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Challenges in cell transplantation for muscular dystrophy.

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Abstract

For decades now, cell transplantation has been considered a possible therapeutic strategy for muscular dystrophy, but failures have largely outnumbered success or at least encouraging outcomes.
In this review we will briefly recall the history of cell transplantation, discuss the peculiar features of skeletal muscle, and dystrophic skeletal muscle in particular, that make the procedure complicated and inefficient. As there are many recent and exhaustive reviews on the various myogenic cell types that have been or will be transplanted, we will only briefly describe them and refer the reader to these reviews.
Finally, we will discuss possible strategies to overcome the hurdles that prevent biological efficacy and hence clinical success.

1. History of cell transplantation for muscular dystrophy

Cultures of embryonic muscle cells able to generate multinucleated, cross-striated myotubes in culture were pioneered by Holtzer, Konigsberg and Yaffe (1-3) in the 60's, with immortal cell lines established in the 70' (4) and for decades they represented an essential tool to study how skeletal muscle develops, as amniote embryos are quite inaccessible. In the same years Mauro (5) identified satellite cells, as mononucleated cells, lying underneath the basal lamina of the muscle fibre but separated from it. Human myogenic cells, derived from satellite cells, were also isolated from both normal and dystrophic muscle and grown in culture (6-8). This naturally led to the idea that these cells could be used to treat muscular dystrophy, but problems of rejection of healthy donor cells or of genetic correction of autologous dystrophic cells were still to be faced.
A landmark paper published in 1989 by Partridge (9) proved that myogenic cell transplantation was possible and led to the significant reconstitution of dystrophin positive muscle fibres in a hind limb muscle of the mdx mouse, the standard animal model for DMD. Based on the enthusiasm caused by this result, several groups in USA and Canada started a series of clinical trials by transplanting myogenic cells isolated from a biopsy of a relative into muscles of DMD patients (10-13). Results showed that the procedure was safe but very inefficient in humans, at variance with what had been observed in mice. This was probably due to the difference in size between human and mouse muscles (2500:1) so that numerous injections would have been needed to distribute the donor cells throughout the whole muscle, given also the limited migratory potency of myogenic cells (14,15) and the limited proliferation of human somatic cells (16). Moreover, mice are syngeneic and thus cell rejection did not occur, whereas few donor cells were detected months after transplantation in patients indicating that they had escaped the host immune surveillance (17). Finally, mouse cells may have survived better than human cells to the massive cell death that follows intra-muscular cell transplantation (18). Throughout the years, work has been carried out to move these

transplantation protocols to the clinic, new cells with different biological features endowed with myogenic potency have been identified, and new clinical trials were consequently conducted, and this will be discussed below.

A simplified scheme illustrating the classic steps in the preparation of myogenic cells as an ATIMP (Advanced Therapy Medicinal Investigational Product) is shown in figure 1

2. How to improve cell isolation, expansion, and characterization.

Skeletal muscle is the most abundant tissue, representing approximately 40% of the body weight. Replacing or repairing it all is a task currently beyond even the most optimistic wishful thinking, also considering that tissue ablation, likely the basis of recent clinical successes in ex vivo gene therapy trials (19), is clearly impossible for muscle or brain at variance with blood and epithelia. Skeletal muscle fibers are multinucleated: this implies obstacles (fibers cannot be expanded in culture and their direct transplantation is feasible only in rodents for research purposes) but also opportunities as one donor derived nucleus may help to correct neighboring dystrophic nuclei. Moreover, given the nature of the tissue, repaired muscle fibers may last for a very long time.

Cell isolation is essentially carried out by proteolytic digestion of the minced tissue or by culturing tissue fragments from which cells migrate (20). Both methods have specific advantages and disadvantages: the first results in the isolation of synchronous and purifiable cells but usually the yield is much lower than what can be obtained after several days when cells grow out from the tissue fragments. In this case however the population is asynchronous, and some cells may have started differentiation while others are still migrating out of the explant. Purification of a specific cell population is then based upon differential expression of different antigens and, while CD56 is universally used to select to select human satellite cells, several groups have developed purification strategies to enrich for sub-populations with higher proliferation and self-renewal potency (21-25).

