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Stefano Biressi, … , Antonio Filareto, Thomas A. Rando

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Review

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# Stem cell therapy for muscular dystrophies

**Stefano Biressi,[1,2] Antonio Filareto,[3] and Thomas A. Rando[4,5,6]**

[1]Department of Cellular, Computational and Integrative Biology (CIBIO) and [2]Dulbecco Telethon Institute, University of Trento, Povo, Italy. [3]Department of Research Beyond Borders, Regenerative Medicine, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Conneticut, USA. [4]Department of Neurology and Neurological Sciences and [5]Paul F. Glenn Center for the Biology of Aging, Stanford University School of Medicine, Stanford, California, USA. [6]Center for Tissue Regeneration, Repair and Restoration, Veterans Affairs Palo Alto Health Care System, Palo Alto, California, USA.

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## Introduction

Muscular dystrophies comprise a heterogeneous group of genetic disorders characterized by progressive muscle wasting and weakness (1, 2). In muscular dystrophies, muscle dysfunction arises from the mutations of genes encoding different cellular components, including proteins associated with the sarcolemma, extracellular matrix, nuclear membrane, and sarcomeric apparatus (3, 4). Different forms of dystrophy differ in terms of age of onset, severity of symptomatic progression, and distribution of affected muscles (1, 3). Depending on the molecular etiology, muscular dystrophies can present clinically relevant defects beyond the skeletal muscle compartment (3). In particular, cardiac involvement is present in several forms of dystrophy (3, 5).

With an incidence of approximately 1 in 5000 male newborns, Duchenne muscular dystrophy (DMD; OMIM 310200) is the most frequent and one of the most severe forms of muscular dystrophy (1, 3). DMD patients typically present with progressive weakness of limb muscles, trunk muscles, and the diaphragm, leading to wasting, kyphoscoliosis, and severe respiratory problems (1, 2). Most patients die in their third decade of life due to respiratory complications (1, 2). Almost all DMD patients have cardiac involvement, and heart failure is the second leading cause of death (5). Cardiac abnormalities, such as dilated and hypertrophic cardiomyopathy, increase with age (6). A rarer (~1 in 20,000 male births) and clinically milder form of dystrophy, Becker muscular dystrophy (BMD; OMIM 300376) has the same causative allele as DMD (1, 3). Onset of BMD symptoms occurs later than in DMD, and the average age of death is in the fifth decade of life (1). Almost 50% of BMD deaths occur due to congestive heart failure and arrhythmias (7).

DMD and BMD are caused by mutations in the *DMD* gene, encoding dystrophin (8). Different types of mutations in the *DMD* gene, which is located on the X chromosome and is the largest known gene of the human genome, cause DMD (8). Whereas DMD patients lack the dystrophin protein because of frameshift mutations, BMD is generally caused by mutations that do not disrupt the translational reading frame (9). A partially functional dystrophin is typically expressed in BMD patients (9).

Dystrophin is a component of a plasma membrane–associated complex called the dystrophin glycoprotein complex (DGC), which acts as a framework to connect the intracellular cytoskeleton to the surrounding extracellular matrix (10). The DGC's crucial role for proper muscle functionality and integrity is demonstrated by the overlap in pathological features between DMD and a number of dystrophies caused by mutations in genes encoding other components of the DGC (4). The most well-studied mechanism that has been proposed to explain the etiology of DGC-related muscular dystrophies is a loss of membrane integrity as a result of disruption of structural proteins (11, 12), but disruption of membrane-associated signaling pathways has also been implicated in pathogenetic processes (13, 14). Whatever the pathophysiological processes, dystrophic muscle exhibits cycles of degeneration and regeneration, accompanied by infiltration of inflammatory cells and progressive accumulation of fibrotic and adipose tissues (15).

Although glucocorticoid treatment is associated with reduced disease progression and multidisciplinary care may further improve patient survival, there is currently no definitive cure for DMD and BMD (16, 17). However, many promising therapeutic strategies are now under active investigation. Among the most well-studied approaches is gene delivery using viral or nonviral vectors, a strategy that seeks to deliver a functional, even if truncated, version of dystrophin to myofibers (18). As an alternative, interventions that alter mRNA splicing (e.g., exon skipping) or translation (e.g., stop codon suppression) have been studied as ways to generate functional dystrophin proteins even in the face of pathological

