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# Stem Cell Based Therapy for Muscular Dystrophies: Cell Types and Environmental Factors Influencing Their Efficacy

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## 1. Introduction

Muscular dystrophies are inherited disorders in which muscle fibers are unusually susceptible to damage, leading to progressive loss of muscle structure and function. Some types of muscular dystrophy affect heart muscles, other involuntary muscles and other organs. The most common form of muscular dystrophy, Duchenne Muscular Dystrophy (DMD), is due to genetic deficiency of the protein dystrophin (Monaco et al., 1985). This protein is one of several partners that interact to link intracellular cytoskeleton to extracellular matrix (ECM) hence consolidating the scaffold necessary for maintaining structural integrity of skeletal muscle fibers. Dystrophin deficiency destabilizes muscle fibers, which become less resistant to contractions leading to muscle fiber necrosis and subsequent regeneration.

Skeletal muscle repair, maintenance and regeneration are mediated by muscle-specific stem cells: the satellite cells (Mauro, 1961), located underneath basal lamina of muscle fibers. In DMD muscles, fat and connective tissue often replace muscle fibers in the late stages of muscular dystrophy, indicating that muscle regeneration does not keep up with fiber loss. Defective muscle regeneration could be due to exhaustion of proliferative capacity of satellite cells (Blau et al., 1983; Webster & Blau, 1990) or to environmental factors that are not conducive to their function. The healing process usually includes sequential and overlapping events of muscle fiber degeneration, inflammatory reaction, regeneration and remodeling of ECM components that require tightly regulated orchestration of the interactive cross-talk that conditions the outcome of the regenerative process. Uncontrolled wound-healing, in response to chronic injury and inflammation, results in tissue fibrosis and scarring which impacts on the efficiency of muscle regeneration, hence contributing to the degradation of muscle function.

At present, we still have no cure for any form of muscular dystrophy, but medications and therapy can slow the course of the disease to allow people with muscular dystrophy to remain mobile for as long as possible. Nevertheless, experimental therapeutic strategies have been initiated in light of basic and technical advances of skeletal muscle biology and pathophysiology. The description of the muscle regeneration process and the identification

of the cells responsible for myofiber regeneration led to stem cell therapy being considered as a potential strategy to alleviate muscle deficiency in DMD patients. Alternatively, the identification of gene defects and the sophistication of molecular biology technologies have opened perspectives for gene therapy, either by providing the deficient gene, or by restoring gene function. Other strategies combining both approaches have been considered and imply the correction of patients' own stem cells before grafting them into the diseased muscles. These promising strategies have been challenged in animal models of muscular dystrophies and although they achieved a certain success, they also identified a number of limitations. Moreover, the failure of myogenic cell grafting to improve muscle function and to restore dystrophin expression in clinical trials of DMD patients, underscored the need to improve the efficiency of cell therapy. Cell environment, which comprises ECM and extracellular matrix deposited factors such as growth factors, cytokines and chemokines, regulate diverse cellular functions. These molecules are metabolized by Matrix MetalloProteinases (MMPs) that play a central role as regulators of tissue microenvironment. In normal situations, precise spatiotemporal repertoires of MMPs balanced by inhibitors, among which are the four Tissue Inhibitors of MatrixmetalloProteinases (TIMPs), regulate extracellular signaling networks and maintain tissue homeostasis. On the contrary, increased MMPs expression or activity has been demonstrated in various disease situations including, practically every known inflammatory disease (Manicone & McGuire, 2008). Such disruption of the dynamic equilibrium between MMPs and TIMPs may affect diverse cellular functions including cell proliferation, migration, adhesion and apoptosis (Holmbeck et al., 2005; Hulboy et al., 1997; Vu & Werb, 2000). In DMD, for example, inflammation and fibrosis are major hurdles in the path of therapeutic strategies (Wells et al., 2002) and their resolution is expected to positively impact on the efficiency of any form of therapy, but more specifically, on the efficiency of cell therapy. Indeed, myogenic stem cells have limited migratory capacity, which is further aggravated by excessive proliferation of connective tissue. Therefore, improving the efficiency of cell therapy could be achieved either by myogenic cells better able to digest accumulated ECM components or, alternatively, by other types of stem cells that can be recruited from a resident or circulating pool and are capable of migrating through one or several tissue barriers to home into skeletal muscles. In this chapter, we consider the use of stem cell therapy to treat muscular dystrophies. By going through the different cell types that have been used, we will try to define the best cell type to use, how to handle and expand these cells before transplantation and the best route of delivery. Moreover, the possibility of using genetically-modified autologous stem cells for transplantation will be presented. This would only be possible if the stem cell had not been deleteriously affected by the dystrophic environment. Finally, we will consider the host environment as a modulator of cell behaviour and the dual role MMPs play in the control of this environment and their impact on transplanted cells migration, differentiation and self-renewal.

## 2. Potential therapies for duchenne muscular dystrophy

Muscular dystrophies are a heterogeneous group of inherited neuromuscular disorders, including X-linked recessive as in DMD, autosomal recessive as in limb-girdle muscular dystrophy type 2, or autosomal dominant as in facioscapulohumeral muscular dystrophy, myotonic dystrophy, and limb-girdle muscular dystrophy type 1 (Emery, 2002). In the last two decades, different types of dystrophies have been genetically characterized. The most

frequent and most severe form, DMD, is a progressive, incurable X-linked recessive disorder that affects 1 in 3500 newborn boys and leads to death in the second or third decade of life (Bushby et al., 2010). DMD patients lack the protein dystrophin while in-frame mutations of the same gene led to expression of a partially functional protein, resulting in the milder Becker muscular dystrophies (BMD). As a result of the absence of dystrophin, muscle fibers of DMD patients undergo necrosis followed by regeneration which, in the long run, fails to keep up with the recurrent cycles of degeneration-regeneration and muscle fibers are lost and replaced by fibro-fatty tissue. Interruption of these cycles can be achieved by dystrophin restoration to the muscle fiber membrane (Meng et al., 2011a). Several strategies can now be used to restore dystrophin to the muscle fibers of affected patients. They include virally-mediated gene therapy, read-through of stop codons, up-regulation of compensatory genes, or skipping of mutated dystrophin exons to give rise to a shorter, but still functional dystrophin protein. However, all of these have possible drawbacks (reviewed (Goyenvall & Davies, 2011; Guglieri & Bushby, 2010; Hoffman et al., 2011; Sugita & Takeda, 2010)). Either gene therapy, or application of antisense oligonucleotides to skip mutated dystrophin exons, requires that the patient has sufficient muscle fibers remaining for treatment. In addition, exon skipping is mutation-dependent and not all patients have mutations amenable to this approach.

### 3. Stem cell therapy for the treatment of DMD

The concept of a cell-based therapy to alleviate loss of muscle structure and function in muscular dystrophy originated with the observation of the intrinsic ability of myogenic stem cells to fuse either with each other to form multinucleated myofibers, or with necrotic muscle fibers to form mosaic fibers. In theory, functional correction could be achieved in DMD by the generation of either hybrid muscle fibers where the donor nuclei provide the missing gene product and/or the regeneration of normal myofibers from the fusion of normal donor cells to replace lost muscle fibers. Cell therapy was the first biologically based approach applied for the treatment of DMD and required the use of animal models to explore the beneficial effects of this therapy. The ability of cultured myogenic cells to regenerate new muscle fibers, that reconstitute the same architectural organization of the original muscle and induce functional recovery, has been validated using an experimental model of irreversible injury to adult rodent muscle associating auto-transplantation of skeletal muscle to X-irradiation (Alameddine et al., 1989; Alameddine et al., 1991; Alameddine et al., 1994).

#### 3.1 Stem cells

Stem cells are defined as cells that can both self-renew and give rise to more differentiated cell type, whereas precursor cells do not have the ability to self-renew. Both cell types present a great advantage for the treatment of muscular dystrophies as they could repair segmental necrosis and also give rise to regenerated muscle fibers to replace those that are lost as a consequence of the dystrophy. They could therefore be effective at later stages of the dystrophy, when muscle fibers have already been lost. Donor cells derived from a normal individual will automatically express dystrophin when they differentiate into a muscle fiber, but the quantity and distribution of dystrophin within the fiber will depend on the number of donor myonuclei and the size of segments of the fiber to which they

contribute. However, the recipient and donor will need to be HLA-matched, so that stem cells from one normal donor could not be used to treat all patients.