Expansion in culture is possible up to very high numbers, however several billions would be needed to fix just a few of the essential muscles of the body. There are obvious differences among the different cell types (satellite cells and subpopulations thereof and other myogenic progenitors) so that specific culture conditions need to be implemented to enhance long term expansion without compromising self-renewal and differentiation potency. For example, expansion of satellite cell-derived myogenic progenitors on plastic surface drastically reduced their engraftment in vivo, when compared to freshly isolated cells (26) that are however too few. A possible solution to this Catch22 situation was offered by the discovery that growing the cells of a soft surface (approximately 12 kP, the natural stiffness of healthy muscle), maintains to a large extent the original engraftment potential (27). However, large quantities of GMP-grade, appropriate biomaterials for clinical use still are still not available using such conditions. Nevertheless, one obvious lesson to learn is that mechanical stress related to adhesion to a stiff substrate is detrimental for myogenic cells and should be avoided as much as possible. Exposure to atmospheric oxygen concentration is another obvious stress that should be avoided, and it is amazing to see how it is still standard procedure to grow and expand myogenic cells in incubators without regulatable nitrogen input in order to reduce oxygen concentration. Oxidative stress affects telomerase and rapidly induces senescence (28-29). Indeed, even low oxygen incubators do not completely solve the problem as cells are exposed to atmospheric oxygen every time they are sub-cultured or simply observed under a microscope. Hypoxic stations may be the final solution for oxidative stress, but their high cost limits their diffusion for the time being especially for large scale cultures under GMP conditions.

Tissue culture media also influence dramatically the proliferative expansion of myogenic cells and a plethora of “stem cell’ media are now commercially available, often at a very high price and invariably with proprietary composition. This may be a problem, as the company selling it may

decide to withdraw it from the market as was the case in the middle of the clinical trial run at San Raffaele in 2011-13 (30).

Quality controls for myogenic cells are more complex than with other cell types more commonly used in the clinics. In addition to standard controls (sterility, endotoxin, mycoplasma, karyotype, tumorigenesis in immune deficient mice and analysis of vector integration sites for virally transduced cells), it is important to know that cells express the expected phenotype, to determine what residual proliferation potential is maintained after in vitro expansion and the ability of terminally differentiating in vitro (as predictor of subsequent in vivo differentiation). SOPs (standard operating procedures) must ensure sufficient safety stringency but also some biological flexibility as it is a common experience that there is significant variability among the same cell type isolated from different individuals.

3. What myogenic cell types shall we transplant?

So far, we have been discussing general features and issues that concern myogenic cells for pre-clinical and clinical in vivo transplantation. But not all myogenic cells are the same and indeed there are many different types, some of which have not been sufficiently characterized to guarantee that they represent a distinct cell type. These cells have been described in detail in several recent reviews to which we refer the reader (30-33). Here we will briefly discuss the three cell types, used in clinical trials, for which a myogenic potency has been unequivocally demonstrated.

Satellite cell-derived myogenic progenitors, also referred to as myoblasts, and subpopulations of them (21-25) are the main myogenic cells present in skeletal muscle, they can be easily expanded in culture and undergo robust myogenic differentiation. They are definitely the cells of choice for treating localized forms of muscular dystrophy or other muscle diseases but are unable to cross the vessel wall and therefore cannot be distributed systemically through the blood circulation to reach all of the body muscles.

Mesoangioblasts are derived from adventitial pericytes of skeletal muscle, can be expanded in culture and differentiate mainly into skeletal and smooth muscle (34). Most importantly they can cross the vessel wall and thus be distributed systemically for treating diffuse forms of muscular dystrophy such as DMD (35). Unfortunately, despite the systemic infusion of large numbers of cells (over a billion in total), the level of engraftment was found to be low (<1%) and though some donor derived dystrophin could be detected, the efficacy in restoring muscle function was minimal (30), thus suggesting that additional strategies are needed to continue clinical experimentation.

