The Libyan International Medical University

Faculty of Basic Medical Science

STEM CELLS BASED THERAPY IN DUCHENNE MUSCULAR DYSTROPHY

Zakaria Bashir Mohammed Eloraibi

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Supervisor: Dr.khadija Mansur
Assisted by : Dr Mohamed haj

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Abstract:

Muscular dystrophies are a group of genetic muscle disorders that cause progressive muscle weakness and degeneration. Within this group, Duchenne muscular dystrophy (DMD) is the most common and one of the most severe. So far, there are very limited therapeutic options available and there is no cure for this disease. But there is a hope with cell therapy especially stem cell - based, which utilize myoblasts, satellite cells, bone marrow cells, mesoangioblasts and CD133+ cells. And finally the human pluripotent stem cells (hPSCs) which hold great potential in treating DMD. hPSCs can be used for autologous transplantation after being specified to a myogenic lineage. We are going to summarize the current methods of hPSCs myogenic commitment, differentiation, and describe the current status of hPSC-derived myogenic cell transplantation.

Introduction:

Muscular dystrophies (MDs) are a group of more than 50 heterogeneous genetic diseases, often presented in children marked by degeneration of skeletal muscle and progressive weakness. The different MDs vary in terms of groups of muscles involved, age at disease onset, progression, and ultimate level of disability. Furthermore, several MDs show compromised physiology of other organs, such as the heart and brain in Duchenne muscular dystrophy (DMD) The most common form of MD is DMD, a fatal disease affecting around 1 in 3,500 to 1 in 5,000 live male births, Boys with DMD usually lose the ability to walk in early teenage years, lose the ability to feed themselves in late teenage years , and die from respiratory insufficiency or cardiomyopathy in early adulthood, Current standard of care includes the use of corticosteroids, cardioprotective treatment, ventilatory support, and physical therapy, However, these treatments have limitations and side effects, and are only able to delay the progression of the disease. No curative therapies are available for DMD.[(1)] In recent years, considerable research effort has been directed to developing new therapeutic options to treat DMD. Exon skipping, gene therapy and cell therapy have received considerable research attention. Antisense oligonucleotide (AON) mediated exon skipping that restores partial but functional dystrophin protein has advanced significantly during recent years with several AONs in clinical trials. Among them, Eteplirsen (for exon 51 skipping, affecting 13% of DMD patients) has been conditionally approved by the FDA in 2016, Gene

therapy that aims to produce a mini-dystrophin in muscle fibers is also in clinical trials with promising initial results, while gene editing to correct the dystrophin gene, may not be far behind. The pathological feature of DMD are due to mutations in the dystrophin gene. As we all know the dystrophin gene is translated into a 427kDa protein, which is part of the Dystrophin Glycoprotein Complex (DGC) that provides a structural and signaling link between the cytoskeleton of the muscle fiber and the extracellular matrix. The dystrophin protein stabilizes the plasmamembrane of the striated muscle fibers in healthy individuals. However in patient with DMD, mutation in the dystrophin gene cause the complete loss of the dystrophin protein, so loss of a functional DGC leads to damage of the sarcolemma upon muscle contraction, which results in loss of sarcoplasmic proteins from the muscle fiber, and extensive damage of the muscle. As a consequence, DMD muscles are subject to chronic cycles of necrosis and regeneration, in the attempt to replenish the damaged fibers with new, functional fibers.Muscle regeneration starts with the activation of the muscle stem cells, the satellite cells. These cells are embedded between the sarcolemma and the basal lamina, Upon activation, satellite cells enter the cell cycle, start to migrate toward the regenerating areas of the muscle, and give rise to more functionally committed cells, the myoblasts, which differentiate to generate new myofibers. Satellite cells usually generate their progeny by asymmetric cell division, when specific, mostly unknown fate determinants are segregated in a polarized manner between the two daughter cells, to generate a new satellite cell and a myoblast. From this well-known mechanism we can say that muscle regeneration in DMD is compromised because the continuous rounds of muscle degeneration and regeneration deplete the pool of satellite cells. In addition, because dystrophin is also expressed in satellite cells, its loss results in distorted polarity of the satellite cells, deficits in their asymmetric division, and precocious differentiation. Consequently, the DMD muscle becomes progressively unable to build new muscle fibers, which further contributes to its wasting. Moreover DMD muscle fibers are gradually replaced by fat and fibrotic tissue, which further hampers the mechanical and physiological activity of the skeletal muscle. In the DMD muscle, chronic inflammation leads to excessive levels of intramuscular reactive oxygen species, which further contribute to the muscle wasting, and hampers the regenerative power of the satellite cells. Muscle regeneration also requires the action of the fibro-adipogenic progenitors (FAPs), a population of muscle interstitial cells. Inflammatory cells regulate activation and proliferation of the FAPs , which, in turn, coordinate the regenerative action of the satellite cells However, in DMD muscles, the continuous rounds of muscle

