) have been investigated as a possible alternative for muscle regeneration. hMADS are $CD44^+$, $CD90^+$ and $CD105^+$, confirming their mesenchymal lineage. Once injected intramuscularly into tibialis anterior of immunocompetent and immunosuppressed

Table 1 Stem cell types for the treatment of chronic or acute skeletal muscle damage

Stem cell types	Markers	Animal models	Muscle damage	Delivery	Ref.
hESCs	$CD73^+/CD56^+$ (sub-fraction)	SCID/Beige mice	Acute	Intramuscular	23
ESCs	$Pdgfr\alpha^+/Flk1^-$ (sub-fraction)	*Rag2*$^{-/-}\gamma c^{-/-}$ mice/*mdx* mice	Acute/chronic	Intra-arterial > intravenous	24
	$Pdgfr\alpha^+/Vegfr2^+$ (sub-fraction)	*KSN* nude mice	Acute	Intramuscular	25
hBMSCs	$VIMENTIN^+/CD44^+$	*NMRI nu*$^{-/-}$ mice/*mdx* mice	Acute/Chronic	Intramuscular	32
hMADS	$CD44^+/CD90^+/CD34^-$/ $HLA\text{-}DR^-/HLA\text{-}I^{low}$	*mdx* mice	Acute/Chronic	Intramuscular	33
Human circulating $CD133^+$ progenitors	$CD133^+/CD34^+/CD45^+/CD90^+$	*scid/mdx* mice	Chronic	Intramuscular/ intra-arterial	37
BM-SP cells	$Cd34^{-/low}/Cd117^+/Sca1^+$	LGMD2F (*Sgcd2*$^{-/-}$) mice	Chronic	Intravenous	38
Satellite cells	$Cd34^+/Pax7^+/Cd31^-/Sca1^-$	*mdx nu*$^{-/-}$ mice	Chronic	Intramuscular	47
	$Cd34^+/Integrin\alpha^+$ (clonal level)	*NOD/SCID* mice	Acute (resident satellite cell withdrawal)	Intramuscular	48
SMPs	$CD45^-/Sca1^-/Mac1^-Cxcr4^+/$ $\beta 1integrin^+$	*mdx* mice	Acute	Intramuscular	49
Muscle-SP cells	$Sca1^+/Cd45^+/Abcg2^+$	*mdx*^{5cv} mice	Chronic	Intra-arterial	54
	$Sca1^+/Abcg2^+/Syndecan4^+/Pax7^+$	*Rosa26* mice/*mdx* mice	Acute/chronic	Intramuscular	55
MABs (human, canine, murine)	$Pdgfr\alpha^+/Pdgfr\ \beta^+/Ng2^+/Alp^+$	*Sgca*$^{-/-}$ mice/*GRMD* dogs/*scid/mdx* mice	Chronic	Intra-arterial	57–59
Myoendothelial progenitors	$Cd56^+/Cd34^+/Cd144^+$	*scid* mice	Acute	Intramuscular	61

Abbreviations: hESCs, human embryonic stem cells; ESCs, embryonic stem cells; hBMSCs, human bone marrow-derived stem cells; hMADS, human multipotent adipose-derived stem cells; BM-SP, bone marrow side population; HSCs, haematopoietic stem cells; SMPs, skeletal muscle precursors; Muscle-SP, muscle side population; MABs, mesoangioblasts

mdx mice, they fuse with host fibres, resulting, 6 months later, in a large number of chimaeric myofibres expressing human *dystrophin*. Interestingly, no differences are reported between immuno-competent and immuno-suppressed hosts and it also seems that hMADS significantly reduce necrosis in the dystrophic muscle.[33] These promising results have since been enhanced by priming the adipose-derived mesenchymal cells by co-culturing with myoblasts[34] or forced *MyoD* expression.[35]