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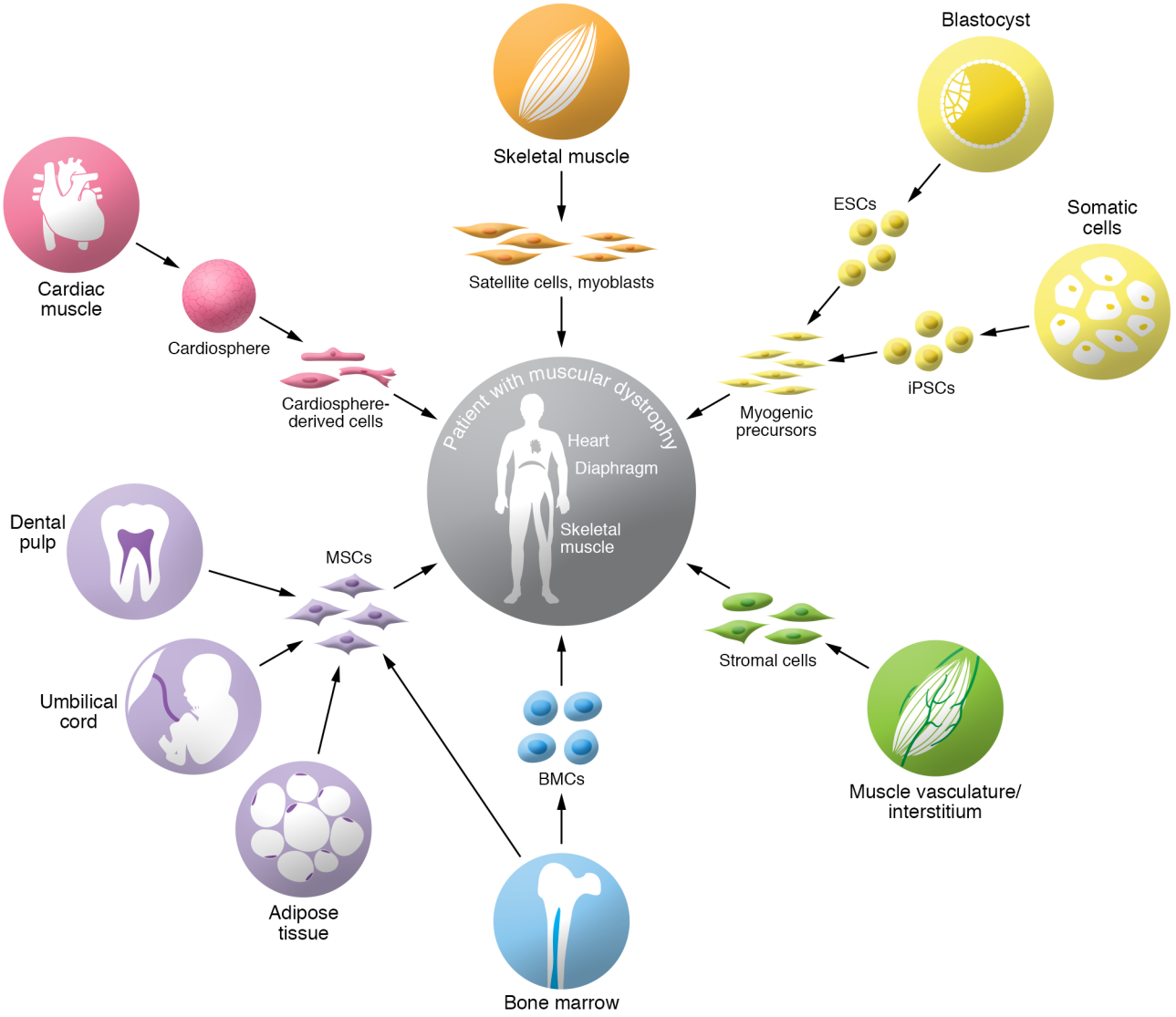


**Figure 1. Overview of candidate cell types for cell therapy in muscular dystrophies.** Different types of progenitor cells derived from skeletal muscle or from nonmuscle tissues have been tested preclinically or clinically for therapeutic cell transplantation in muscular dystrophies. The identity of the progenitor cells and their tissue of origin are indicated. MSCs, mesenchymal stem cells; iPSCs, induced pluripotent stem cells; ESCs, embryonic stem cells; BMCs, bone marrow–derived cells.

gene mutation (19). More recently, with the advent of gene editing strategies such as transcription activator–like effector nucleases (TALENs), zinc finger nucleases, and CRISPR/Cas9, investigators have sought to directly correct endogenous mutations in *DMD* and restore its normal reading frame (20). Several pharmacological and genetic approaches striving to reconstruct the DGC complex through overexpression of DGC components, such as utrophin, are also under investigation (21, 22). Focusing more on downstream pathogenic mechanisms, pharmacological interventions have targeted pathological consequences of dystrophin mutations including myofiber necrosis, inflammation, fibrosis, ischemia, mitochondrial dysfunction, and aberrant histone deacetylation (22).

This Review focuses on a therapeutic approach that has been envisioned and tested for decades, namely cell-based therapy (Figure 1). The promises of cell therapy are multiple. On one hand, using genetically normal or genetically corrected cells, cell therapy is a gene delivery approach to introduce normal copies of the *DMD* gene (or other therapeutic genes in non-DMD patients) into myofibers by cell fusion. As a consequence, development of gene correction/complementation strategies is crucial for the successful application of cell therapy, particularly when autologous cells are used as vehicles. In addition, delivery of muscle stem or progenitor cells holds promise to sustain or enhance muscle repair, and possibly populate the muscle stem cell niche for future regenerative demands. Finally, as with many cell-based therapies, cellular vehicles often deliver trophic or even immunomodulatory factors to a tissue that produce a therapeutic benefit. In fact, a growing body of evidence indicates that some progenitors evaluated in cell therapy approaches exert at least part of their regeneration-promoting role through paracrine signals (23). Here, we provide an overview of past attempts and current strategies to use cell-based therapies aimed at ameliorat-

ing both the skeletal and the cardiac defects in patients affected by muscular dystrophies.

## Cell therapy in dystrophic skeletal muscle

### Myogenic stem and progenitor cells from skeletal muscle

*Satellite cells*. Skeletal muscle tissue possesses a tremendous capacity for effective self-repair of acute damage (24). Muscle stem cells (MuSCs), also known as satellite cells, are responsible for the muscle regenerative process (25). MuSCs are marked by expression of the transcription factor PAX7 and are required for productive muscle repair (26–28). MuSCs are actively maintained in a quiescent state in adult muscle (29), but can quickly divide following muscle injury, enter the cell cycle, and give rise to proliferating myoblasts that ultimately differentiate and fuse in the process of tissue repair. Activated MuSCs also undergo self-renewal to maintain the population of adult MuSCs (30).

Many studies have demonstrated that transplantation of both mouse and human MuSCs not only promotes regeneration, but transplanted MuSCs also maintain the ability to repopulate the stem cell compartment (31–35). However, several obstacles, such as loss of potency with ex vivo expansion and limited in vivo migration after transplantation, currently hinder the use of these cells for clinical application. As discussed below, strategies aimed at improving these aspects of MuSC transplantation are under active investigation in the preclinical setting (Figure 2).