Although many different stem or precursor cells have been shown to contribute to muscle regeneration in animal models, many of these give rise to only limited amount of muscle, for example haematopoietic stem cells (Ferrari et al., 1998) and mesenchymal stem cells (Chan et al., 2006; Meng et al., 2010). Satellite cells are the archetypal skeletal muscle stem cell, but they are by definition quiescent cells underneath the basal lamina of muscle fibers and it would be impossible to obtain enough of them for therapeutic application. However, the progeny of satellite cells, muscle precursor cells or myoblasts, could be prepared in sufficient quantity for transplantation. In this review, therefore, we will focus on cells that can be expanded in culture and that have been shown to contribute to muscle regeneration—myoblasts, cells derived from blood-vessel associated pericytes (termed mesoangioblasts) and skeletal muscle-derived AC133+ cells. There are several recent reviews on stem cells to treat muscular dystrophies, which cover most of the cell types that have been studied (Meng et al., 2011a; Negroni et al., 2011; Palmieri et al., 2010; Skuk & Tremblay, 2011; Tedesco et al., 2010).

### 3.2 Models and markers

To investigate the potential contribution of a particular cell type to muscle regeneration, the standard experiment is to graft the cells into an animal model of DMD and measure their contribution to skeletal muscle fibers, which may be quantified by either counting the number of dystrophin-positive fibers, or measuring the amount of dystrophin on western blot. Several animal models of DMD exist but the most widely-used is the dystrophin-deficient *mdx* mouse (Bulfield et al., 1984) used in 1,940 pubmed publications, as of 21 July 2011. Other models of DMD include the golden retriever muscular dystrophy (GRMD also known as Canine X-linked Muscular Dystrophy CXMD) dog and zebrafish (reviewed (Banks & Chamberlain, 2008; Collins & Morgan, 2003)). Mouse and dog models have been used to investigate different potential therapies for DMD, including precursor/stem cell transplantation (Nakamura & Takeda, 2011). However, when grafting cells from one donor to another, the host must either be immunodeficient or immunosuppressed. Therefore, *mdx* mice with different types of immunodeficiency have been used as hosts in cell transplantation experiments, including *mdx* *nu/nu* (Boldrin et al., 2009; Collins et al., 2005; Partridge et al., 1989), SCID *mdx* (Benchouir et al., 2007; Dellavalle et al., 2007; Torrente et al., 2004), Rag<sup>-/-</sup> *mdx* (Gerard et al., 2011), or *mdx* mice immunosuppressed with FK506 (Kinoshita et al., 1994b), but to what extent these different hosts are comparable, being on different genetic backgrounds and having different mechanisms and degrees of immunodeficiency, has not been ascertained (reviewed (Meng et al., 2011b)). Immunodeficient mice are more convenient to work with than mice that have to be immunosuppressed and seem to permit greater donor-myoblast-derived muscle regeneration (Partridge et al., 1989). However, it is important to consider the effect of the immunological system on donor-derived muscle regeneration, as DMD patients will not be immunodeficient. Non-dystrophic mice or monkeys, whose muscles have been injured to mimic the degeneration and regeneration that occurs in dystrophic muscles, have also been used as recipients to test cell transplantation (Cooper et al., 2001; Morgan et al., 2002; Sacco et al., 2008; Skuk et al., 1999), as have mice that model different types of dystrophy, e.g. sarcoglycan (Sampaolesi et al., 2003) and dysferlin-deficient mice (Diaz-Manera et al., 2010).



A major consideration when using both dystrophic and non-dystrophic animal models to test stem cells is that the host muscle usually has to be injured in some way to enhance donor cell engraftment. This is surprising, as the muscle fiber degeneration and regeneration that is already occurring in dystrophic muscle would be thought to be sufficient to promote donor stem cells to contribute to muscle regeneration. However, muscle fiber necrosis is often focal and only cells located nearby contribute to regeneration (Yokota et al., 2006). Therefore, if the transplanted stem cell is located a distance away, it may not either receive the correct signals, or be able to migrate to the damaged fibers. As many of these injury regimes are very severe, for example, cryoinjury (Brimah et al., 2004; Irintchev et al., 1997; Negroni et al., 2009) or use of snake venoms (Lefaucheur & Seville, 1995; Silva-Barbosa et al., 2005) to induce degeneration and regeneration in host muscles, they could not be used in patients. Even in dystrophin-deficient *mdx* *nu/nu* host mice, satellite cells contribute little, if any, to muscle regeneration (Boldrin et al., 2009; Collins et al., 2005) although myoblasts contribute to muscle regeneration to a greater extent (Morgan et al., 2002; Partridge et al., 1989). This poor contribution of donor cells to muscle regeneration is likely to be due to the fact that *mdx* mouse muscles, in contrast to those of DMD patients, regenerate very well. We therefore blocked muscle regeneration in *mdx* muscles by applying local high doses of radiation, to obtain a model more similar to DMD, in which the muscle degenerates and atrophies but does not regenerate (Morgan et al., 1990; Pagel & Partridge, 1999; Wakeford et al., 1991). If host muscle is irradiated with 18 Gy before donor cell grafting, satellite cell and myoblast contribution to muscle regeneration is significantly augmented (Boldrin et al., 2009; Collins et al., 2005; Morgan et al., 2002). This may be due to prevention of competition from local host stem or satellite cells, as irradiated *mdx* muscles do not regenerate (Pagel & Partridge, 1999; Wakeford et al., 1991) unless a severe injury, e.g. injection of snake venom notexin, is imposed on them, which evokes rare radiation-resistant stem cells to regenerate (Gross & Morgan, 1999; Heslop et al., 2000). Other models that could be used to test whether a wholly or partially emptied satellite cell niche is necessary for efficient donor muscle stem cell engraftment include Pax7 knockout mice (Seale et al., 2000) that lack satellite cells, or the *mdx* mouse that also lacks telomerase (mTR) activity and therefore shows a reduction in the regenerative capacity of myogenic stem cells (Sacco et al., 2010).

Another important consideration is the marker(s) to be used to assess the contribution of donor cells to regenerated muscle fibers and/or satellite cells (reviewed (Meng et al., 2011a)). As the aim is to produce dystrophin in host muscle fibers, it is sensible to quantify dystrophin restoration in the host muscles (Partridge et al., 1989). Because “revertant” fibers that spontaneously express dystrophin are present in animal models of DMD (Hoffman et al., 1990) and in DMD patients (Arechavala-Gomez et al., 2010) and because clusters of revertant fibers increase in number with time (Hoffman et al., 1990; Yokota et al., 2006), revertant fibers must be controlled for, particularly if dystrophin is being used alone as a marker of donor-derived muscle fibers and especially in time course studies. If grafting human cells into mouse, a human-specific dystrophin antibody (e.g. Novocastra Dys3 (Brimah et al., 2004)) may be used, that will not identify mouse revertant fibers. Because of the existence of revertant fibers, many groups use a second marker of either muscle fibers or cells of donor origin, e.g. by using donor cells from genetically-modified mice, e.g. myosin 3f nLacZ-E, that is expressed in myonuclei of donor origin, Myf5 nLacZ/+ that is expressed in satellite cells of donor origin (Collins et al., 2005), or ubiquitously (Morgan et al., 2002) or muscle-specifically (Kinoshita et al., 1994a) expressed  $\beta$ -gal or GFP. Donor cells may

alternatively be marked in culture with constructs expressing a marker protein (Blaveri et al., 1999; Cousins et al., 2004; Diaz-Manera et al., 2010; Morgan et al., 2002). However, caution should be used in interpreting results, as GFP is notoriously difficult to use in skeletal muscle (Jackson et al., 2004), some markers spread further along a mosaic muscle fiber than others (Blaveri et al., 1999) and others are switched off in vivo (Boldrin et al., 2009).

### 3.3 Contribution of locally-delivered donor stem cells to muscle regeneration.

A large body of evidence can be found in the literature to illustrate the contribution of myoblasts to skeletal muscle regeneration, although the number of donor-derived muscle fibers is limited (Figure 1). However, the host muscle environment that permits regeneration from myoblasts of mouse and human origin appears to be different, although a comparative experiment to establish this point has not been performed: human myoblasts form more muscle within host muscles that have been cryoinjured prior to grafting (Brimah et al., 2004), whereas mouse myoblasts form significantly more muscle in irradiated host muscles (Boldrin and Morgan, manuscript in preparation).

