NEW STEM CELL THERAPIES FOR DUCHENE MUSCULAR DYSTROPHY

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1. Abstract

Muscular dystrophy refers to a heterogeneous group of inherited degenerative neuromuscular disorders characterized by progressive wasting and weakness of the skeletal muscles and, in some cases cardiac muscle. Muscular dystrophies lead to loss of motility, and, in the most severe forms, progressive paralysis and assisted ventilation. Duchenne muscular dystrophy (DMD) is the most common and severe form, affecting 1 in 3,500 newborn males and causing death in the late teens or early 20s. DMD arises from mutations in the dystrophin (*DMD*) gene, which encodes the protein dystrophin that links the actin cytoskeleton to the extracellular matrix in muscle fibres. The absence of dystrophin results in constitutive membrane instability and death of muscle cells. Damaged muscle fibres undergo repeated cycles of segmental necrosis, degeneration and subsequent regeneration, during which muscle stem cells, termed satellite cells, become activated to regenerate muscle fibres. However, the regenerative process is largely inefficient due to persistent inflammation and the substitution of myofibres by a non-functional mass of adipose cells and fibrotic tissue. Improved medical assistance has increased the lifespan of patients but still there is no efficacious therapy and steroids are the only palliative treatment available. In recent years, increased attention has been given to cell-based therapies in which immune suppression in the treatment of muscular dystrophy is avoided by transplanting autologous cells bearing a functional dystrophin gene. In this project, we hypothesize that a fruitful cellular therapy strategy to combat muscular dystrophy requires at least:

1. The efficient rescue of the primary defect in the mutated cells
2. The possibility of easily obtaining a sufficient number of cells for transplantation
3. An efficient engraftment into the diseased host tissue

Thus, our synergistic objectives are:

a) To optimize strategies to obtain genetically corrected mouse- and human-derived cells from dystrophic mice and DMD patients respectively. We will test the ability of these cells to produce dystrophin efficiently, to be amplified ex-vivo to obtain the necessary cell numbers, and to self-renew in vivo to provide a long-lasting treatment.

b) To improve the therapeutic efficiency of cell-based therapies by targeting the altered host environment. The presence of chronic inflammation and excessive extracellular matrix deposition (fibrosis) in dystrophic muscles currently represents a major obstacle for the successful engraftment of donor cells. Migration to the damaged areas, survival and self-renewal of the transplanted cells is severely hampered by their interactions with the altered cellular and extracellular matrix components of the target tissue. We aim at interfering with inflammation and fibrosis as a necessary step for the success of cell mediated therapies in muscular dystrophy.

2. Results obtained

A) Optimize strategies to obtain genetically corrected dystrophic mouse- and human-derived cells

We have tested the hypothesis that a minority of genetically corrected cells that are able to produce dystrophin will fuse with an excess of mutated DMD cells (not producing dystrophin). The corrected nucleus will produce U7 small nuclear RNA (snRNA), which allows exon skipping, and will also enter neighbouring nuclei upon cell fusion during regenerative myogenesis, thus amplifying the therapeutic effect of corrected cells.

Identification of proper cell lines. We obtained primary myogenic cells from UCL and Newcastle banks carrying a skippable mutation of exon 51. U7 snRNA transduction in these cells induced exon 51 skipping and the production of a shorter form of dystrophin. However, lack of cell fusion prevented spreading of the snRNA to neighbouring nuclei and excluded investigating whether trans-correction of dystrophin nuclei may occur.

We also used immortalized dystrophic myoblast lines with a skippable exon 51 mutation, (provided by Dr. V. Mouly, Institute of Myologie, Paris). When mixing transduced cells with an excess of uncorrected DMD immortalized myocytes at ratios that mimic an engraftment of 3% and 10%, (closer to what may be achieved after in vivo transplantation with an optimised protocol and starting at an earlier patient age), hybrid myotubes were generated with high levels of dystrophin RNA. These results

provide evidence that novel dystrophin can be produced at a higher than expected level through exon skipping.