*Myoblasts*. Initial studies in dystrophic *mdx* and *dy/dy* mutant mice, modeling DMD and merosin-deficient congenital muscular dystrophy, respectively, demonstrated that normal myoblasts, obtained through in vitro expansion of MuSCs, can locally remediate the genetic defect after intramuscular injection (36, 37). Substantial effort was therefore invested to enhance the efficacy of myoblast transplantation. The survival and mobility of precursor cells, the extent of distribution of proteins produced by transplanted myoblasts, and the influence of the host environment, including the host immune response elicited by grafted cells, were investigated in different animal models (38). Furthermore, it was shown that some transplanted myoblasts could also survive as muscle precursor cells (39).

Initial observations in mice were rapidly followed by a series of clinical trials in which allogenic myoblasts were expanded in culture and transplanted in the muscles of DMD patients (40). These early studies reported variable expression of donor cell–derived dystrophin and myofiber chimerism, but were inconclusive in terms of functional improvement (41–43). Although discouraging in terms of efficacy, these early studies demonstrated the overall safety of the procedure and revealed the requirement of appropriate immunosuppression (44).

In subsequent years, a series of studies using both murine and human myoblasts, and mainly the *mdx* model, disclosed several determinants of myoblast engraftment efficacy (45). Myoblast transplants were also performed in large-animal models to more accurately model human physiology (46, 47). These studies led to an optimization of transplantation protocols and to further clinical trials in dystrophic patients (Table 1). Major improvements consisted of use of tacrolimus for immunosuppression and increased density of myoblast injection sites (48, 49). Indeed, delivery of small volumes of cell suspension through closely spaced (1–2 mm) injections resulted in robust levels of dystrophin expression (48, 49). Data from one patient undergoing myoblast transplantation in a portion of the gastrocnemius muscle showed that donor-derived dystrophin was induced in over 30% of the myofibers at the injection site (49). More recently, a series of studies in nonhuman primates have revealed additional determinants, such as the needle size, cell number, and injection volume (50). These have led to an ongoing phase I/II clinical trial evaluating the effectiveness of high-density delivery of myoblasts in preserving muscle strength in DMD patients (ClinicalTrials.gov NCT02196467) (Table 1).

Notably, a clinical trial reported positive results of myoblast transplantation in patients affected by oculopharyngeal muscular dystrophy (OPMD; OMIM 164300), a form of dystrophy characterized by the selective involvement of the pharyngeal and eyelid muscles. In this study (51), autologous myoblasts obtained from unaffected muscle groups were transplanted into the constrictor muscles of the pharynx of OPMD patients. Most of the patients exhibited stabilization of symptoms up to 2 years after transplantation, and a cell-dose-dependent effect on swallowing was apparent (51).

It is worth noting that the relatively limited amount of muscle tissue affected in OPMD patients allows for focal cell delivery. By contrast, in other types of dystrophies, such as dystrophinopathies, a vastly greater muscle mass is affected, requiring different strategies for cell delivery (1, 2). Unfortunately, intra-arterial delivery of myoblasts has proven to be ineffective (52).

*Mesoangioblasts*. An additional cell type, named mesoangioblasts (MABs) for their ability to differentiate into different mesodermal lineages (including skeletal muscle) and their association with blood vessels, became highly attractive for cell therapy in DMD owing to their ability to efficiently egress a blood vessel wall from the circulation into interstitial muscle tissue, making them a candidate for systemic delivery (53, 54). Originally identified in the wall of embryonic dorsal aortae in mice, MABs were also isolated and characterized as a pericyte-derived population of cells from skeletal muscle of adult mice, dogs, and humans (55, 56).

Several studies indicated that MABs represent a promising approach to cell therapy in murine models of different muscular dystrophies (54, 55, 57–59). Furthermore, genetically corrected MABs delivered intra-arterially into dogs with golden retriever muscular dystrophy (GRMD) restored dystrophin expression in 5%–50% of skeletal myofibers in select hind-limb muscles and improved muscle morphology and function (56). In addition to their direct involvement in skeletal muscle regeneration, MABs also have paracrine effects and can modulate immune cell function (60). Transplanted MABs also contribute to the MuSC pool (54, 59, 61).

These studies led to a phase I/IIa trial (EudraCT 2011-000176-33) in five DMD patients (62). The clinical trial involved four intra-arterial infusions of HLA-matched MABs at doses consistent with those administered to dystrophic dogs in preclinical tests (56). Two months after the final infusion, muscle biopsies were collected and analyzed, revealing minimal donor cell engraftment and very modest increase of dystrophin levels (62). Disease progression in one patient was stabilized for more than 2 years, but