Different muscle injury models used for intra-muscular grafting of putative muscle stem cells may also give rise to discrepancies between groups. Some groups have grafted cells into muscles of non-dystrophic mice that had been cryo-injured immediately prior to grafting (Brimah et al., 2004; Cooper et al., 2001; Ehrhardt et al., 2007), but others grafted cells into muscles of *mdx* SCID mice that had been injected 48 hours previously with cardiotoxin, (Dellavalle et al., 2007) or into cryo-injured muscles of immunodeficient Rag2<sup>-/-</sup> gamma chain-non-dystrophic mice (Pisani et al., 2010). Vauchez et al. grafted into muscles of non-dystrophic SCID mice, injuring the muscles prior to grafting by a combination of irradiation and notexin (Vauchez et al., 2009); Zheng et al. grafted cells into muscles of SCID mice that had been injured by cardiotoxin one day previously (Zheng et al., 2007). How these different injury regimes mimic the dystrophic muscle environment and to what extent the local environment, genetic background and immunological status of the host mouse affect muscle stem cell behavior are important to determine, for the identification of robust methodologies which could reliably be used for therapeutic trials in muscular dystrophies.

Interestingly, although they contributed to much muscle regeneration after intra-arterial injection, pericytes only gave rise to very small numbers of muscle fibers after intra-muscular transplantation: CD56<sup>+</sup>/ALP<sup>-</sup> cells (satellite-cell derived myoblasts) gave rise to more muscle than CD56<sup>+</sup>/ALP<sup>+</sup> cells (pericytes), but CD56<sup>-</sup>/ALP<sup>-</sup> cells, taken to be fibroblasts, made very few donor muscle fibers (Dellavalle et al., 2007). Meng et al. also found that both CD56<sup>+</sup> and CD56<sup>-</sup> skeletal muscle-derived cells contributed to muscle regeneration, CD56<sup>+</sup> cells making significantly more muscle than either CD56<sup>-</sup>, or non-fractionated cells after intra-muscular transplantation. CD56<sup>+</sup> cells contributed predominantly to nuclei inside the basal lamina of muscle fibers, i.e. within muscle fibers and/or satellite cells. But CD56<sup>-</sup> or non-sorted cells contributed to significantly more nuclei outside the basal lamina, confirming that there were more non-myogenic cells within CD56<sup>-</sup> cell population (Meng et al., 2011b). Zheng et al. showed that human skeletal muscle-derived CD56<sup>+</sup> cells that also expressed CD34 and CD144 (myoendothelial cells) contributed to more muscle regeneration than did CD56<sup>+</sup>/CD34<sup>-</sup>/CD144<sup>-</sup> cells (myoblasts) (Zheng et al., 2007) in contrast to the findings of Meng suggesting that pericytes, rather than endothelial cells, are the major CD56<sup>-</sup> contributor to muscle regeneration (Meng et al., 2011b).

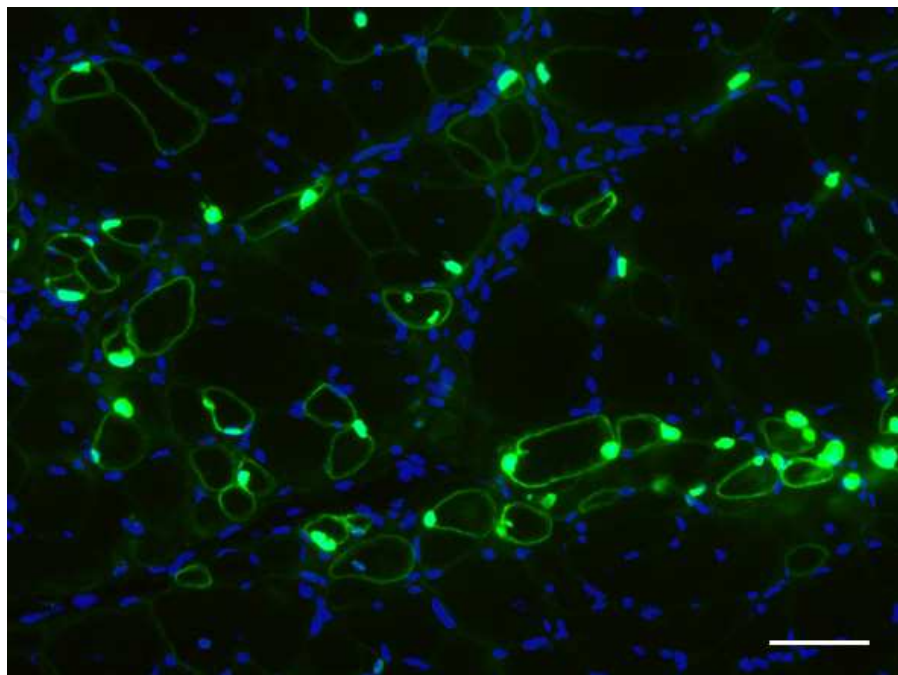


Fig. 1. 7  $\mu$ m transverse cryosection of *mdx* nu/nu host *tibialis anterior* muscle, that had been cryoinjured and grafted with  $5 \times 10^5$  human skeletal muscle-derived stem cells 4 weeks previously. Stained with antibodies to human spectrin and human specific lamin a/c, that recognise muscle fibers and nuclei of human origin respectively. Counterstained with DAPI. Bar= 50  $\mu$ m. (Courtesy of Dr Jinhong Meng).

### 3.4 Contribution of systemically-delivered donor stem cells to muscle regeneration

The contribution of blood vessel-derived cells (both from skeletal muscle and embryonic dorsal aorta) to skeletal muscle regeneration *in vivo* after their systemic delivery has been demonstrated in several publications (Dellavalle et al., 2007; Sampaolesi et al., 2003; Sampaolesi et al., 2006)(reviewed (Sancricca et al., 2010)), however these promising findings could not be replicated by others (Meng et al., 2011b). For long-term efficacy, it would be useful to know whether a grafted pericyte self-renews to give more functional pericytes and if so, what contribution these have to further muscle regeneration. Another stem cell that is promising for systemic delivery to skeletal muscle is the AC133+ cell, derived from either blood (Torrente et al., 2004), or skeletal muscle (Benchaouir et al., 2007).

### 3.5 Death and proliferation of grafted cells

Donor myoblasts die on intra-muscular grafting (Beauchamp et al., 1999; Skuk et al., 2002; Smythe et al., 2000) possibly as a result of one or a combination of various factors: cell dissociation, trophic factor withdrawal, oxidative stress, excito-toxicity, hypoxia and, possibly, anoikis (reviewed (Gerard et al., 2011; Skuk & Tremblay, 2011)) and much effort has been expended to prevent this death (reviewed (Skuk & Tremblay, 2011)). Recent experiments have indicated that the number and density of cells transplanted into one site intra-muscularly may also be critical factors influencing their survival and proliferation (Pellegrini & Beilharz, 2011) and that more cells may not give rise to more muscle fibers of donor origin (Pellegrini & Beilharz, 2011; Praud et al., 2003; Rando & Blau, 1994), possibly



because cells in the centre of dense pellets undergo more apoptosis. But another theory is that the cells that die are irrelevant, as those that survive proliferate extensively under appropriate environmental conditions, to reconstitute the host muscle (Beauchamp et al., 1999). It is however unclear whether other types of muscle stem cell undergo death after transplantation, or if they proliferate within the grafted muscles.

### 3.6 Signals inducing muscle stem cells to contribute to muscle regeneration

Intra-arterial injection of mesoangioblasts has shown that only a very small percentage reach downstream skeletal muscles, most being trapped in the filter organs. To enable cells to exit blood vessels, the vessels must express the appropriate adhesion molecules for that cell type. Molecules that have been shown to be important for mesangioblast extravasation into skeletal muscle include HMGB1, SDF-1 and TNF- $\alpha$  (Palumbo et al., 2004). Expression of the adhesion molecules L-selectin and alpha 4 integrin on mesoangioblasts improved their migration into skeletal muscle (Galvez et al., 2006). Pre-treatment with nitric oxide was shown to augment the positive effects of TNF- $\alpha$ , TGF- $\beta$  and VEGF on mesangioblast migration (Sciorati et al., 2006).

Once the donor stem cells have entered the muscles, they must migrate to sites of injury, proliferate to give a pool of muscle precursor cells and then differentiate to form muscle fibers, either by fusing with each other or by repairing necrotic segments of dystrophic fibers. This will require them to respond to a new series of signals, which might be more appropriate for satellite cells than for stem cells.