The experimental approach was next applied to iPS lines carrying mutations of exon 51 (in collaboration with Dr. K. Anastassiadis, University of Dresden). Despite efficient correction, these cell lines differentiated into mono- or binucleated myocytes, avoiding determination of a possible trans-correction effect. To solve this problem, skin fibroblasts from DMD patients (gift of Dr. Y. Torrente, University of Milan) were efficiently converted into myogenic cells by inducible MyoD-ER expression upon exposure to tamoxifen. Both lines were efficiently corrected but formed only very small myotubes and were used as correcting tools.

We succeeded in using an immortalized (with telomerase and CDK4) myogenic cell line from Dr. V. Mouly, which readily differentiated into large multinucleated myotubes. This line was used as the DMD receiving myogenic cells in co-cultures with a minority (at 1:10 or 1:30 dilution) of either WT or DMD cells (immortal myoblasts of the same line, iPS derived, DMD, genetically corrected, myogenic cells or DMD fibroblasts), also genetically corrected. We found that dystrophin mRNA accumulation in the cocultures, reached values of 55% and 28% respectively, which demonstrates that dystrophin synthesis also occurred in nuclei other than the transduced ones.

Dystrophin protein was analysed by immunofluorescence and WB. Despite the fact that genetically corrected DMD cells express only 60% of the WT level, its expression remains almost constant at progressive dilution, with protein levels above 30% of the WT, which is the threshold of therapeutic range for dystrophin. Similar successful results were obtained with iPS derived, DMD myogenic cells or MyoD converted DMD fibroblasts, both transduced with the same lentiviral vector.

To implement these experiments, we have developed a system with optimised culture conditions, using a double layer of matrigel that allowed good maturation of the myotubes. Moreover, cells were readily innervated by motor neurons formed addition of fragments of mouse embryonic spinal cord, which unexpectedly induced twitching of the cultures.

Finally, we tested the efficiency of the strategy in vivo. We implanted into immunodeficient SCID/bg mice matrigel plugs loaded with human myogenic cells. This allows proper dosing of the ratio of corrected and non-corrected cells. The plugs with cells were implanted subcutaneously in the abdomen of the mice and were analysed after one month. The results indicate the formation of bundles of mature muscle fibres with elevated expression of dystrophin, comparable with the amount synthesized by muscle fibres deriving from all corrected cells. This is explained by the larger number of nuclei that a mature muscle fibre contains in comparison with a myotube. We foresee an immediate clinical translation and will be thus validated in patients in the next two years.

Specific objectives achieved

- Production and validation of lentiviral vectors.
- Transduction of primary, IPS lines and immortalised human myogenic cells.
- Detection of exon skipped dystrophin production by RT-PCR, IF and WB and its enhanced production in cocultures of dystrophic corrected and non corrected cells.
- Development of optimised in vitro culture systems for mature corrected myotubes.
- Efficient correction of dystrophin mutation and production of therapeutic levels of dystrophin in a mouse model in vivo.

B) Assessment of the self-renewal potential of mouse WT and HAC-corrected mesoangioblasts

Mesoangioblasts (MABs) are blood-vessel-associated stem/progenitor cells able to differentiate into skeletal muscle. MABs can migrate across the blood vessel wall, enabling intra-arterial delivery as a potential clinical therapy for neuromuscular disorders. Our work answers three important questions: B.1) Are MABs 'stem cells', capable of multiple rounds of regeneration and self-renewal?; B.2) Is this maintained in genetically corrected MABs?; B.3) Is it conserved in human MABs?

B.1. Self-renewal capacity of MABs. To answer this question, GFP^+-MABs from donor mice were transplanted into scid/mdx mice. GFP^+ cells and dystrophin-positive myofibres were detected in recipient host mice after 1 month. MAB-derived satellite cells (GFP^+/ SM/C-2.6$^+$) were isolated for further transplantation experiments. The self-renewal capacity of donor was determined in clonogenic assays and by retransplantation in scid/mdx mice. Our results show that clonally expanded MABs

which had entered the satellite cell compartment are able to successfully regenerate dystrophic muscle.