A sub-population of circulating, haematopoietic stem cells expressing CD133, which constitutes another interesting and easily isolatable cell pool, has been reported to express early myogenic markers.[36] Intramuscular or intra-arterial injection of genetically corrected $CD133^+$ progenitors, isolated from both peripheral blood and muscles of DMD patients, results in a significant recovery of muscle morphology, function and re-expression of human *dystrophin* in *scid/mdx* mice.[37]

In contrast, several studies have reported of absent or incomplete muscle repair by mesenchymal or haematopoietic stem cells. On being intravenously injected into a mouse model for LGMD2F (mice lacking δ-sarcoglycan), bone marrow side population cells engraft and fuse into skeletal fibres, but do not restore δ-sarcoglycan expression.[38] Green fluorescent protein-positive bone marrow (GFP^+ BM) cells, delivered through retro-orbital injection, fuse with $\sim$3% of fibres in the tibialis anterior of treated *mdx* mice, but almost no dystrophin expression is detected in GFP^+ myofibres. However, where dystrophin is detected, its expression is spatially more limited than in revertant fibres.[39] These data have been confirmed in another study, in which it has been demonstrated that $\geqslant$80% of BM-derived muscle-incorporated nuclei in the transplanted dystrophic mouse are 'silent'. Incorporated nuclei fail to express myogenic factors, including *dystrophin*, and this 'silencing' is still retained even in the presence of strong chromatin-remodelling agents, such as 5′-azacytidine.[40] It has also been demonstrated that haematopoietic cell transplantation (HCT) alone result neither in any skeletal fibre regeneration nor in expression of dystrophin or other muscle genes.[41] Nevertheless, an interesting role for HCT in muscle regeneration may come from immunotolerance effects towards allogeneic myoblast engraftment. DMD dogs have been successfully treated using both peripheral HCT and freshly isolated myoblasts from the same healthy donor. Donor myoblast-related *dystrophin* expression increased up to $\sim$7% of wild-type levels and was maintained for at least 24 weeks, without any pharmacological immunosuppression.[42]

Finally, bone marrow-derived multipotent adult progenitor cells (MAPCs) were observed to durably repair muscles in ischaemic limbs, by efficient revascularization of necrotic tissues.[43]

Local myogenic-committed progenitors. Satellite cells are quiescent unipotent myoprecursors, located between the fibre and the basal lamina; during embryogenesis, they form during the second wave of myogenesis and, after contributing massively to the first post-natal muscle growth, they stop proliferating and reach their niche.[44] They can also be re-activated on muscle damage, re-entering cell cycle and contributing to the formation of new muscle fibres.[45] Given their natural commitment, it's easy to imagine satellite cells as a major candidate for muscle regeneration in muscular dystrophies. On single fibre transplantation into radiation-ablated *mdx* tibialis anterior, donor satellite cells multiply and expand, re-populating the satellite cell pool and differentiate into functional myofibres.[46] $Pax7^+$ $CD34^+$ GFP^+ satellite

cells, isolated from *Pax3*$^{GFP/+}$ mice diaphragms, have also been demonstrated to be a good cell model for *mdx* irradiated muscle treatment, resulting in the restoration of *dystrophin* expression in many skeletal fibres and contributing to the resident satellite compartment.[47] Injections were administered intramuscularly and, notably, two major problems arose; satellite cells have a very low migration capability and, furthermore, cells showed impaired engraftment capability when expanded *in vitro*, even if for few days.[47]

To test the regeneration capability of satellite cells at a clonal level, single-cell dilutions of CD34$^+$ integrinα^+ luciferase-expressing satellite cells were injected into the skeletal muscle of a NOD/SCID mouse depleted of resident satellite cells by 18 Gy irradiation. It was shown by *in vivo* imaging that a single satellite cell can reconstitute the satellite compartment and, on further damage, can rapidly re-enter a new proliferation wave, generating new myofibres.[48]

Recently, interesting findings resulted from a prospective isolation of skeletal muscle precursors (SMPs), consisting of a CD45$^-$ Sca1$^-$ Mac1$^-$ Cxcr4$^+$ β1-integrin$^+$ subset within the endogenous satellite compartment. When injected into cardiotoxin-injured muscles in immunodeficient *mdx* mice, SMPs robustly contributed to muscle regeneration (up to 94%) by fusing with pre-existing fibres or stimulating *de novo* myogenesis. Muscle histology and contractile force in treated mice were significantly better than those in untreated mice. Furthermore, SMPs contributed greatly to the endogenous satellite population, undergoing new cycles of re-activation on subsequent induced damage.[49] However, as freshly isolated SMPs were injected, without *in vitro* expansion, the migration capability remains restricted in the areas surrounding the intramuscular injection site.