In order to have long term benefit, the donor stem cells muscle repopulate a stem cell niche within the muscle and must retain the properties of a functional muscle stem cell within this niche. It is not clear if pericytes within their niche contribute to muscle regeneration, so the best niche to occupy would be the satellite cell niche. However, efficient repopulation of any niche, for example, the satellite cell niche, would only be possible if it were emptied as a consequence of the dystrophy and if the niche environment remains permissive for donor-derived stem cell function. As satellite cell of donor origin are most commonly found on fibers containing myonuclei of donor origin, they may not be called upon to regenerate, as the fiber on which they are situated will already have been strengthened by the new dystrophin and may not undergo further necrosis, at least at the sites where dystrophin is expressed. A means of activating these cells and drawing them towards more distant areas of injury is therefore required to enable them to respond to future muscle fiber necrosis elsewhere within the muscle.

### 3.7 Autologous cell transplantation

An attractive proposition for treating muscular dystrophies is to use genetically-corrected autologous stem cells. The use of autologous stem cells should circumvent the need for immunosuppression, although tissue culture components or expression of novel protein isotypes *in vivo* may evoke an immunological reaction. But, if the stem cells are skeletal-muscle derived, their function may be impaired by either the primary genetic defect, or secondary environmental consequences of the primary defect.

In some muscular dystrophies, the gene responsible is not expressed in satellite cells (reviewed (Morgan & Zammit, 2010)); for example, dystrophin is not expressed in satellite

cells (or other types of muscle stem cell), so satellite cells in DMD muscles would therefore be expected to have normal function. However, the satellite cells may have undergone many divisions in their previous attempts to repair the dystrophic fibers and could therefore be close to senescence (Decary et al., 1996; Decary et al., 1997; Webster & Blau, 1990). They would consequently be of little use for autologous therapy, as they would undergo insufficient divisions *in vitro* to be genetically modified and then to proliferate following transplantation. But although human myoblasts are exhausted in DMD (Decary et al., 1996; Decary et al., 1997; Webster & Blau, 1990), *mdx* satellite cells do not appear to suffer the same consequence of dystrophin deficiency (Bockhold et al., 1998). Recent evidence has indicated that *mdx* satellite cells are highly functional following transplantation into irradiated *mdx* nu/nu muscles (Boldrin and Morgan, unpublished observations). So although satellite cells may not be lost in DMD (reviewed (Boldrin et al., 2010)), their function is compromised, which may be due to telomere shortening leading to reduced proliferative capacity, or a change in the timing or extent of differentiation. However, caution must be taken when comparing *mdx* and DMD cells, as there are differences in telomere biology between mice and humans: inbred mouse strains have extremely long telomeres (20–150 kilobases) compared with humans (up to 15 kilobases) (Bekaert et al., 2005) and telomerase activity is lower in human compared to mouse cells (reviewed (Mather et al., 2011)).

It is unclear whether skeletal muscle-resident cells other than satellite cells contribute to muscle regeneration in muscular dystrophies, or even to maintenance and repair of normal muscle. If they had not actively contributed to the cycles of degeneration and regeneration that occur in DMD, they would be capable of many more divisions than the satellite-cell derived myoblasts and therefore be a more attractive candidate for autologous therapy. However, if they do not contribute to muscle regeneration in DMD, why do they not do so? And why would they be effective after transplantation, if they are not functional *in situ*? Possibly they are not recruited to muscle fiber maintenance and regeneration when they are in their natural niche *in vivo*, but do so after they encounter the site of muscle damage after either intra-muscular or systemic injection.

An ideal autologous stem cell would be derived non-invasively, e.g. from the peripheral blood, or a skin biopsy. However, to date there is only one report of blood-derived stem cells that make reasonable amounts of muscle after their systemic delivery (Torrente et al., 2004).

### 3.7.1 Genetic modification of autologous cells.

Genetic correction of autologous stem cells has been successfully used as a therapeutic option in other conditions and encouraging preclinical results have also been recently obtained in animal models of DMD (Meregalli et al., 2008). However key questions that need to be resolved before this approach could be used in DMD include the optimal vector configuration and the safety profile of the gene delivery methodology. Lentiviral vectors efficiently infect quiescent cells, including stem cells (S. Li et al., 2005) and give long-term, heritable, gene expression because they integrate into the host genome. Drawbacks with lentiviral vectors include possible gene silencing, or mutagenesis (Wilson & Cichutek, 2009), due to the site at which the virus inserts into the host genome. Although lentiviruses integrate preferentially into active transcription sites (Ciuffi, 2008) the development of third generation lentiviruses with advanced SIN design (Bokhoven et al. 2009), physiological

promoters and cell-specific envelope proteins (Rahim et al., 2009) and enhancer-less regulatory elements, e.g. the ubiquitously acting chromatin opening element (UCOE) (Montini et al., 2006; Zhang et al., 2007) should circumvent these problems.

A lentiviral vector has been used to insert a 6.8 kb dystrophin mini gene (S. Li et al., 2005), to give rise to a shorter dystrophin protein in regenerated muscle fibers. While these engineered mini-dystrophins appear to retain most of the functional properties of full-length dystrophin, they nevertheless miss important domains, such as the nitric oxide synthase anchoring domain (Lai et al., 2009). Considering the cloning capacity of lentiviral vectors (up to 10kb (M. Kumar et al., 2001)), it should be possible to further optimise a vector so that it accommodates most of the functionally relevant coding region of dystrophin. An optimal dystrophin construct in a lentiviral vector could be used to treat patients with different mutations, in contrast to the U7 constructs, which, although they can be placed in a lentiviral vector and induce dystrophin expression in stem cells *in vitro* and following their transplantation *in vivo* (Quenneville et al., 2007), are mutation-specific.

#### **4. Matrix Metalloproteinases: modulators of microenvironment and cell function in skeletal muscles**

The Matrix Metalloproteinases (MMPs) are a large group of zinc-dependent extracellular endopeptidase proteinases within the Metzincin superfamily of protease that also includes a disintegrin and metalloproteinases (ADAM) and ADAM with thrombospondin motifs (ADAMTS). MMPs family comprises 23 members in humans that share common modular domains and form 5 main sub-groups based on their structure and substrate: collagenases, gelatinases, matrilysins, stromelysins and membrane-type (Figure 2). With the exception of membrane bound MMPs, the other members of the group are secreted in the extracellular space where they are present in latent forms and become activated by other proteases or in response to signaling events. Their activity is regulated at the transcriptional and post-transcriptional levels as well as by their physiological inhibitors, Tissue Inhibitors of Matrix Metalloproteinases (TIMPs). Collectively, they are able to degrade all components of the ECM. Initially confined to the degradation of ECM, their function has progressively evolved and they are now regarded as major regulators of tissue environment and cell functions (Murphy, 2010; Rodriguez et al., 2010). They modulate cell proliferation, adhesion, migration and signaling (Fanjul-Fernandez et al., 2010).

##### **4.1 Matrix Metalloproteinases in remodeling muscles**

The adult skeletal muscle is a very stable tissue yet it is endowed with a high capacity to adapt to modification of functional demands, trauma or disease. In normal situations, the dynamic equilibrium between MMPs and TIMPs maintains homeostasis of ECM that provides a dynamic support and stores a number of growth factors that are liberated during ECM remodeling. In response to remodeling situations, dysregulation of this balance occurs in favor of MMPs, to allow necessary hydrolysis of ECM which results in the liberation of neo-epitopes from basement membrane components, as well as various growth factors and signaling molecules that modulate cell response to environmental modifications. Such imbalance may be temporary and the equilibrium is restored upon the disappearance of remodeling stimuli.