Next mdx-derived MABs, genetically-corrected with a human artificial chromosome containing the entire dystrophin locus (DYS-HAC), were tested in their capacity to self-renew in vivo upon serial transplantation. Explanted muscles showed dystrophin-positive myofibres, indicating that genetically-corrected mdx- DYS-HAC MABs are clonally expandable and can engraft dystrophic muscle after multiple rounds of self-renewal. Satellite cells of mdx-DYS-HAC-corrected MABs can be serially transplanted for at least 4 rounds and the clonogenic capacity of cells was unaltered by multiple rounds of serial transplantation.

B.2. Validation using freshly isolated and iPS cell-derived mouse mesoangioblasts

By serially transplanting primary, freshly isolated MABs we have:

- Established colonies of tissue non-specific alkaline phosphatase (TNAP) creERT2 mice and cre-inducible Rosa26-YFP and Rosa26-TdTomato reporter lines. These mice allow fresh isolation of myogenic pericytes to generate donor-derived myofibres and cells with stem cell markers in host mice upon transplantation.
- Characterized the dynamics of SM/C2.6, a surface marker linked to the self-renewal potential of the aforementioned populations.
- Discovered that mouse iPSC-derived mesoangioblast-like cells upon transplantation in dystrophic mice generate donor-derived myofibres as well as an SM/C2.6^{+} and SM/C2.6^{-} population of mononuclear cells, similarly to their primary/native counterpart.

B.3. Characterization of HAC-corrected muscle- and iPS cell-derived mesoangioblasts from DMD patients

Human myogenic progenitors, including myoblasts and mesoangioblasts from DMD patients with and without genetic correction were analysed in their proliferative potential to withstand clonal cell expansion after HAC transfer. We have used lentivirally delivered excisable hTERT and Bmi1 cDNA transgenes to extend human cell proliferation, enabling transfer of novel DYS-HACs into DMD satellite cell-derived myoblasts and pericyte-derived mesoangioblasts. The reversibly immortalised and genetically corrected cells maintained a stable karyotype and did not undergo tumorigenic transformation. Human cells remained myogenic in vitro, and engrafted and differentiated murine skeletal muscle upon transplantation. Finally, we have also

explored an alternative strategy to test engraftment of human muscle progenitors, with the aim of translating it in the future to genetically corrected cells (e.g. with the DYS-HAC).

We have tested human myoblasts engraftment and differentiation capacities in dystrophic mouse skeletal muscles after treatment with Dll4 (a notch signalling activator) and PDGF-BB, which in mouse myoblasts induces MABs properties, including improved migration, whilst preserving their myogenicity. In contrast to experiments with mouse cells, we did not observe an increase in the expression of hLamin A/C+ hSpectrin+ cells in response to Dll4 & PDGF-BB treatment. Unfortunately, only one of the transplanted populations could be quantified, as the in vitro expansion of the other population negatively impacted on its differentiation capacity. Moreover, human SCs/myoblasts showed heterogeneity of response to the treatment, likely due to intrinsic genetic variability amongst non-isogenic populations.

Future work on this aspect will focus on systematically testing this phenomenon in vivo using unexpanded human biopsy-derived cells and iPS cell-derived myogenic progenitors. In this context, we have also validated transgene-free, small molecule-mediated protocols to derive human myogenic progenitors from iPS cells which could be amenable both to genetic correction and to the aforementioned Dll4 & PDFG-BB pro-migration treatment. Specifically, we have already tested a protocol published by Caron et al. (Stem Cells Transl Med 2016) and validated its efficacy in generating myogenic progenitors and terminally differentiated myotubes.