One of the major concerns about satellite cells is that, probably, they are not a homogeneous population. As argued by Collins *et al.*,[46] the variable engraftment rate of satellite cells from a single myofibre transplantation could be due to the functional heterogeneity of the satellite cell pool and of their niche of origin. Satellite cell heterogeneity is still a contentious issue and has been extensively reviewed in several studies[50,51] and is still open for debate.[52]

Muscle side population (SP) cells are defined as Sca1$^+$ CD45$^+$ cells, able to rapidly efflux the Hoechst dye 33342, and are being investigated as potential myogenic progenitors. They are associated with the muscle vasculature and are spontaneously committed towards the haematopoietic lineage. On co-culture with myoblasts, they can form myotubes *in vitro* and, if injected intramuscularly into crushed tibialis of a *scid/bg* immunodeficient mouse, can give rise to up to 1% of regenerating fibres.[53] The efficiency of SP-mediated muscle regeneration has been increased to 5–8% by injection into the femoral artery of *mdx*^{5cv} DMD mice, resulting in *Pax7* and *desmin* expression by donor cells, after extravasation and recruitment to inflammation sites.[54] Impressive results have been obtained with the identification of a rare subset (0.25%) of SP cells, characterized by both satellite- and SP-related markers, such as Sca1$^+$/ABCG2$^+$/Syndecan4$^+$/Pax7$^+$, and found in the typical satellite compartment, under the basal lamina. Once sorted from the mononuclear fraction of the hind limb, they can grow in association with single muscle fibres and can robustly undergo myogenic differentiation *in vitro*. On intramuscular injection in the presence of 1.2% $BaCl_2$, these satellite-SP cells have been shown to efficiently compete with endogenous satellite cells in regenerating the wild-type muscle. Injected cells resulted in 30% fibre regeneration and, strikingly, in up to 75% reconstitution of the endogenous satellite cell pool. The newly generated satellite cells were able to undergo new rounds of proliferation and muscle repair on subsequent injuries. Furthermore, the same long-term effects have been proven through injections into dystrophic *mdx*^{4cv} tibialis anterior muscles, producing up to 70% regenerating fibres.[55] However, $BaCl_2$-induced muscle damage is not a widely used and accepted regeneration model, and this must be taken into consideration when interpreting these findings.

Recently, a new type of vessel-associated muscle-derived stem cells has been investigated as a suitable potential model for chronic muscle therapy, namely mesoangioblasts (MABs). They can be isolated from the dorsal aorta of E9.5 embryo[56] or from adult skeletal muscle of mice, dogs and humans.[57–59] MABs are CD34$^+$, Sca1$^+$, PDGFRα^+, PDFGRβ^+, NG2$^+$ and ALP$^+$, thus supporting the idea that they are a sub-group of the pericytic population.[60] They show high proliferation rates *in vitro*, without transformation potential, and display, in *in vitro* and embryonic chimaera systems, multipotent differentiation capability towards myogenic, osteogenic, chondrogenic and adipogenic lineages. On intra-arterial injection into inflammated muscles of *αsg-KO* mice or DMD Golden Retriever dogs, they are capable of consistently regenerating (up to 50%) the muscle architecture, *sarcoglycan/dystrophin* expression and electrophysiological properties of wasted dystrophic muscle.[57,58] Similarly, good results have been achieved through human MAB transplantation into *scid–mdx* immunodeficient dystrophic mice.[59]

Treatment of dogs with MABs resulted in very good and long-term results in some animals, in terms of general motility restoration and whole muscle regeneration, whereas other animals did not show a good engraftment and any clinical improvement.[58] This confirms the general idea that background variability in dystrophies in larger organisms, such as dogs or humans, has to be evaluated for effective cell therapies.