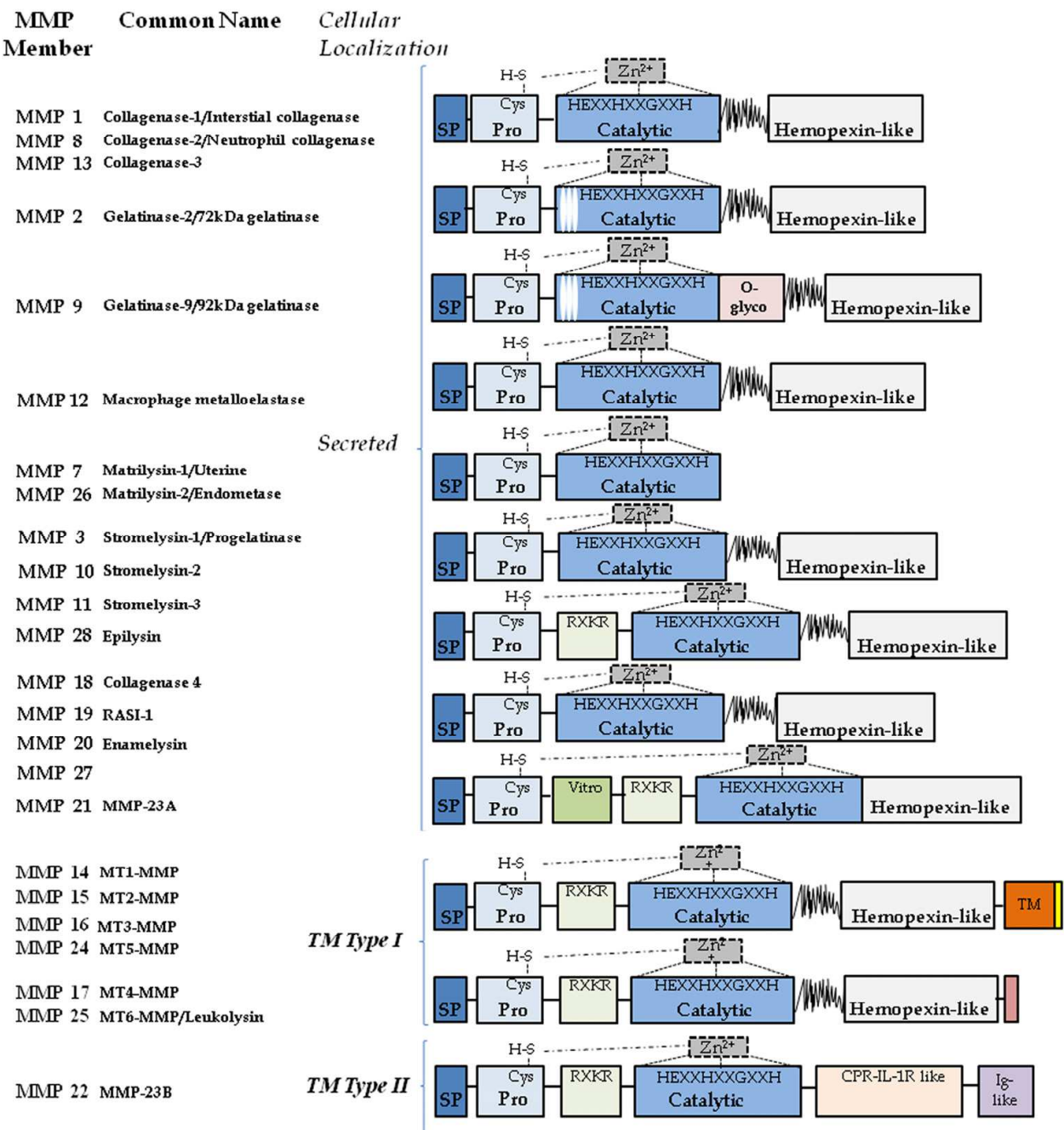


Fig. 2. Structural domains and nomenclature of the matrix metalloproteinases, A: Schematic representation of modular domains composing MMPs which are translated as inactive zymogens with an amino terminal signal peptide (SP), a pro-domain which folds over the zinc ion, in the catalytic site, to maintain latency (Pro), a catalytic domain that carries zinc at the active site, a hemopexin that confers the specificity to and interaction with the substrates or inhibitors (TIMPs) and presents the substrate to the catalytic site via a highly flexible hinge domain. Membrane-type (MT-) MMPs are anchored to the membrane either with a hydrophobic domain and a short cytoplasmic tail (Type I transmembrane protein) or with a glycosyl-phosphatidyl-inositol (GPI) domain. Gelatinases A and B also contain three collagen-binding fibronectin type II repeats within the catalytic domain and MMP-9 has an additional Serine-Threonine and proline rich O-Glycosylated domain. Some MMP have a furin-like motif



between the pro- and catalytic domains that allow their activation before they are secreted or localize to the membrane. MMP-22 has a cysteine/proline rich, interleukin-1R domain and an Immunoglobulin-like domain. Compiled from (Bourboulia & Stetler-Stevenson, 2010; Fanjul-Fernandez et al., 2010; Rosenberg, 2009; Sternlicht & Werb, 2001).

In skeletal muscles, normal muscle development, limb immobilisation, electrical stimulation and muscle injury are all remodeling situations characterized by MMPs/TIMPs dysregulation. However, the nature of MMPs that is upregulated and the time frame of this upregulation depend, a great deal, on the model used. Immobilization or unloading, that result in muscle fiber atrophy, induce upregulation of both MMP-2 and MMP-9 and downregulation of TIMPs (Berthon et al., 2007; Giannelli et al., 2005; Reznick et al., 2003; Stevenson et al., 2003; Wittwer et al., 2002) (Berthon et al., 2007; Giannelli et al., 2005; Reznick et al., 2003; Stevenson et al., 2003; Wittwer et al., 2002 ) but only MMP-2 is active (Liu et al., 2010). Whereas a single bout of degeneration-regeneration, induced by experimental injury to the muscle, also results in upregulation of these two proteases but the time frame and intensity differ according to the type/extent of injury (Ferre et al., 2007; Frisdal et al., 2000; Kherif et al., 1999). In a cardiotoxin injury model that induced massive myofiber necrosis, gelatinase activity progressively increased and peaked at day 7, when muscle fiber formation was the most active. It then returned to normal at later stages. This increase was due to simultaneous and consecutive steps of gelatinases regulation- both expression and activation. Within hours after tissue injury, MMP-9 was induced in the tissue that expressed only MMP-2 in the normal situation. It correlated with inflammatory cells infiltration of necrotic muscle fibers that exhibit high gelatinase intracellularly, in contrast to pericellular localization of gelatinase in normal muscles (Figure 3). Simultaneously, MMP-2 expression and activation decreased within the first 24 hours and was followed by a progressive reconstitution of these forms afterwards. MMP-9 transcripts localized to

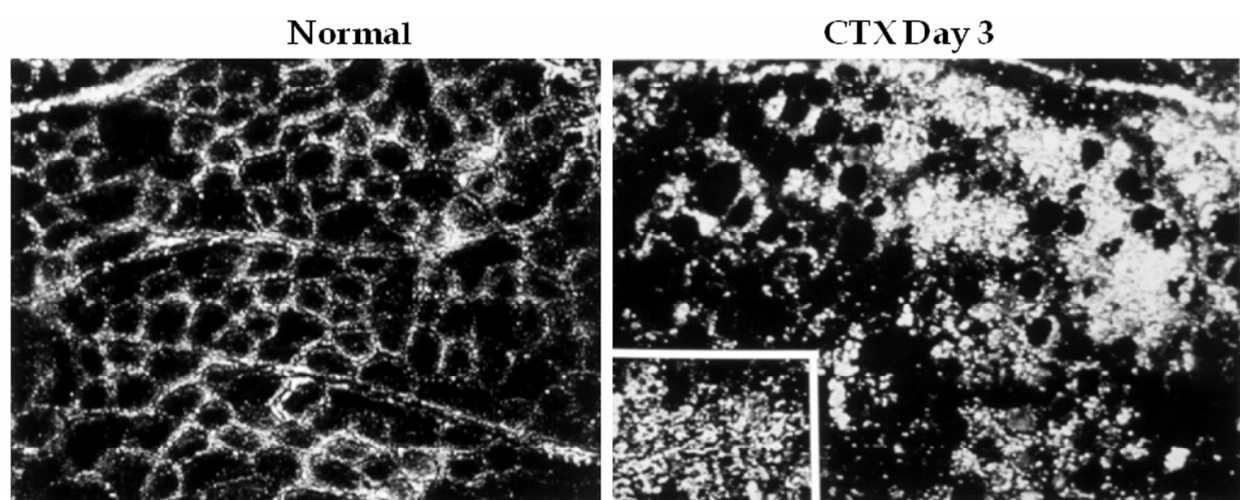


Fig. 3. *In situ* zymography of normal and cardiotoxin injured muscle 3 days after injury. In normal muscles, gelatinase activity (shown in white) localized to the endomysium and mononucleated cells present at the vicinity of muscle fibers. In injured muscles, gelatinase activity localizes to inflammatory cells. At early time points when necrotic muscle fibers are invaded by inflammatory cells, gelatinase activity is detected within degenerating muscle fibers that are invaded by inflammatory cells. Original magnification, X40 (Alameddine, Unpublished results).

inflammatory cells and mononucleated cells at satellite cell position (Kherif et al., 1999). The possibility that myogenic cells upregulate MMP-9 has been confirmed recently. When exposed to debris of damaged myotubes, the myogenic cells upregulated MMP-9, monocyte chemoattractant protein (MCP)-1 and other factors necessary for angiogenesis, tissue regeneration, and phagocyte recruitment (Dehne et al., 2011). Latent MT1-MMP (63kDa), which contributes with TIMP-2 to MMP-2 activation, is processed to its active (50kDa) form that retained its ability to process MMP-2 and was preceded by TIMP-2 decrease (Barnes et al., 2009). MMP-3 and TIMP-1 transcripts were also shown to be upregulated within the first 24 hours following cold injury. TIMP-1 started to decrease 72 hours post injury and early increase was followed by a decrease of active MMP-3 (Urso et al., 2010).