C. Targeting the altered host environment to improve the therapeutic efficiency of cell-based therapies

We postulated that an exacerbated Th2-type-dependent immune response favours muscle dystrophy progression since it correlates with the development of fibrosis. We therefore proposed that modification of Th2-type-dependent signals during the process of muscle regeneration would lead to an altered inflammatory phenotype that would affect fibrosis development, particularly in the context of muscle dystrophy. We have used several relevant mouse models and have explored in detail the modifications of the inflammatory cells that may influence regeneration and the fibrotic phenotype.
In the context of these studies we have:

- established a standard assay to identify and isolated different inflammatory cell populations from regenerating murine muscles. This protocol allows distinction of $CD11b^{+}$ $F4/80^{+}$ Ly6C high $MHC2^{-}$ pro-inflammatory monocytes and two populations of macrophages ($CD11b^{+}$ $F4/80^{+}$ Ly6C low MHC2 high and $CD11b^{+}$ $F4/80^{+}$ Ly6C low MHC2 low).

- described the kinetics of these inflammatory populations during the course of regeneration and in the muscles of dystrophic mdx mice at different stages of the disease.

- characterized the inflammatory profile of the different cell populations in terms of their pattern of cytokine expression in regenerating muscles.

- investigated the effects of the absence of a typical Th2-type cytokine during muscle regeneration and in the context of muscle dystrophy.

- We have also analysed mechanistically how modifications of Th1/Th2 balance, by modifying IL-6 signalling influences muscle regeneration. Surprisingly, we have found that fibro/adipogenic progenitors are not only responsible of the presence of fibrosis in muscular dystrophy, but their IL-6 production is also required for the proper proliferative activity of muscle stem cells and correct muscle regeneration. IL-6 trans-signalling predominates over classic IL-6 signals during muscle regeneration.

- Explored the effects of several compounds to diminish fibrosis in skeletal muscles: Among them, we have used imatinib. This drug is a tyrosine kinase inhibitor which has been shown to target PDGFRα, a major membrane receptor that controls the fibrogenic activity of mesenchymal fibro/adipogenic progenitor cells (FAPs). Despite a reduction in the number of FAPs in regenerating muscles, we also detected a general reduction in the number of infiltrating inflammatory cells, which affected macrophages. More importantly, negative effects of the treatment were evident by a reduction of the total number of satellite cells and specifically in their proliferating fraction. Therefore, due to these deleterious actions on the regenerative capacity of muscle, we have decided to test alternative treatments, such as Wnt7a protein, as a possible antifibrotic compound for muscle dystrophy.

In vitro studies show that Wnt7a modulates of the immune response of macrophages negatively affecting the expression of both pro- and anti-inflammatory genes with these settings. In vivo, Wnt7a treatment to mice induced a reduction of pro-inflammatory macrophages at day 3 after injury and more anti-inflammatory macrophages. In the same experiments, a small tendency to reduce the number of FAPs by Wnt7a treatment was observed. When dystrophic mdx mice were treated with this compound, no significant differences in myofibril size or in fibrosis extension were detected, indicating no major impact of the treatment in either the disease progression or regenerative capacity. To characterize the effects of this drug at the cellular level in more detail, we isolated macrophages, satellite cells and FAPs from Wnt7a and vehicle-treated muscles and we analysed expression of pro- and anti-inflammatory cytokines and pro-fibrotic markers, but we did not find any significant differences for the molecules analysed.

Finally, we have explored the antifibrotic role of miR-21 systemic inhibition in vivo in dystrophic mice with the aim of using it as a palliative or combinatorial treatment for DMD based on our previous positive results obtained by local administration. Experimental and control groups of DBA-mdx mice which have already developed prominent fibrosis (12 months old) were treated systemically with control and miR-21 specific inhibitor for two months. In spite of a reduction of endogenous miR-21 levels to non-dystrophic levels, no positive effects were found. These results demonstrate that efficient systemic inhibition of miR-21 in dystrophic mice with LNA inhibitor does not ameliorate muscle degeneration or fibrosis deposition at this age in DBA-mdx mouse model.