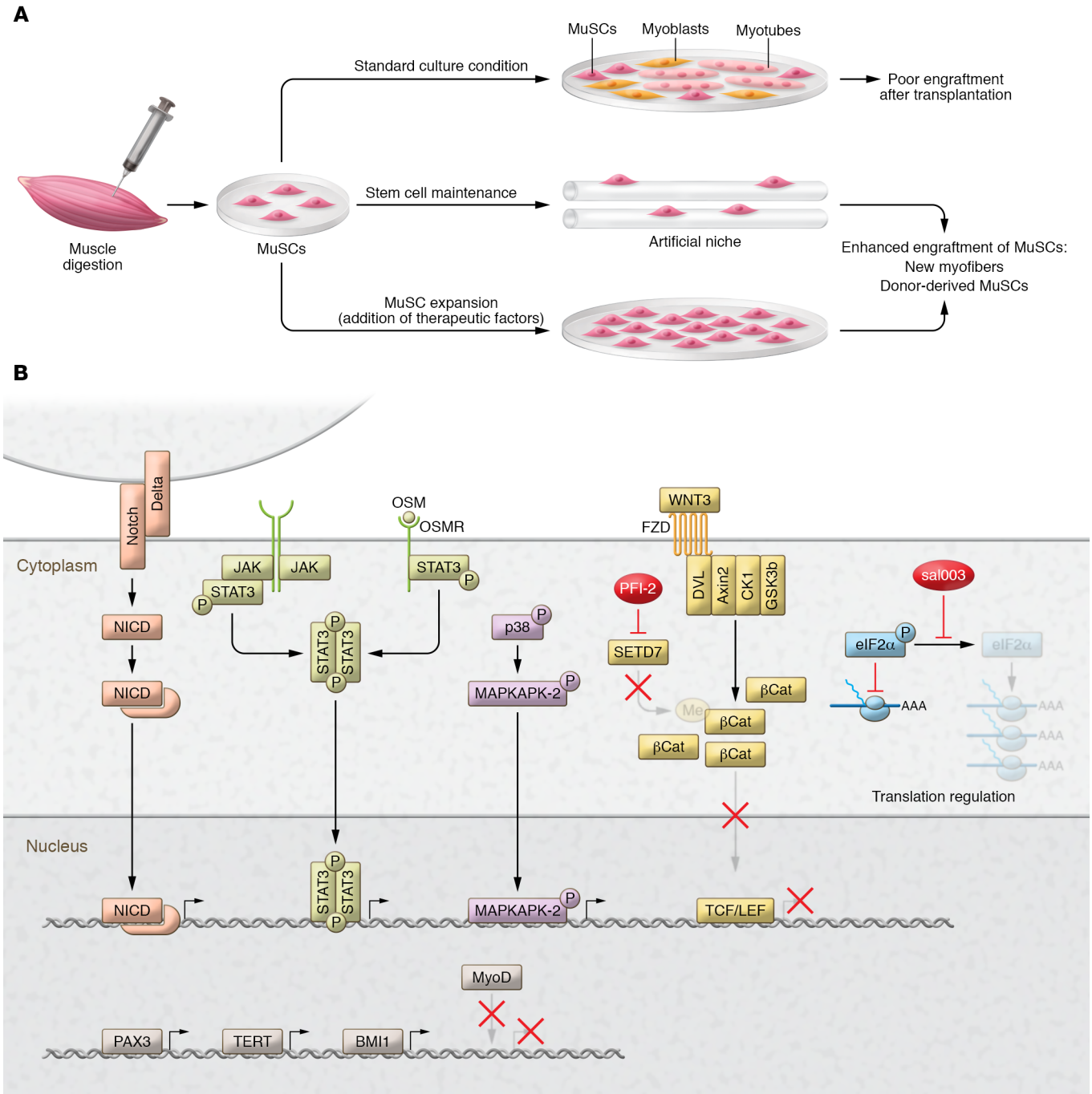


**Figure 2. Preservation of potency during in vitro culturing of myogenic cells for cell therapy applications.** (**A**) Expansion of MuSCs isolated from enzymatically digested skeletal muscles under standard culturing conditions on plastic dishes (upper path) selects for myoblasts with poor regenerative and engraftment capability. Two main strategies have been proposed to preserve the in vivo regenerative potential of MuSCs during in vitro culturing and resulted in productive engraftment in preclinical studies. One strategy (middle path) consists of the manipulation of artificial culturing substrates with bioengineering techniques to mimic the niche in which MuSCs reside and stimulate quiescence. The second strategy (lower path) consists of the adoption of culturing conditions favoring the expansion of myogenic progenitors with preserved regenerative potential. (**B**) Expansion of myogenic progenitors with preserved regenerative potential can be achieved through the addition to the culture medium of factors able to modulate the activity of fundamental signaling pathways, such as the Notch, JAK/STAT, oncostatin M (Osm), and p38 MAPK signaling pathways (34, 138–140, 142); Setd7-dependent epigenetic modifications (143); eIF2α-dependent translational control (141); or the genetic alteration of regulators of myogenic lineage progression and proliferation.

no clear correlation with dystrophin expression was apparent. One patient developed atrial fibrillation and a thalamic stroke detected by MRI, highlighting the need for stringent cardiovascular monitoring when intra-arterial delivery of cells is used. Although this clinical trial failed to demonstrate clear efficacy, it provided information essential for future cell therapy trials (Table 2).

*Other cell populations with myogenic potential from muscle interstitium.* The search for myogenic cells suitable for cell therapy applica-

**Table 1. Selected clinical trials for muscular dystrophies employing myoblasts**

| Donor | Cell therapy approach: Route | Cell therapy approach: Immun. sup. | Dx / no. of patients | Time point (mo) | Outcome: Efficacy | Outcome: Side effects | Reference or trial ID |
|---|---|---|---|---|---|---|---|
| Parents | i.m. | Tacrolimus (± steroids) | DMD 9 | 1 | • % total DYS+ fibers at injection site: 3.5%–21.2% (8/9); 1 fiber/muscle section (1/9); noninjected site: 0–4.6% (9/9);<br>• % donor-derived DYS+ fibers at injection site: 6.8%–13.5% (5/6); 1 fiber/muscle section (1/6); noninjected site: <0.1% (6/6);<br>• presence of donor-derived nuclei (2/2);<br>• increased DYS in semiquantitative RT-PCR (9/9) and qPCR (3/3);<br>• α-sarcoglycan reassembly (3/3) | Weight increase (1/3); diarrhea (3/9); local immune cell infiltration (8/9) | 48 |
| Father | i.m. | Tacrolimus | DMD 1 | 1 | • % donor-derived DYS$^+$ fibers at injection site: 27.5%; noninjected site: 0%;<br>• increased DYS in RT-PCR<br>• 70% increase in metacarpal flexion strength<br>• increased DYS in immunofluorescence;<br>• 70%–100% increase in metacarpal flexion strength | Thumb paresthesia | 49 |
| Allogeneic | | Tacrolimus | DMD 10 | 2-18 | Recruiting | | NCT 02196467 |
| Autologous (unaffected muscle) | i.m. | | OPMD 12 | 24 | • stabilization of pharyngeal propulsion;<br>• improved upper esophageal sphincter function (6/12);<br>• dose-dependent improvement in drinking test | | 51 |
| Autologous (unaffected muscle) | i.m. | | OPMD 10 | | Ongoing | | NCT 02878694 |

The number of patients with a specific parameter is indicated as a fraction of total patients considered. DYS, dystrophin; Immun. sup., immune suppression; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR.

tions in muscle diseases resulted in the identification of several candidates, mainly consisting of stromal cells that can be isolated from skeletal muscles (63). Muscle side population (SP) cells are a population of myogenic progenitors that can be isolated from murine and human muscle based on their ability to extrude Hoechst dye (64). Muscle SP cells, similarly to bone marrow–derived SP cells (65), can home to the skeletal muscles of dystrophic mice after systemic delivery, fusing with limited percentages of host myofibers (66). Moreover, muscle SP cells can give rise to MuSCs upon transplantation (67). Although SP cell engraftment appears to be insufficient to be therapeutically relevant in terms of dystrophin complementation, evidence suggests that SP cells may influence the myogenic program through paracrine mechanisms (68).