CD133 cells are also myogenic and expandable in culture even though their ability to be systemically distributed has not been analyzed. They were tested in a proof of principle trial published in 2007 (36) but since then no other trials using these cells have been reported.

Finally, iPS-derived myogenic progenitors (37) have not yet been tested in clinical trials although these cells hold great promise for a number of reasons: they are highly myogenic, can be autologous, genetically corrected in culture and available in virtually unlimited numbers for subsequent transplantation rounds. Moreover, both ES or iPS cells can be genome edited (by deleting HLA antigens) to create an immune-privileged, universal donor cell (myogenic in this specific case) that would be administered to all patients with the same disease/mutation from the same bank (38) after quality controls that would be extremely difficult if not impossible for a patient specific product. More importantly, this would cut the cost of these therapies making them affordable, not a secondary issue, also for therapies that have clearly been successful as in the case of Strimvelis (39). However, the problem of systemic delivery may persist (systemic delivery has not been tested for these myogenic progenitors) but one solution may be to differentiate them towards mesoangioblasts, as previously shown (40).

4. Post-transplantation survival, migration, differentiation, and replenishing of the progenitor pool.

Once finally inside the dystrophic muscle, the myogenic progenitors (those that made it) will face a number of additional problems, all contributing to a final poor outcome. First of all, the majority of transplanted cells die within the very first day. This is too soon to be due to an adaptive immune response but is currently attributed to anoikosis for cells intra-muscularly injected as a suspension (41), though interaction with inflammatory cells, present in the dystrophic muscle may further reduce the fraction of cells that had managed to adhere to the extracellular matrix (42). Through the years, much work has been dedicated to enhance survival, migration and proliferation of transplanted cells: addition of extracellular matrix proteins or biomaterials was found to enhance survival (43, 44), expression of growth factors or metalloproteinase increased proliferation and migration (45, 46, 47, 48), or even co-injecting cells that may help transplanted myogenic progenitors (49, 50, 51).

In the case of systemically delivered cells, this massive cell death is less, but the overall outcome is not much better. Only 40-50% of intra-arterially injected cells are retained in downstream micro-vasculature (not only of skeletal muscle but also of bone, dermis etc.), the remaining reaching the venous circulation and ending in liver, lung and other capillary filters (Sampaolesi & Cossu, unpublished results). To extravasate mesoangioblasts exploit the system professionally used by leukocytes, but time-lapse microscopy in vivo indicated that they are much slower than leukocytes and this probably reduces their efficiency. Once inside the muscle, cells still have to face macrophages and other inflammatory cells, usually present in large numbers in the different muscular dystrophies, that may further reduce the number of survivors.

Next, survivors need to hopefully proliferate to restore at least in part their total number and migrate through a fibrotic tissue and reach spots of active regeneration, towards which cells are probably chemo-attracted (52), in order to fuse with regenerating fibre to which they contribute the protein product whose absence caused muscle degeneration. Whether cells fusing with many more resident dystrophic myoblasts will be able not only to produce enough protein to prevent further degeneration but also to restore a fully function is still not a fully answered question. Whether transplanted myogenic progenitors may also contribute to replenish the satellite cell pool is another important question: the answer is probably yes (53), but the extent to which they do so may impact on the long-term outcome of transplantation.

Since muscle pathology progresses with patient age, another important consideration is the age for intervention. Especially for early onset muscular dystrophies such as DMD, cell transplantation (as well as gene therapy) should be conducted in pediatric patients, ideally at diagnosis or shortly thereafter. Unfortunately, very young children may be more susceptible to adverse events (54); therefore, regulatory agencies, for evident safety reasons, usually recommend older patient for gene and cell therapy. In this way the chance of success is reduced resulting in a negative attitude of funding bodies to support further experimentation.

In the meanwhile, strategies were developed not only to improve engraftment (see above) but also to delay the progression of the disease, and this would have significant benefit for any cell or gene therapy, buying time for the patient by maintaining a muscle of better quality for transplantation. Although the idea of combinatorial therapies is shared by the scientific community, translating the concept into clinical trials, testing different variables in a handful of patients, is practically impossible as it will be discussed below.