#### 4.2 Matrix Metalloproteinases in dystrophic muscles

In dystrophic *mdx* and CXMD muscles, MMP-2 and MMP-9 are found in muscle extracts whereas only MMP-2 is found in normal muscles (Fukushima et al., 2007; Kherif et al., 1999). MMP-9 is upregulated in both muscles and serum of *mdx* mice (H. Li et al., 2009) throughout their lifespan (Alameddine et al., unpublished results). MT1-MMP, TIMP-1 and TIMP-2 are also upregulated in CXMD muscles and gelatinase activity localized to necrotic fibers and endomysium, demonstrated by *in situ* zymography (Fukushima et al., 2007). Differences in MMP expression and activity patterns detected in different adult *mdx* muscles - gastrocnemius, soleus and diaphragm- led Bani (Bani et al., 2008) to hypothesize that the microenvironment of distinct skeletal muscles may influence a particular kinetic pattern of MMP activity, which ultimately favors persistent inflammation and myofiber regeneration at different stages of the myopathy in *mdx* mice. Gene array analysis revealed profound modification of mRNA levels of several MMPs and other associated proteins in gastrocnemius and *tibialis anterior* muscles of *mdx* mice. MMP-3, -8, -9, -10, -12, -14 and -15 Adamts2 and Timp-1 mRNA levels are increased, MMP-11 as well as Adamts1, Adamts5, Adamts8 and Timp-2 and Timp-3 were downregulated (A. Kumar et al., 2010).

In DMD muscles, TIMP-1, TIMP-2 and MMP-2 transcripts are upregulated and MMP-2 activity is increased (von Moers et al., 2005). TIMP-1 levels, usually increased in the serum and plasma of patients with fibrotic diseases, are elevated in serum, plasma, and muscle extracts of muscular dystrophy patients and animal models. It correlated with TGF $\beta$ 1 levels in DMD and in congenital muscular dystrophy (CMD) patients but not with Becker muscular dystrophy patients (Sun et al., 2010). In muscle tissue from dystrophin deficient and LAMA2-mutated muscular dystrophy patients, pro-fibrotic TGF- $\beta$ 1 is increased partly through a positive autocrine feedback loop and is released from decorin that is degraded by MMP-2. DMD fibroblasts have been shown to produce more soluble collagen, biglycan, decorin, TGF- $\beta$ 1 and MMP-7 and less MMP-1 than normal fibroblasts. TGF- $\beta$ 1 is known to modulate the ability of cells to synthesize various ECM components and was shown to modify the protein pattern produced by DMD fibroblasts upon their transformation to myofibroblasts. It increased MMP-7 thought to contribute to fibrosis (Fadic et al., 2006; Simona Zanotti et al., 2010; S. Zanotti et al., 2007).

In Emery-Dreifuss muscular dystrophy, screening of MMP-2, MMP-9 and MT1-MMP levels in the serum showed an increase of MMP-2 levels in both the autosomal and X- linked forms, suggesting it may serve as biomarker for the detection of cardiac involvement in patients with no subjective cardiac symptoms (Niebroj-Dobosz et al., 2009).

### 4.3 MMPs and inflammation in the development of fibrosis

End-stage DMD muscles are characterized by an almost complete disappearance of muscle fibers and their replacement by fibro-fatty tissue. Although DMD is not a fibrotic disease *per se*, their muscle biopsies are generally characterized by excessive production, deposition, and contraction of extracellular matrix. This accumulation results from factors, produced in diseased muscles, that influence the normal balance between production and/or hydrolysis of ECM components. Clearly, structural and functional changes of tissue microenvironment in dystrophic muscles are not equivalent to those that accompany normal muscle development. The permanent induction of wound-healing response with its inflammatory component may be essential contributors to the development of fibrosis in dystrophic muscles. Acute or chronic inflammation includes exudation of plasma proteins, recruitment of leukocytes and activation of cell and plasma derived inflammatory mediators as well as increased expression of MMPs (Manicone & McGuire, 2008). When inflammation is continuous or excessive, it is thought to contribute to tissue injury, organ dysfunction or chronic disease states. Inversely, decrease of MMP activity has been incriminated in the development of fibrotic conditions. Decreased MMP activity may result from dysregulation of the balance between MMPs and TIMPs. Upregulation of MMPs or downregulation of TIMPs activity could be applied for resolution of tissue fibrosis (reviewed (Hemmann et al., 2007)).

Experimental evidence shows that inflammatory cells such as macrophages, eosinophiles and T lymphocytes, the major infiltrating cell types, contribute to increased fibrosis (J. Morrison et al., 2000). Inflammatory cells produce cytokines/chemokines that regulate MMPs expression. In their turn, MMPs modulate the activities of cytokines and their receptors at the cell surface. The list of validated ECM components, growth factors (receptors and binding proteins) and cytokines/chemokines substrates is compiled in table 1.

Studies using gene microarrays have demonstrated that dystrophic muscles are characterized by an inflammatory “molecular signature”, in which CC chemokines are prominent (Y. W. Chen et al., 2000; Y. W. Chen et al., 2005; Porter et al., 2003; Porter et al., 2002). Similarly, CC chemokines are greatly upregulated in normal skeletal muscles after experimental injury (Hirata et al., 2003). CC chemokine receptors (CCRs 1, 2, 3, 5) and ligands (macrophage inflammatory protein-1 $\alpha$ , RANTES) are expressed at higher levels in dystrophic than in wild-type muscles across age groups (6, 12, and 24 wk). Moreover, chemokine ligand expression and muscle inflammation are significantly higher in dystrophic diaphragms than in limb muscles of the same animals. *In vitro*, CCR1 is constitutively expressed by myotubes formed from primary myoblasts derived from diaphragm muscles. Stimulation of myotubes by proinflammatory cytokines (tumor necrosis factor- $\alpha$ , interleukin-1 $\alpha$ , interferon- $\gamma$ ) found within the *in vivo* dystrophic muscle environment, upregulates CCR1 in *mdx* and wild-type myoblast cultures, and also increases expression of its ligand RANTES to a significantly greater degree (Demoule et al., 2005).

In damaged muscles, various cytokines and growth factors are also released during necrosis and regeneration of muscle fibers. The most widely documented pro-fibrotic agent that is over-expressed in dystrophic muscles is TGF- $\beta$ . It is upregulated in dystrophic muscles, after invasion of the damaged muscle by inflammatory cells (Y. W. Chen et al., 2005; Zhou et al., 2006) that were shown to express TGF- $\beta$  mRNA although these cells may not be the sole contributors to its production (Bernasconi et al., 1999; Gosselin et al., 2004).