3. Relevance and future applications

Duchenne muscular dystrophy (DMD) is the most common and severe form, affecting approximately 1 in 3,500 newborn males and the usual lifespan is 25 years. DMD leads to continuous muscle weakness, degeneration, and wasting. Finally, there follows premature demise in affected individuals due to respiratory and/or cardiac failure. DMD arises from mutations in the dystrophin (*DMD*) gene, which encodes the protein dystrophin that links the actin cytoskeleton to the extracellular matrix in muscle fibres. The absence of dystrophin results in constitutive membrane instability and death of

muscle cells. Damaged muscle fibres undergo repeated cycles of segmental necrosis, degeneration and subsequent regeneration, during which muscle stem cells (satellite cells) activate to regenerate muscle fibres. However, in diseased muscles, the regenerative process is largely inefficient due to persistent inflammation and the chronic substitution of myofibres by a non-functional mass of adipose cells and fibrotic tissue. For decades, researchers have tried to find effective therapy methods, but still there is currently no cure for DMD patients.

Part of the results obtained with the development of this project has direct and immediate clinical applicability. We carried out 15 years of pre-clinical work in three mouse and one dog model, that showed the safety and efficacy of this protocol. Thus, we completed a first in-man trial, based upon repeated intra-arterial administrations of HLA-matched donor mesoangioblasts (from a sibling) in five DMD patients. The trial showed safety but minimal efficacy, even though in the youngest patient we detected donor derived dystrophin in the range detected by trials with oligonucleotides for exon skipping. Differences with pre-clinical models were mainly due to the advanced age of patients (chosen for safety reasons), the ongoing treatment with steroids (which inhibits mesoangioblast adhesion to the endothelium), the lower cell dose, and the different posture between human and other mammals so that targeting leg muscles only is not sufficient to maintain posture and ambulation. To reach clinical efficacy we are developing a three-arm strategy by: a) implementing each step of transplantation, b) running a proof of principle trial, and c) performing a detailed pharmacodynamics of transplanted cells. The results reported here represent the core of the protocol implementation as we expect to increase dystrophin production with this experimental strategy. In addition we have data on enhancing mesoangioblasts binding to the muscle endothelium (supported by the MRC) and on enhancing crossing of the blood vessel. The clinical trial, to start soon has been supported by the Wellcome Trust.
Our data provide evidence of multiple synergistic approaches to overcome major hurdles in developing complex ex vivo gene transfer into clinically relevant human muscle progenitors for DMD gene and cell therapy. By performing important groundwork in mouse models of muscle regeneration we have established the foundation for testing human DMD genetically corrected muscle progenitors and also performed relevant work to enhance their migration in vivo as well as deriving them from iPS cells without the use of transgenes. (which could potentially cause insertional mutagenesis). Future work will focus on translating the findings of this project

combining DYS-HAC genetic correction, reversible immortalisation (when primary cells will be used), safely-derived iPS cell-derived myogenic progenitors and treatment to improve cell migration both locally and systemically.

Finally, our results clearly indicate that modulation of the environmental Th1/Th2 balance during regeneration and in muscle dystrophy affects the course of the disease and the presence of fibrosis. We have attempted to modulate pharmacologically the accumulation of muscle fibrosis with different compounds. First we used imatinib, a tyrosine kinase inhibitor that targets mesenchymal fibro/adipogenic progenitor cells (FAPs), which are responsible for the development of fibrosis. This compound was effective in reducing FAP numbers, but also had undesirable effects since it also reduced the number of muscle stem cells, which are directly involved in reconstituting mature muscle fibres during regeneration. Moreover, FAPs also provide some beneficial factors during regeneration. As an alternative, we tested the effects of Wnt7a and systemic miR-21 inhibition which has shown to have beneficial effects in other contexts. In both cases we found no significant positive effects in the experimental conditions used. As a general conclusion from these studies, the exploration the effects of new compounds has yet to be implemented as does setting the ideal conditions for fibrosis reduction that can facilitate the efficacy of gene and cell mediated therapies in muscular dystrophy.

4. Publications

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