5. Recent and ongoing trials and how to improve them.

For Duchenne Muscular Dystrophy at the time of writing this review two trials using intra-muscular injection of donor myoblasts have been completed (55,56) and one is ongoing (NCT02196467). These trials aim at showing engraftment, differentiation and functional amelioration of specific muscles, under a regime of immune suppression. The results of the completed trials have shown that endpoints were met, but it is unclear how this therapeutic strategy will allow one to treat all of the muscles essential for posture (abdominal and lower back muscle), breathing (diaphragm, intercostal) and ambulation (hip abductor, gluteus maximus). In addition, life-long immune suppression would be required for a long-lasting effect since heterologous and not autologous myoblasts were used. Though this is common for organ-transplantation, still it would be preferable to avoid it if possible.

In 2015 we reported the results of a first in man trial (30) based upon four consecutive (at escalating doses) intra-arterial administrations of HLA-matched donor mesoangioblasts (from a sibling) in five DMD patients. The trial showed safety but minimal efficacy, even though we detected, in the youngest patient, 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 (that inhibits mesoangioblasts adhesion to the endothelium), the lower cell dose and the difference in posture between humans and other mammals, so that targeting leg muscles only is not sufficient to maintain posture and ambulation. As mentioned above, the overall engraftment was too low to predict that some methodological improvement and a younger patients' age may lead to the threshold of clinical efficacy. Moreover, significantly increasing the cell dose would increase the costs and the risk of adverse vascular events. Thus, we developed an autologous, cell-mediated exon skipping approach. DMD patient derived mesoangioblasts are transduced with a lentivector expressing a snRNA engineered to skip exon 51 (57). The transduced mesoangiobalsts will be transplanted in a single muscle of the same patient and will fuse with the resident dystrophic myoblasts forming a new muscle fibre. Since the snRNA is produced by the donor nucleus, assembles in the cytoplasm and then enters all the neighbouring dystrophic nuclei present in the same myofiber, this mechanism should amplify several folds the production of dystrophin. Preliminary in vitro and in vivo data show that this is indeed the case and a new proof of concept trial, based upon intra-muscular injection of autologous, genetically corrected mesoangioblasts, should start in Manchester, but is being delayed due to the COVID pandemics.

Another disease which affects primarily a few small well defined muscles (pharyngeal and eyelid muscles leading to dysphagia and ptosis) leaving other muscles unaffected, making it an ideal target for autologous cell therapy, is Oculo-Pharyngeal muscular dystrophy OPMD. OPMD is a late onset autosomal dominant genetic disease caused by an abnormal trinucleotide repeat expansion in the PABPN1 (58,59). A phase I/IIa clinical trial, based upon autologous, phenotypically normal, skeletal myoblasts, isolated from unaffected muscles of OPMD patients and transplanted into the affected pharyngeal muscles demonstrated safety with no adverse side effects. A dose dependent functional improvement in swallowing was observed in this safety study (Clinical Trials.gov NCT00773227). This resulted in improved quality of life in patients treated with the highest dose (60). Since the transplanted cells still contain the mutation a 'silence and replace' gene therapy approach has been developed (61) and will be taken into the clinic in the near future by Benitec Biopharma as a phase I cell and gene therapy approach for OPMD.

It must not be forgotten that clinical trials using myoblasts have also been carried out to treat urinary and fecal incontinence (62)

6. Conclusions

We have learned a lot in these twenty years, and definitely more from failures that from the few successes. Still much remains to be learned both from pre-clinical work on relevant animal models and from exploratory clinical trials.

Indeed, a matter of serious consideration concerns the decision of moving into clinical experimentation on the basis of available data. On the one hand pre-clinical data are never too many but on the other, there is information that can only be obtained from patients and adverse events that may not manifest in animals. In general, the field of myogenic cell transplantation has been cautious and has moved into trials only after convincing pre-clinical evidence. Still a better understanding of the biology of the cells to be transplanted, more information on dose dependence, an accurate pharmacokinetics in a large animal models and long term follow up could only enhance the chance of success in subsequent trials.