Furthermore, another class of myogenic precursors has been isolated from endothelial population of adult human muscle, through FACS-mediated prospective isolation of CD56$^+$ CD34$^+$ CD144$^+$ cells. These myoendothelial progenitors, on injection into injured muscle of *scid* mice can achieve muscle engraftment and fibre neo-formation at an higher degree than CD56$^+$ canonical myoprecursors.[61]

Cell Conditioning and Priming

To increase engraftment specificity and differentiation potential, cell transplantation can be combined with pre-injection cell conditioning or with genetic manipulation.

Cell migration limitations are often a major cause of low engraftment efficiencies in skeletal muscle, which is a very complex tissue with severely limited cell motility, particularly in the presence of large fibrotic or necrotic areas. Thus, some attempts have been made to assist migration by conditioning cells with migration-enhancing soluble factors or by

overexpressing commiting/mobilizing proteins before applying them to the degenerated muscle.

A lot of interest has been shown in *Notch* signalling, as a potential enhancer of myogenic commitment. Rat BMSCs, transfected with a plasmid for the intracellular domain of *Notch1* (NICD) and injected locally or intravenously into injured muscles of rats or nude mice, account for a very high level of regenerating fibres (up to 89%).[62] Given that NICD is the active signalling form of the receptor, it could be possible that finer genetic tools to activate *Notch* signalling could enhance myogenesis by donor cell injection, although recently it has been demonstrated that a temporal switch from *Notch* to *Wnt3a* signalling activation is necessary during normal adult myogenesis.[63]

Encouraging *in vivo* results have come from cell therapy experiments involving soluble factor-dependent cell conditioning. Hmgb1, a cytokine secreted by activated macrophages and monocytes, is able to increase the recruitment of MABs out of the vessels into the muscle. Heparin–Sepharose beads, loaded with Hmgb1 and injected into the femoral artery, were shown to promote the *trans*-endothelial migration of intra-arterial-injected embryonic MABs into non-injured tibialis anterior of wild-type mice.[64] Pre-treatment of MABs with mobilization cytokines, such as SDF1 and TNFα, highly increase MAB homing into dystrophic muscle of α*sg-null* mice, thus reducing approximately 50% the aspecific homing into filter organs. Their regenerative effect was magnified by TNFα priming and α4-integrin overexpression.[65] Furthermore, improving the angiogenic potential in necrotic areas could help cell therapy. For example, tendon fibroblasts expressing placenta growth factor (PIGF, an angiogenic factor) and matrix metalloproteinase 9 (MMP9), injected intramuscularly into aged α*sg-KO* mice, result in a dramatic increase in the extension of regenerated dystrophin$^+$ muscle areas after intra-arterial delivery of wild-type MABs.[66] Such results corroborate the idea that a deeper knowledge of cytokines and angiogenic factors regulating the inflammation-dependent recruitment of myoprecursors will serve to improve the benefits mediated by cell therapy.

Genetic Manipulation for Autologous Cell Therapy

Correction of the dystrophic genotype in the transplanted cells could allow the usage of autologous cells, instead of heterologous wild-type cells, thus avoiding immunosuppressive drugs.