A population of cells endowed with myogenic potential was identified in the interstitium of murine skeletal muscle through expression of the stem cell marker PW1 and absence of PAX7 and was therefore named PW1$^+$PAX7$^-$ interstitial cells (PICs) (69). PICs do not derive from MuSCs, are myogenic in vitro, and contribute to muscle regeneration, as well as generating MuSCs, following transplantation (69). Notably, a subpopulation of interstitial cells that express PW1 and myogenic factor 5 (MYF5), but not PAX7, was also reported in the CD56$^+$ fraction of cells isolated from human muscles (70).

Beyond these, other populations of cells with myogenic potential have been identified based on molecular markers, adhesion properties, or other functional characteristics. These have been studied primarily by single laboratories and include muscle-derived stem cells (MDSCs), MuStem cells, SMALD$^+$ cells, and smooth muscle–mesenchymal cells (71–74). All have been shown to contribute to new muscle formation upon transplantation (72–74). Further molecular characterization is necessary for each of these, as well as identification of the nature and localization of the cells of origin in vivo.

## Cells from non–skeletal muscle tissues

*Mesenchymal stem cells*. Over the last two decades, different stromal cell types known to exhibit peculiar paracrine/secretory functions have been tested as potential treatments for muscular dystrophies. These mesenchymal stromal cells, often referred to as "mesenchymal stem cells" (MSCs), are under evaluation as potential cellular therapies for different pathological conditions. However, uncertainties remain because of the extreme heterogeneity of such cells obtained from different tissues and based on different criteria (75). Furthermore, it is unclear how paracrine effects are likely to be effective, long-term treatments of chronic, degenerative diseases like muscular dystrophies. Nevertheless, studies using both intramuscular and systemic delivery in different dystrophic mouse models have examined the potential therapeutic efficacy of MSCs of different tissue origin (76–81). Although many studies demonstrate the ability of MSCs to engraft in skeletal muscle, their ability to enhance muscle contractile force is unclear (82). Studies have also suggested the potential of transplanted MSCs to give rise to MuSCs (76, 79). Notably, in some of these studies, MSCs derived from nonmurine tissues were suc-

**Table 2. Selected cell therapy clinical trials for DMD involving nonmyoblast cell types**

| Cell type | Cell therapy approach | | | | No. of patients | Time point (mo) | Outcome | | Reference or trial ID |
|---|---|---|---|---|---|---|---|---|---|
| | Donor | Genetic correction | Route | Immun sup | | | Efficacy | Side effects/ Adverse events | |
| MABs | HLA-matched siblings | No | i.a. | Tacrolimus (+ steroids) | 5 | 2 | • DNA chimerism: 0–0.69%<br>• modest amount of donor-derived DYS in 1 patient<br>• transient functional stabilization (2/5) | Atrial fibrillation, thalamic stroke (1/5); cutaneous reticulum (2/5) | 62 |
| MABs | Autologous | Yes (exon-skipping snRNA) | i.a. | | 5 | | Ongoing | | EudraCT 2019-001825-28 |
| MSCs | Allogeneic (umbilical cord–WJ) | No | i.m. and i.a. | | 9 | 2, 9, 24 | • increased DYS+ fibers after injection (9/9)<br>• increased DYS in qPCR (8/8);<br>• reduction in CK levels (7/9 after 24 mo)<br>• improved pulmonary function | | 87 |
| MSCs | Autologous (BM) | | | | 20 | | Recruiting | | NCT 03067831 |
| CDCs | Allogeneic | No | i.c | Steroids | 13 | 6,12 | • decreased cardiac fibrosis and improved inferior wall systolic thickening (MRI)<br>• preservation of upper limb performance (8/9 lower-functioning patients) | Transient immune response against donor HLA antigens (1/13); atrial fibrillation (5/13); cTn increase (13/13) | 91 |
| CDCs | Allogeneic | No | i.v, | | 20 | | Ongoing | | NCT 03406780 |

The number of patients with a specific parameter is indicated as a fraction of total patients considered. BM, bone marrow; CK, creatine kinase; cTn, cardiac troponin; DYS, dystrophin; i.a., intra-arterial; i.c., intracoronary; Immun. sup., immune suppression; qPCR, quantitative PCR; snRNA, small nuclear RNA; WJ, Wharton jelly.

cessfully transplanted into immune-competent mice, supporting the idea that MSCs have immune-evasive properties (83).

Local and intravenous injections of human adipose-derived stromal cells were able to engraft in muscles of GRMD dogs and express human dystrophin (84). Stromal cells obtained from human dental pulp were administered to GRMD dogs, resulting in significant engraftment in muscles but only modest human dystrophin expression after systemic multiple deliveries (85). These findings support the idea that MSCs may exert antiinflammatory or paracrine effects (23).

The effects of intravenous administration of human umbilical cord–derived MSCs (UC-MSCs) were studied in one pediatric and two adult BMD patients (86). Although histology of muscle biopsies did not reveal any improvement, the clinical examination reported gait improvement in the pediatric patient (86). In a subsequent study, nine DMD patients received a combination of intramuscular and systemic administration of allogeneic UC-MSCs (NCT02484560) (Table 2). Pulmonary function, a readout of diaphragmatic activity, was improved in all patients (87). Variable induction of dystrophin expression was reported in all patients, and although limb muscle strength was not significantly different between pre- and post-treatment, the majority of the treated patients exhibited a reduction in creatine kinase levels (87).