At the time of writing other trials are planned for mitochondrial myopathies and congenital malformations and more will follow. However, it is crucial that good planning, careful analysis of the results and, most important novel approaches to overcome the current hurdles, examples of which have been provided above, are put in place with the hope to move the field towards clinical efficacy in the future.

7. Credit author statement

GC wrote the ms, FG, GBB and VM, discussed, commented and revised it.

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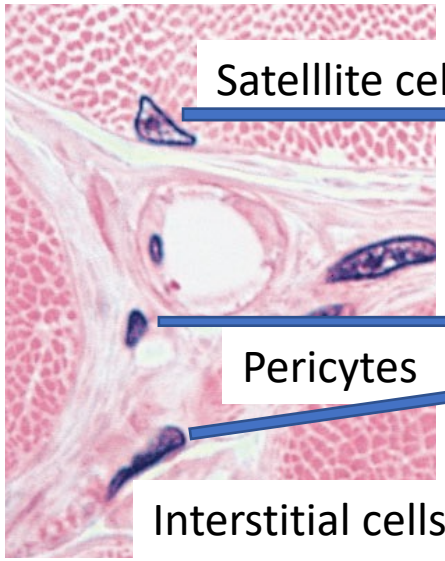
10. Figure legend

Figure 1. The development of a myogenic cell based ATIMP, from cell isolation expansion, correction and characterization to quality controls, pre-clinical testing, GMP preparation and transplantation.

Lentivector-mediated
gene correction

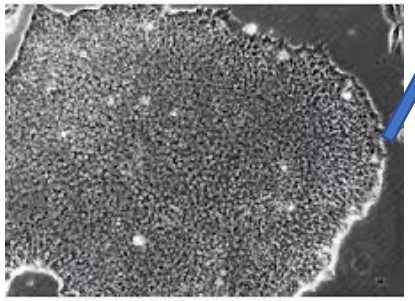


Satelllite cells

Pericytes

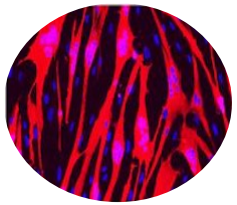
Interstitial cells

In vitro expansion



iPS cell derived

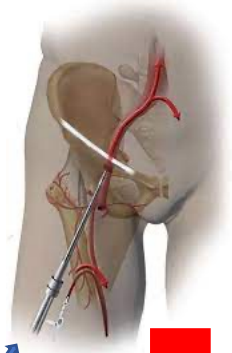
Differentiation



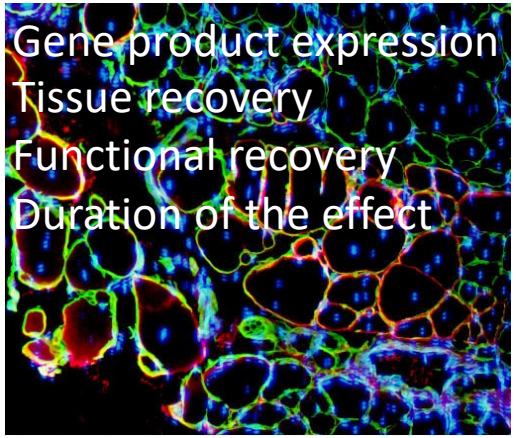
Therapeutic gene
expression

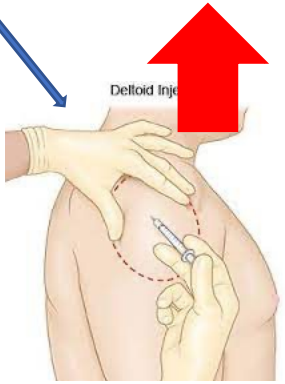
Pre-clinical models

GMP vector
Cell factory
Quality control
Regulatory
approval



Systemic





Local