Enzyme	Enzymes ECM substrates	Growth factors & Cytokines/Chemokines
<b>Secreted-type MMP</b>		
<b>Collagenases</b>		
Interstitial collagenase MMP-1	Aggrecan; Collagens I,- III, VII, VIII, X, XI; Entactin; Fn; Gelatins; Ln; Link protein; Tenascin; Vn; Perlecan;	CTGF; IL1- $\beta$ ; IGFBPs; MCP-1, MCP-2, MCP-3, MCP-4; TNF- $\alpha$
Neutrophil collagenase MMP-8	Aggrecan; Collagens I- III; Gelatins; link protein	LIX/CXCL5
Collagenase-3 MMP-13	Aggrecan; Collagens I-III,VI, IX, X, XIV; Fibrillin; Fn; Gelatins; Osteonectin; Ln; Perlecan	CTGF; MCP-3/CCL7, TGF- $\beta$ ; SDF-1/CXCL12
Collagenase-4 MMP-18	Collagen I	
<b>Gelatinases</b>		
Gelatinase A MMP-2	Aggrecan; Collagens I, III-V, VII, X- XI; Decorin; Elastin; Entactin; Fibrillin; Fn; Fibulins; Gelatins; Ln; Link protein; Osteonectin; Tenascin;	CTGF; FGFR1; CX <sub>3</sub> CL1; IL1- $\beta$ ; IGFBPs; MCP-3/CCL7; TGF- $\beta$ ; TNF- $\alpha$ ; SDF-1/CXCL12
Gelatinase B MMP-9	Aggrecan; Collagens III, IV-V, XI; Decorin; Elastin; Entactin; Fibrillin; Gelatins; Ln; Link protein; Osteonectin; N-telopeptide of collagen I; Vn	MCP-3; CCL11; CCL17; Fractalkine; GRO-alpha; IGFBP-3; IL1- $\beta$ ; IL-2R $\alpha$ ; IL-8/CXCL8; Kit-L; LIF; TGF- $\beta$ ; TNF- $\alpha$ ; SDF-1/CXCL12; VEGF
<b>Stromelysins</b>		
Stromelysin-1 MMP-3	Aggrecan; Collagens III-V, VII, IX- XI; Decorin; Elastin; Entactin; Fibrillin; Fn; Gelatins;Ln; link protein; Osteonectin; Perlecan; Tenascin; Vn;	CTGF; HB-EGF; IL1- $\beta$ ; IGFBPs; MCP-1, MCP-2, MCP-3, MCP-4; IL-1 $\beta$ ; TGF- $\beta$ 1; TNF- $\alpha$ ; SDF-1/CXCL12;
Stromelysin-2 MMP-10	Aggrecan; Collagens III-V; Elastin; Fn; Gelatin; Link protein	
<b>Matrilysins</b>		
Matrilysin-1 (MMP-7)	Aggrecan; Collagens I, IV; Decorin; Elastin; Entactin; Fn; Fibulins; Gelatins; Ln; Link protein; Osteonectin; Osteopontin; Tenascin; Vn; Syndecan-1,	CTGF; Fas-L; HB-EGF; IGFBP-3; TNF- $\alpha$ ; RANKL
Matrilysin-2 MMP-26	Collagen IV; Fn; Fibrinogen; Gelatin; Vn	



<b>Furin-activated MMP</b>		
Stromelysin-3 MMP-11	Aggrecan; Fn; Gelatins; Ln;	IGFBP-1
Epilysin MMP-28	Unknown	
<b>Other secreted-type MMP</b>		
Metalloelastase MMP-12	Aggrecan; Collagen I, IV; Elastin; Entactin; Fibrillin; Fn; Gelatin; Ln; Osteonectin; Vn;	TNF- $\alpha$
RASI-1 (MMP-19)	Aggrecan; Collagen I, IV; COMP; Fn; Gelatin; Ln; Tenascin;	IGFBP-3
Enamelysin (MMP-20)	Aggrecan; Amelogenin; COMP; Gelatin;	Unknown
MMP-21	Unknown	Unknown
MMP-27	Unknown	Unknown
<b>Membrane-anchored MMP</b>		
<b>Type I transmembrane-type MMP</b>		
MT1-MMP MMP-14	Aggrecan; Collagens I-III, VI; Entactin; Fibrillin; Fn; Gelatins; Ln; Osteonectin; Vn;	CTGF; IL-8; MCP-3/CCL7; TNF- $\alpha$
MT2-MMP MMP-15	Aggrecan; Entactin	
MT3-MMP MMP-16	Collagen III; Fn; Gelatins	
MT5-MMP MMP-24	PG	
<b>GPI-linked MMP</b>		
MT4-MMP MMP-17	Gelatin;	
MT6-MMP MMP-25	collagen IV; Fibrin; Fn; Gelatin; Ln	
<b>Type II transmembrane-type MMP</b>		
MMP-23	Gelatins	

CCL11, CC chemokine ligand 11; CCL17, CC chemokine ligand 17, COMP, cartilage oligomeric matrix protein; CTGF, connective tissue growth factor; Fas-L, Fas ligand; FGF, Fibroblast Growth Factor,, FGFR1, Fibroblast growth factor receptor 1; Fn, fibronectin; HB-EGF, heparin-binding epidermal growth factor like growth factor; IGFBP, insulin-like growth factor binding proteins; IL1- $\beta$ , interleukin-1 $\beta$ ; IL-2R $\alpha$ , Interleukin 2 receptor; IL-8, interleukin 8; Kit-L, kit ligand; Ln, laminin; LIF, Leukimia inhibitory factor; LIX-CXL, lipopolysaccharide induced CXC chemokine, MCP-, monocyte chemotactic protein-; PCPE, Procollagen C protein enhancer; PG, proteoglycan; Pro, proteinase type; SDF-1/CXCL12, Stromal cell derived factor, TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TGF- $\beta$ , transforming growth factor  $\beta$ ; RASI-1, rheumatoid arthritis synovium inflamed-1; RANKL, receptor activator for nuclear factor  $\kappa$  B ligand.

Table 1. Validated MMPs substrates that include ECM and non-ECM proteins. Of the long list of non-ECM substrates, only growth factors, receptors and cytokines/chemokines have been extracted because of the role they play in the modification of tissue environment and the modulation of cell functions (Manicone & McGuire, 2008; C. J. Morrison et al., 2009; Shiomi et al., 2010; Sternlicht & Werb, 2001).

TGF- $\beta$  is thought to play a prominent role in the pathogenesis of muscle fibrosis. Its short-term neutralization by decorin administration resulted in a 40% decline in type I collagen mRNA expression in *mdx* mice. *In vitro*, it stimulates collagen synthesis and inhibits collagen degradation in fibroblasts (Grande et al., 1997; Ignatz & Massague, 1986; Sharma & Ziyadeh, 1994). Myoblast stimulation by TGF- $\beta$ 1 induced autocrine production of TGF- $\beta$ 1, downregulation of myogenic proteins, production of fibrosis-related proteins and phenotypic transformation of myogenic cells to fibroblast/myofibroblast cell types *in vitro* (Yong Li et al., 2004). TGF- $\beta$  treatment of myogenic cells also upregulated Connective Tissue Growth Factor (CTGF) (Maeda et al., 2005) incriminated in various fibrotic diseases. CTGF is overexpressed in dystrophic muscles and is thought to contribute, with TGF- $\beta$ , to the development of fibrosis (Sun et al., 2008). Interestingly, both factors are validated MMPs substrates and could be modulated through MMPs action.

Tumor necrosis factor-(TNF- $\alpha$ ), also upregulated in muscular dystrophy (Porreca et al., 1999) may exert direct adverse effects on skeletal muscle function and regeneration potential. Blockade of TNF- $\alpha$  by inhibitory antibodies reduced necrosis and contractile dysfunction in response to eccentric exercise (Piers et al., 2011; Radley et al., 2008). *In vitro* TNF- $\alpha$  has been shown to stimulate collagen synthesis in fibroblasts (Lurton et al., 1999) hence contributing directly to muscle fibrosis. *In vivo*, short-term pharmacological blockade of TNF- $\alpha$  in *mdx* mice significantly reduced the level of both TGF- $\beta$ 1 and type I collagen mRNA (Gosselin et al., 2004). Whether TNF- $\alpha$  mediates muscle fibrosis directly or indirectly (by upregulating the expression of TGF- $\beta$ 1) remains an open question. However, TNF- $\alpha$  induces MMP-9 upregulation in myogenic cells (Torrente et al., 2003).

Concomitance between inflammation and upregulation of MMPs in mouse models or human diseases with inflammatory conditions, led several groups to propose MMPs as potential therapeutic targets in pathological conditions with aberrant MMP expression and activity (reviewed (Clutterbuck et al., 2009)). Inhibition of MMPs has been recently investigated in *mdx* mice. MMP-9 inhibition either by the administration of nuclear factor-kappa B inhibitory peptide, gene deletion or by L-arginine treatment was reported to reduce muscle injury, inflammation, fibrosis and decrease pro-inflammatory cytokine release (Hnia et al., 2008; A. Kumar et al., 2010; H. Li et al., 2009). However, whether this inhibition is acting directly on the development of fibrosis or through prevention of muscle fibers necrosis and tissue scarring remains an open question.