*Cardiosphere-derived cells*. Recent evidence suggests a promising role of a cardiac population of stromal cells, referred to as cardiosphere-derived cells (CDCs) (88). Preclinical studies, which evaluated beneficial effects in dystrophic heart as the primary outcome, demonstrated that CDCs delivered systemically in *mdx* mice improved also the skeletal muscle phenotype (89, 90). However, these effects appeared to be due not to genetic restoration of dystrophin, but rather to effects possibly related to exosomes secreted by the transplanted cells. Intriguingly, in a clinical trial of the intracoronary injection of CDCs in 25 DMD patients, a measure of upper limb strength was greater in the 13 treated patients compared with the 12 controls (91). These findings have led to an additional clinical trial (NCT03406780) in which CDCs were injected intravenously in a cohort of mostly (80%) nonambulatory DMD patients (Table 2).

*Bone marrow–derived/blood-borne cells*. Despite initial enthusiasm that bone marrow–derived hematopoietic cells might serve as a source of myogenic progenitors and that bone marrow transplantation could then be a treatment for muscular dystrophies (92, 93), long-term studies in which bone marrow transplantation was performed in *mdx* mice found a negligible effect: only 0.09% of the myofibers became chimeric during the entire lifespan of the mice (94). These negative results were confirmed in a study performed in dystrophic dogs (95). Inefficient contribution of cells of the hematopoietic lineage into myofibers was also observed in DMD patients (96, 97).

The inefficiency of bone marrow cells in complementing dystrophin expression in dystrophic patients led to a search for alternative sources of circulating myogenic progenitors. A population of cells expressing CD133 and exhibiting myogenic poten-

tial when cocultured with myogenic cells was identified in the blood and muscle of DMD patients (98, 99). CD133$^+$ cells were shown to ameliorate the dystrophic phenotype upon transplantation in different dystrophic mouse strains (98–100). Intramuscular and intra-arterial delivery of genetically corrected CD133$^+$ cells, isolated from DMD patients and transduced with lentiviral vectors expressing antisense oligonucleotides with exon-skipping ability, resulted in dystrophin expression and a recovery of muscle function in *mdx/SCID* mice (99). Similarly corrected, autologous, muscle-derived CD133$^+$ cells were shown to increase the number of dystrophin-positive fibers when injected intra-arterially in GRMD dogs (101).

## Pluripotent stem cells

Pluripotent stem cells (PSCs), comprising embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are particularly appealing in regenerative medicine because they can be propagated indefinitely in the undifferentiated state but retain the capacity to differentiate to all somatic tissues (102, 103). Protocols to obtain muscle progenitors from PSCs are creating possibilities for cell therapy for DMD (104).

Initial studies aimed at the generation of myogenic progenitors involved the formation of embryonic bodies (EBs) and led to poor myogenic differentiation, which was attributed to spontaneous differentiation into heart progenitor cells (105). To increase differentiation along the skeletal myogenic lineage, forced overexpression of the transcription factor MyoD was attempted, but the resulting increase in differentiation remained suboptimal and failed to produce myogenic self-renewing cells (106). Strategies relying on antibodies recognizing myogenic and mesenchymal markers and selective media compositions were used to isolate a minority of engraftable myogenic progenitors appearing in ESC cultures (107, 108). Notably, generation of mouse myogenic precursors endowed with in vivo regenerative potential was also achieved by the expressing of a central regulator of embryonic myogenesis, PAX3, during EB-PSC differentiation (109). Similar approaches based on overexpression of PAX7 were subsequently used to generate human skeletal myogenic precursors from both ESCs and iPSCs (110, 111). These PSC-derived myogenic progenitors were shown to promote substantial muscle regeneration and to seed the MuSC compartment when delivered systemically or intramuscularly to *mdx* mice (109, 110, 112, 113).

As an alternative to transcription factor transfection, directed differentiation of PSCs to form myogenic progenitors has met with success. The first study to reprogram, differentiate, genetically correct, and transplant human iPSC–derived myogenic cells from patients with muscular dystrophy involved the differentiation of cells toward MAB-like cells (114). Human and mouse myogenic progenitors have been generated from PSCs by the inclusion of factors important for muscle development in the medium (115, 116). Notably, those culture conditions resulted in production of PAX7$^+$ cells resembling quiescent MuSCs. When transplanted in vivo in a preclinical model of DMD, the myogenic cells form large foci of myofibers (115).

It was also demonstrated that genetically corrected DMD myogenic cells derived from human PSCs (hPSCs; using CRISPR/Cas9 technology) could be directed to differentiate and restore dystrophin in immune-deficient *mdx* mice to levels approaching those observed in cells directly isolated from fetal tissues (117, 118). Work from different laboratories further expanded both gene correction strategies and myogenic cell induction/selection protocols applied to PSCs (111, 119–122).

Notably, a recent study reported the production of myogenic progenitors from teratomas derived from murine PSCs (123). This protocol relies on isolation of myogenic progenitors from teratomas developed from PSCs injected in injured dystrophic muscle. Importantly, the ability to colonize host muscle after intramuscular injection appears to be greater in teratoma-derived cells than in myoblasts and PAX-modified PSCs. Teratoma-derived cells can also give rise to functional MuSCs (123). Further studies will address whether teratoma-derived myogenic cells are suitable for systemic delivery and whether they can be derived from human teratomas.

*Direct reprogramming to myogenic progenitors*. Protocols involving overexpression of myogenic regulatory factors have been proposed to efficiently derive myogenic progenitors from sources different from PSCs, in a process of direct reprogramming that is applicable to fibroblasts from DMD patients (124). This approach was used to generate myogenic progenitors for testing in transplantation paradigms in *mdx* mice (125). Direct reprogramming of accessible autologous cell types may represent a clinically relevant alternative to PSC-derived cells.