degeneration and regeneration increase the FAPs' differentiation into adipocytes or fibroblasts, the accumulation of which further compromises the mechanical features of the muscle. Muscle commitment and differentiation are mainly controlled by the regulated spatio-temporal expression of a set of four proteins (Myf5, MyoD1, Myogenin, and Mrf4) termed the muscle regulatory factors (MRFs). The MRFs are transcription factors that drive the expression of a multitude of genes regulating establishment and maintenance of the myogenic fate. In embryonic development, myogenic cells originate from mesodermal precursors that colonize the paraxial mesoderm (PM), and that initially become part of the anterior area of the primitive streak (PS). The initial mesodermal differentiation is controlled by specific signaling proteins and molecules emanating from the anatomical regions surrounding the developing PM. Somitogenesis is the most important step towards the complete determination of the muscle progenitors. Similar to formation of the PM, it is controlled by extracellular gradients of specific signaling molecules and proteins such as FGF8 and Wnt3 which emanating from the caudal portion of the embryo.[(2)(3)]

Cell therapy is based on the heterologous, or autologous, transplantation of cells, with the goal of regenerating the damaged tissue or organ of the patient, and replenishing specific stem cell populations.[(4)]

Materials and Methods

In the first study which was in 2015, it was first decided to differentiate the PSCs into the myogenic linage to induce them to a PM, NMPs-like fate several groups successfully generated PM cells by treating mouse and human PSCs with the inhibitor of the glycogen synthase kinase3-β (Gsk3-β) CHIR99021. They found out that treating the cells with CHIR99021 mimics the addition of Wnt in the culture medium, so they knew that the induction of a PM fate can be further supported by supplementing the culture media of CHIR99021- treated PSCs with FGF. The next step they did was inducing the differentiation of the NMP-like cells into PSM-like cells, PSM cells express serially Tbx6 and Msgn1, so the activation of the Wnt signaling with CHIR99021 is sufficient to induce the expression of these two PSM markers in the PSCs.[(5)]

The second study which was conducted in 2016, they treated the same types of cells with CHIR99021 alone robustly induced the PSM in serum-free media which indicate that the initial activation of the Wnt pathway by CHIR99021 is sufficient to differentiate the PSCs to the PSM stage. From this step onward, the cell culture conditions used to induce the full myogenic commitment *in vitro* vary among the different protocols, including further treatments to increase the muscle programming efficiency, via the addition of hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1) and FGF2 to the culture medium. When in was treated in such a way mESCs generate Pax7+ myogenic cells, which give rise to Myogenin+ myoblasts and fuse into myosin heavy chain (MyHC)+ myotubes that show contractile activity in vitro. A simplified protocol of muscle commitment has recently been devised in the labs by treating normal, and DMD-derived, hiPSCs (DMD-hiPSCs) with a Notch inhibitor (DAPT), after an initial treatment with CHIR99021.[6]

Results

The first study showed that FGF and activin to be dispensable for the acquisition of the NMP fate probably because the activation of the Wnt signaling in the PSCs results in the production of FGFs by the cells These findings suggest that intracellular activation of Wnt signaling is sufficient for PSCs to acquire the PM/NMP fate, and the activation of the Wnt signaling is sufficient to induce the expression of these two PSM markers in the PSCs. For example, Chal et al showed that by inhibiting BMP and activating Wnt, both mouse and human ESCs can differentiate into PSM progenitors.[5]

In 2016, a defect was indentified in myotube formation in the DMD-hiPSCs caused by the up-regulation of the BMP and TGF-β signaling in the DMD myoblasts. The addition of a TGF-β inhibitor into the medium significantly improved the fusion of the muscle programmed DMD-hiPSCs. Increased myogenic linage differentiation of the healthy hPSCs was also observed by using different TGF-β inhibitors on CHIR99021 pre-treated hiPSCs in vitro, or by treating mice transplanted with muscle programmed, genetically corrected DMD-hiPSCs.[6]

Discussion

In the case of DMD, the main goal is to reconstitute the satellite cell pool with dystrophin competent cells, and thereby restore muscle function due to the presence of dystrophin expressing muscle fibers. The source of the therapeutic cells can be healthy, histocompatible donors, or genetically corrected autologous cells. Thus far, a number of different cell types have been applied in transplantation experiments in DMD animal models, and in DMD clinical trials. Experimental transplantation dates back to 1989, when research from groups led by Kunkel and Partridge pioneered the intramuscular (IM) transplantation of normal neonatal mouse myoblasts into mdx mice, a dystrophin deficient DMD mouse model. Subsequent experiments in humans and mice showed that IM injected normal human or mouse myoblasts formed new dystrophin+ fibers, with the partial reconstitution of a normal muscle morphology However, these early successful results in mice did not extend into the clinic. Following transplantation of muscle stem cells harvested from healthy immunocompatible donors, only small percentages of normal dystrophin were detected in patient's biopsies post transplantation from different studies. With the exception of one study, DMD patients receiving heterologous, partially immune-compatible, human myoblasts, did not show any functional improvements of the transplanted limb. These results can be explained by immune-rejection, the limited number and scarce migration of injected cells, and by massive cell death after transplantation.