A widely used strategy relies on lentiviral transduction of muscle-regenerating cells to allow integration and expression of the disrupted gene. To correct DMD, several alternatives of the *dystrophin* gene (which is too long to be introduced in a lentiviral vector) exist, such as *micro-* or *mini-dystrophin*. These are shorter isoforms of the native protein, which retain a partial functionality, thus providing, in principle, a possible molecular rescue on differentiation towards newly formed myofibres. *mdx*^{5cv} mice, injected intravenously with autologous muscle-SP cells that were previously transduced with human micro-dystrophin (hμDYS)-expressing lentiviruses, show some skeletal fibres positive for the human version of the lacking protein.[67] Similarly, very good results came from transplantation of hμDYS-transduced human pericytes into *mdx–scid* mice.[59] In GRMD dog treatment, autologous MABs, transduced with human *micro-dystrophin*, induced a quite widespread expression of dystrophin and other proteins of the sarcoglycan complex in analyzed muscles and a partial recovery of the histological architecture. However, treated dogs had poorly restored general motility and force.[58] These results may support the idea that, especially in higher organisms, mini- or micro-genes are not so feasible, or, at least, show highly variable and only short-term effects. Genetic correction can be addressed also for treatment of other genetic defects resulting in dystrophy, as when α*sg-KO* MABs, carrying a lentiviral α*sg* cDNA construct under a constitutive promoter, are injected into LGMD2D mice, there is extensive fibre regeneration, mobility and muscle force recovery.[57]

Conclusions and Perspectives

Stem cell-based therapy is the most attractive approach for the treatment of DMD and other muscular dystrophies, and research in this direction has moved rapidly in past few years. Experiments in small and large animal models are paving the way for clinical experimentation, but it would be imprudent to predict a 'cure' from these first attempts. Nevertheless, several clinical trials have been started or planned, involving myoblast or perycyte injection from HLA-matched donors and, given the growing variety of possible myogenic progenitors in literature, the number will increase during the near future. Other clinical trials are focusing on gene- or antibody-based strategies, such as adeno-associated viruses carrying γ*-sarcoglycan* or μ*-dystrophin* and antibody-triggered myostatin blockade.[21]

Furthermore, encouraging results have come from clinical trials related to exon-skipping technology. Specific exons carrying mutations can be skipped by antisense oligonucleotides administration to restore the reading frame and result in the production of internally deleted, but functional dystrophin. Recently, two clinical trials involving two different drugs, AVI-4658 (developed by the MDEX Consortium, United Kingdom and manufactured by AVI BioPharma, Bothell, WA, USA) and PRO051 (developed by University of Leiden, The Nederlands in collaboration with Prosensa B.V.), were performed on Duchenne patients. In both trials, biopsy data showed that injections of antisense oligonucleotides, to skip exon 51, into dystrophic muscles, successfully induced new dystrophin production, with no adverse events. These pioneering studies are now followed by randomized controlled trials of systemic therapies both in The Netherlands and the United Kingdom.

Therefore, it is reasonable to expect encouraging results that may also drive a combination of the stem cell- and gene-based therapies. It is critical to better understand the biological properties of stem cells and their paracrine role in the treatment of muscular diseases, to realize the potential positive effects of these new cures.

The scientific community largely accepts the presence of adult stem cells in all tissues but their origin is still controversial. We and other authors suggest pericytes as a source of stem cells present in skeletal and cardiac muscles.[59,60] They are influenced by their surrounding when maintaining

a specific cell commitment, although this has to be clarified in pathological tissues.

Interestingly, other research groups have identified mesenchymal cells as stem cells for all tissues,[68] raising questions regarding the possibility that the primary source of cell plasticity is confined to the bone marrow. It could be possible that cells move from bone marrow towards pericyte compartment, to adopt a specific cell fate, influenced by local niche. To further elucidate their origin, it is necessary to generate transgenic animals to track endogenous stem cells during muscle development and regeneration.

Despite the fact that current regulatory restrictions will defer the clinical translation of new approaches, that are successful in animal models, several trials have been started. Moreover, the actual huge costs of GLP–GMP (good laboratory and manufacture practice) stem cell technology limit the feasibility of cell therapy treatment for patients affected by muscular dystrophy. However, strategies to lower costs are being investigated to develop treatments that are available for large numbers of patients. In conclusion, it is critical to better understand the biological properties of stem cells and their paracrine role in the treatment of muscular diseases, to realize the potential positive effects of these new cures. Therefore, it is reasonable to expect encouraging results from the on-going trials that may also drive a combination of stem cell- and gene-based therapies for the treatment of muscular dystrophies.

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