Extreme precaution has to be taken into consideration regarding MMPs inhibition in muscular dystrophy particularly as animal models of MMP gain- or loss- of function and clinical trials of MMP inhibition in cancer patients have unraveled the dual role an individual MMP could exert, depending on tissue type or stage of the disease (protective/detrimental) (reviewed (Fanjul-Fernandez et al., 2010)). In skeletal muscles, the beneficial effect of certain MMPs has been documented, underscoring the necessity for better knowledge of the role MMPs are playing in muscle diseases (Alameddine manuscript in preparation). Indeed, Mmp-2 gene ablation has been shown to impair the growth of muscle fibers by downregulating VEGF and nNOS (Miyazaki et al., 2011). Moreover, proteinases upregulation, during satellite cells activation, is essential for dismantling the satellite cells niche (Pallafacchina et al., 2010) and MMP-1 has been shown to reduce muscle fibrosis (Kaar et al., 2008).

4.4 MMPs favor cell migration

Myogenic cells have been reported to express various MMPs -MMP-1, -2, -3, -7, -9, -10, -14 and -16, either constitutively or after treatment with growth factors, cytokines or phorbol esters (Balcerzak et al., 2001; Caron et al., 1999; El Fahime et al., 2000; Guérin & Holland, 1995; Kherif et al., 1999; Lewis et al., 2000; Lluri & Jaworski, 2005; Nishimura et al., 2008; Ohtake et al., 2006). Cytokines and growth factors differentially modulate MMPs expression in myogenic cells. Treatment of adult mouse myoblasts by soluble serum fibronectin, PDGF-BB, TGF- $\beta$  or IGF-1 had no effect on the expression of MMP-9 expression, whereas TNF- $\alpha$  and b-FGF reproducibly induced the expression of MMP-9 expression 30- and 10-folds. Other MMPs, such as MMP-1 and MMP-2, were not significantly affected by any of these growth factors (Allen et al., 2003; Torrente et al., 2003).

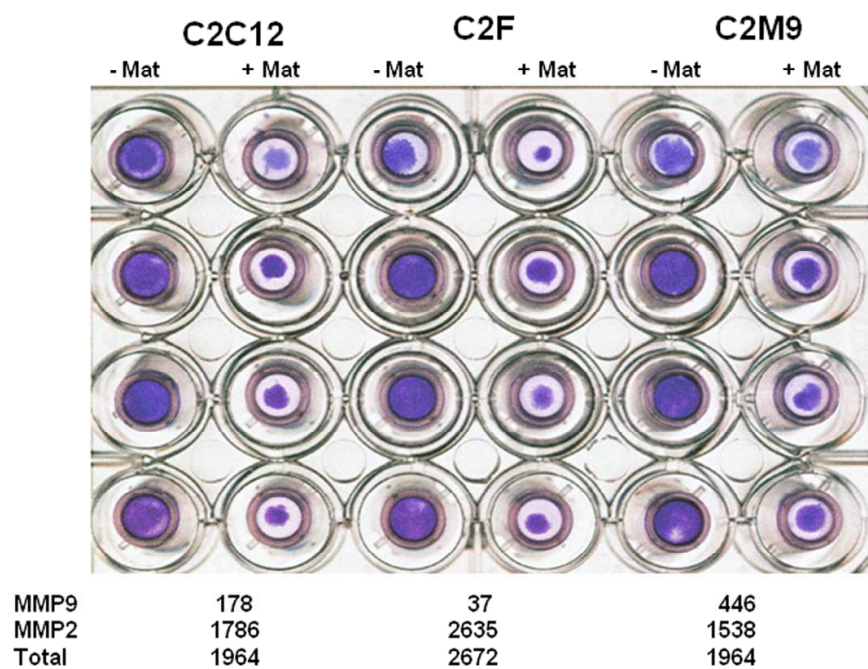


Fig. 4. Invasion assay establishing the correlation between migratory capacity of myogenic cells and MMP-9 expression levels. Three different cell types, C2C12 and 2 variant clones, expressing different levels of MMP-2 and MMP-9, that were quantified in the same zymography gels with Image J, were assayed in a two chamber migration assay with (+Mat) or without (-Mat) growth factor reduced Matrigel as substrate. The invasive capacity is measured by the ratio between cells that migrated through Matrigel and those diffused through the porous membrane. MMP-2, MMP-9 and total gelatinase values are expressed in arbitrary units. Invasive capacity of C2M9 was > to C2C12>C2F cells.

The role MMPs/TIMPs play in myogenic cells migration and potentially in cell fusion has been confirmed by overexpression and inhibition studies. Myoblasts overexpressing MMP-7 had a higher propensity to form myotubes than parental controls and generated more fibers when transplanted into a single site (Caron et al., 1999). MMP-1 enhanced C2C12 myoblast migration in a wound healing assay *in vitro* by increasing the expression of migration related marker proteins such as N-cadherin,  $\beta$ -catenin, latent MMP-2 and TIMP-1 (Wang et al., 2009). C2C12 cells stably transfected with MMP-2 and MMP-14 cDNA significantly increased the number of myonuclei without affecting the number of myotubes formed (Echizenya et al.,

2005). MT1-MMP has been proposed as a major MMP checkpoint regulator of myotube formation, as shMT1-MMP partly inhibited muscle cell fusion at a specific stage (Ohtake et al., 2006). MMP-9 and TIMP-1 have also been suspected to play a role in myogenesis *in vitro*. MMP-9 expression in human myogenic cells favored their migration on fibronectin and its inhibition by a blocking antibody decreased two dimensional cell migration (Lewis et al., 2000). Cells overexpressing MMP-9 have also better three dimensional migratory capacities (Figure 4). They exhibit higher migration when seeded on top of a Matrigel gel that better mimics ECM and their migration is inhibited in the presence of a specific MMP-9 inhibitor (Morgan et al., 2010). Of relevance to this review is that these cells have also higher engraftment capacities. Upon transplantation in a single site in irradiated and non-irradiated muscles of *mdx* nu/nu mice, they formed more dystrophin positive muscle fibers over larger areas, indicating they migrated better in a dystrophic environment (Morgan et al., 2010).

## 5. Conclusion

Although promising, there are several challenges to be overcome before stem or precursor cells could be used to treat muscular dystrophies. Apart from reliably and reproducibly identifying and purifying the cells of interest, their characteristics have to be maintained on expansion in culture: attempts at re-creating the niche *in vitro* may facilitate the retention of stem cell characteristics (Cosgrove et al., 2009; Gilbert et al., 2010). Encouraging results from one laboratory should be independently confirmed, before any particular stem cell is considered for therapeutic application.

Systemic delivery would involve turning a cell into a leukocyte to cross the blood vessel endothelium (Springer, 1994) and then switching on survival, migration, proliferative and myogenic regulatory factors once the cells are within the muscle. Even if it does not prove possible to treat muscles body-wide, transplanting stem cells locally into a small, vital, muscle, e.g. in the finger, may prove more practicable and although not life-saving, would improve the quality of life of DMD patients.

But for successful local as well as systemic delivery of stem cells to skeletal muscle, the inhospitable muscle environment remains a major hurdle. Studies on the factors and signaling pathways that hinder donor cell survival, proliferation and migration within both normal and dystrophic muscle and how these may be modified to augment the regenerative capacity of transplanted cells, remain vital for the successful use of stem cells in neuromuscular diseases. More importantly, elucidation of the role MMPs in general and individual MMPs in particular, play in the modulation of the dystrophic microenvironment and stem cell response to this environment warrants further study. In light of our present knowledge, it is tempting to propose that MMPs are temporally upregulated to permit migration and fusion of stem cells, then down-regulated, after donor-derived muscle has been formed, to reduce inflammation and fibrosis and thus improve muscle function. However, it is not clear whether the presence of either stem cells of normal origin, or muscle fibers expressing dystrophin, are sufficient to prevent the uncontrolled wound healing response that occurs in dystrophic muscles.

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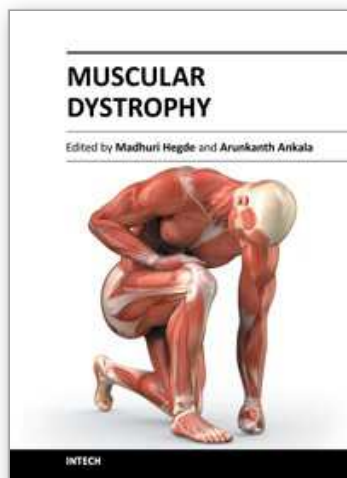
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## **Muscular Dystrophy**

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With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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