## Cell therapy in dystrophic heart

Over the past two decades, more than 200 trials based on cell transplantation have been performed in patients affected by cardiovascular diseases. These trials have involved different cell types, such as skeletal myoblasts, bone marrow–derived cells, MSCs, and cardiac-resident progenitors (126). However, the efficacy of these cells remains controversial, and the absence of a general consensus has limited the transition of these therapies from investigational to general practice (127). In particular, the poor ability to differentiate into cardiomyocytes and the resulting risks of promoting ventricular arrhythmias are limiting the application of myoblast transplantation (128).

The ideal cell type to use in DMD heart would generate new cardiomyocytes expressing dystrophin, which would confer long-term protection against the disease process. Similar to what has been reported for skeletal muscle, restoring 15%–20% of normal levels of dystrophin appears to be sufficient to prevent cardiac disease progression (129). Preclinical studies in dystrophic mice have investigated the cardiac effects of cells that had previously been tested in skeletal muscle. Bone marrow transplantations in *mdx* mice suggest a limited contribution of bone marrow–derived cells to the cardiomyocyte pool (92). Aorta-derived MABs, which can be induced to differentiate into beating cardiomyocytes in vitro, can delay the onset of cardiomyopathy upon transplantation into the hearts of severely dystrophic mice (53, 130). Intriguingly, a population of MABs, able to spontaneously differentiate into functional cardiomyocytes and referred to as cardiac MABs, was isolated from ventricles of postnatal hearts (131). Although not yet tested in dystrophic settings, cardiac MABs efficiently generate new myocardium

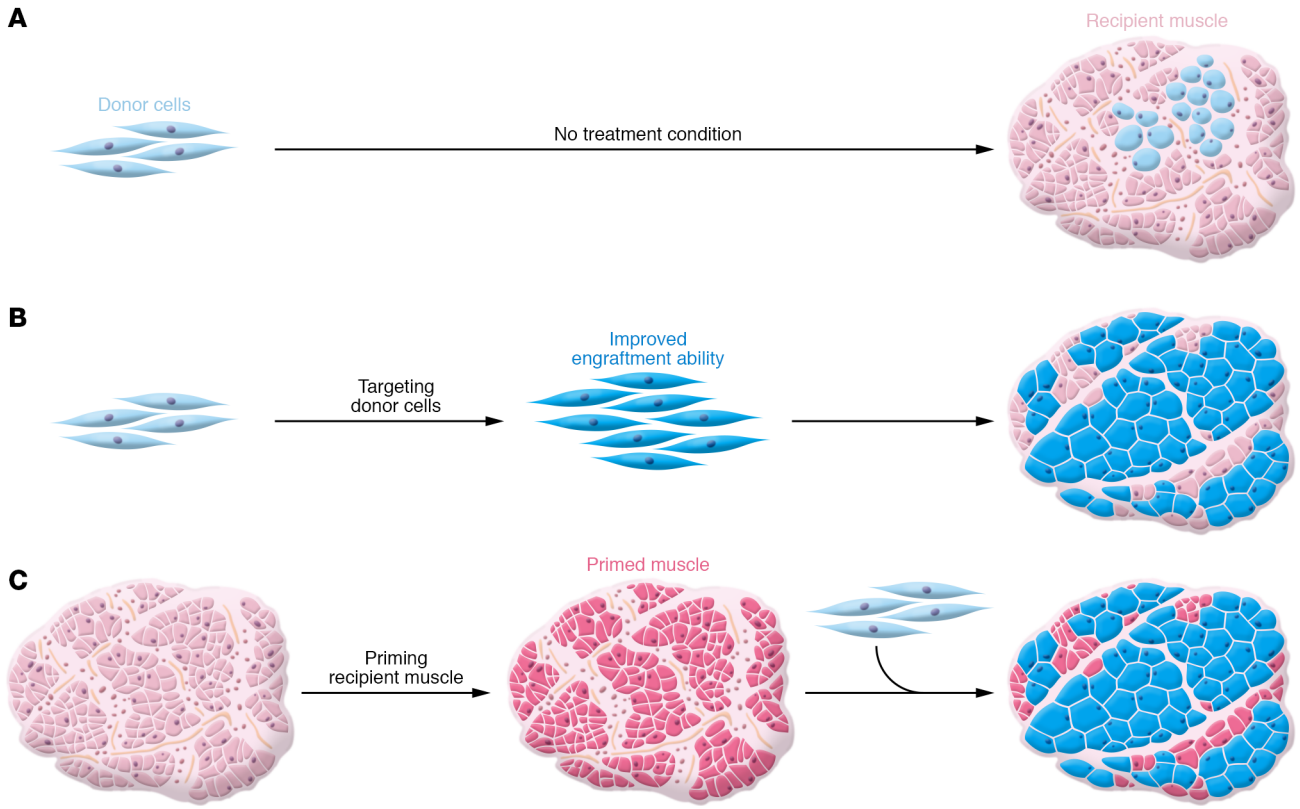


**Figure 3. Strategies aimed at increasing in vivo engraftment of transplanted cells. (A–C)** Approaches to enhance productive engraftment in cell transplantation experiments include targeting the cells to be transplanted and priming the recipient tissue. Productive engraftment is schematized by a transverse section of a transplanted muscle showing an increased proportion of fibers replenished by the transplanted cells (marked as blue fibers) compared with "No treatment condition," as well as a reduction in the dystrophic pathology.

in ischemic murine hearts (131). More recently, the observation that iPSCs may be obtained from DMD patients, genetically corrected, and differentiated to functional cardiomyocytes has paved the way for future investigations for the treatment of dystrophic cardiomyopathy (117). Finally, CDCs were shown to improve cardiac myopathy after intra-cardiac or intravenous delivery in *mdx* mice (89, 90).

Recently, a phase I/II randomized, controlled, open-label trial (NCT02485938) assessed the safety and efficacy of intracoronary allogenic CDCs in DMD patients with established cardiomyopathy (91). CDC administration appeared safe and demonstrated signs of efficacy. The sustained changes in scar and regional function are reminiscent of the responses to CDC observed in a recent trial of ischemic cardiomyopathy (132). The results of a recently completed follow-up phase II double-blind, placebo-controlled clinical trial (NCT03406780), in which CDCs were infused intravenously, await publication.

## Challenges and future perspective

To date, only MuSC-derived myoblasts, MABs, $CD133^+$ cells, MSCs, and CDCs have been evaluated in clinical trials, and only in studies involving a limited number of dystrophic patients (Table 1 and Table 2). Despite some positive indications in terms of safety or functional/histological recovery, these therapeutic options are still preliminary. Many obstacles continue to limit the clinical use of cell therapy in dystrophic settings.

An important limitation is that MuSCs lose their regenerative potential when expanded ex vivo (35, 133). Similarly, $CD133^+$ cells, SP cells, and MABs appear to be defective when isolated from diseased muscles (134–136). Since the first pioneering studies on myoblast transplantation, there has been much interest in developing culturing conditions to preserve the regenerative potential of transplanted cells (45). Most studies were initially centered on myoblasts and MuSCs, but findings can potentially be extended also to other cell types (Figure 2). A promising approach uses bioengineering principles to more closely mimic, in terms of tissue rigidity, the natural microenvironment, where MuSCs are maintained (137, 138). Alternative approaches rely on modulation of signaling pathways that maintain stemness and suppress terminal myogenic differentiation (35, 139–143). Additionally, epigenetic regulators have been studied as potential ways to enhance the therapeutic potential of MuSCs (144). Genetic manipulation was also shown to be a suitable approach, as exemplified in a study in which reversible cell immortalization was obtained with excisable hTERT and Bmi1 transgenes, in both DMD MuSCs and MABs (145). Additionally, different reports suggest that interfering with genes controlling the myogenic program may preserve muscle cell regenerative potential (146, 147). Finally, lowering oxygen tension close to physiological levels was shown to improve the self-renewal of myoblasts and transplantation efficiency in *mdx* mice (148).

Poor engraftment is another obstacle hindering the success of cell transplantation approaches, which depend on different

parameters such as homing to the degenerating skeletal muscle, intramuscular diffusion, survival, and proper functional maturation of the transplanted cells. Attempts have been made to modulate key molecular determinants controlling these parameters (Figure 3). The amount of donor muscle formed reportedly increased after exposure of donor myogenic cells, before or during their implantation, to factors altering the activity of signaling pathways initiated by FGF, IGF, IL-4, Wnt7a, and TGF-β superfamily members (149–153). Other approaches attempted to increase the survival of grafted cells by preconditioning cells with stressful stimuli or interfering with signaling pathways controlling cell death (154, 155). Several injectable biomaterials have also been shown to improve survival and the maturation of cells transplanted in dystrophic muscles (156, 157).

Several strategies targeting the recipient tissues reportedly increase engraftment (Figure 3). Exercise or induction of mild local damage was shown to improve transplantation efficiency of different cell types (158–160). Promoting neovascularization enhances engraftment of myoblasts, MABs, and MDSCs (161–163). Modifying extracellular matrix or inhibiting profibrotic molecules improves muscle engraftment (161, 164–167). The inflammatory state of recipient muscles also seems to play a key role in controlling transplantation outcome, as intramuscular coinjection of macrophages with myoblasts enhanced their engraftment (168). Moreover, intramuscular overexpression of the cytokine HMGB1 and the antiinflammatory action of a nitric oxide–releasing derivative of flurbiprofen increase the efficacy of MAB therapy (169, 170).

One of the greatest challenges for cell therapy in muscular dystrophies is effective systemic delivery. The selection of a proper route of administration appears to be crucial. Arterial delivery seems to be an absolute requirement to escape filter organs, which are likely to prevent any intravenously delivered cells from reaching muscles throughout the body, and to ensure muscle targeting with consequent genetic complementation (54, 171). Another important aspect influencing the outcome of systemic delivery is the ability of the transplanted cells to cross the blood vessels and colonize the recipient muscles. Notably, promising attempts were recently made to enhance transendothelial migratory ability in MuSC-derived myoblasts (172). Furthermore, specific molecules have been shown to modulate the muscle homing of systemically injected $CD133^+$ cells, MDSCs, SP cells, and MABs (159, 173–175). These initial studies call for a systematic investigation of the molecular and cellular events controlling these processes.

An additional determinant of productive cell therapy is the control of the immune response against transplanted allogenic cells or against autologous cells expressing a foreign therapeutic gene (176). The exact mechanisms underlying rejection are still under active investigation, but increasing evidence suggests a major role for T lymphocytes (101, 177, 178). The optimization of protocols to minimize the immune response will be crucial for the success of cell therapy in dystrophies and will require careful evaluation of the immunogenic properties of the specific cell population to be transplanted (44, 81, 84, 179), selection of effective immunosuppressant drugs that are not toxic to the transplanted cells (180, 181), and identification of the optimal dosage and duration of immunosuppression to balance efficacy and undesired side effects. Indeed, evidence suggests that transient immunosuppression might be sufficient to ensure the long-term retention of transplanted cells (182, 183).

For most of the cell populations that have been tested, the relative contribution of paracrine effects versus gene complementation is unknown. An understanding of these issues will facilitate the definition of effective cell therapy protocols. The experience gained with recent preclinical and clinical studies will lead to improved clinical trial design (184). These optimizations, together with the recent progress in gene correction strategies, will pave the road for future evaluation of cell therapy in muscle diseases.

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Address correspondence to: Thomas A. Rando, Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, California 94305-5235, USA. Phone 650.849.0444; Email: rando@stanford.edu. Or to: Stefano Biressi, Department of Cellular, Computational and Integrative Biology, University of Trento, Via Sommarive 9, Povo, 38123 Trento, Italy. Phone: 39.0461.28.5290; Email: stefano.biressi@unitn.it.

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