Satellite cells have a strong therapeutic advantage over myoblasts, because of their self-renewal capability, which maintains their stemness. Indeed, mouse muscles transplanted with a single mouse muscle fiber, containing around seven satellite cells, or even with a single mouse satellite cell, showed a much better muscle engraftment than was the case with transplanted myoblasts. Similarly, human satellite cells isolated from muscle biopsies, and transplanted into immunecompromised mice, also on a mdx background, led to stable engraftments, formation of mouse fibers expressing human markers, and colonization of the mouse satellite cell niche, the latter being a key feature for the regeneration of a chronically damaged muscle. However, many practical limitations and safety concerns still affect the use of human satellite cells in the clinic. New protocols have been designed to overcome these hurdles, for example by using hydrogels or hypoxia conditioning[7], by expressing proteins regulating cell migration, and by elongating the telomeres to increase cell proliferation. However, despite the promising potential of muscle cell

transplantation and numerous efforts to optimize cell culture conditions in a lab setting, the use of myoblasts or satellite cells to treat DMD in the clinic has not been realized yet. To overcome the therapeutic limitations of satellite cells, researchers sought, and found, other cell populations with myogenic capability, such as those inside the bone marrow (BM). The finding of circulating cells with myogenic potential increased the scientific interest in identifying new types of non-muscle cells with the potential to contribute to muscle regeneration. That led to the discovery of mesoangioblasts, Originally isolated from the embryonic dorsal aorta of the mouse embryo, mesoangioblasts contribute to postnatal muscle development, and are considered the developmental precursors of pericytes, perivascular cells resident in the adult muscle, While mesoangioblasts derived from mouse embryo aorta express myogenic markers such as MyoD1, and endothelial markers such as VE-cadherin and CD31, pericytes express neither, rather they express markers such as NG2, PDGFRβ and CD146, Nevertheless, pericytes can differentiate into muscle when exposed to low serum conditions, or when co-cultured with myoblasts, The potential plasticity of pericytes was also confirmed by their ability to leave the perivascular niche, and adopt the fate of the recipient local tissue. This evidence further suggested their therapeutic use for DMD. Indeed, the intra-vascular injection of mouse mesoangioblasts, or of human pericytes, in both mouse and canine models of DMD and of limb-girdle muscular dystrophy, demonstrated their ability to colonize the muscle. In a phase I/IIa clinical trial, five DMD individuals were injected intra-arterially with donor HLAmatched normal mesoangioblasts. The trial showed in one individual a band corresponding to the full-length dystrophin by immunoblotting that could not be explained by revertant fibers, but no functional improvement in any of the patients. All patients had undetectable or extremely low immunological responses against dystrophin protein domains.

CD133+ cells, first isolated from human peripheral blood, are multipotent stem cells with the capacity to repopulate the BM, and differentiate into endothelial cells. CD133+ cells have also myogenic potential, as they express myogenic markers, and can give rise to satellite cells and to dystrophin positive myofibers after IM or intra-arterial transplantation into immunocompromised dystrophic mice. In 2007, a clinical trial of IM transplantation of DMD autologous CD133+ cells showed increased muscle vascularization, but no integration of the donor cells in the muscle fibers pluripotent stem cells, Vertebrate pluripotent stem cells (PSCs) retain their ability to differentiate into the three germ layers of the embryo: ectoderm, mesoderm, and endoderm. Typical PSCs are

the embryonic stem cells (ESCs), and the induced PSC(IPSCs) The generation of iPSCs opened up new avenues in stem cell therapy, and solved many problems associated with use of ESCs. For example, while human ESCs (hESCs) can only be isolated from the inner cell mass of an early embryo, which incurs numerous technical and ethical problems, human iPSCs (hiPSCs) can be generated from somatic cells, thereby allowing for the possibility of designing autologous, patient-specific, cell therapeutic strategies.

IPSCs initially were generated by transducing mouse or human somatic fibroblasts with lentiviruses expressing the four "Yamanaka" factors (Oct3/4, Sox2, c-Myc, and Klf4), subsequently to reduce the risk of DNA mutagenesis and to improve the rate of reprogramming, protocols were created to model the iPSCs for clinical purposes For therapy of muscular dystrophies, hiPSCs hold great promise. Transplantation of therapeutic cells differentiated from hiPSCs generated from the patient's own cells will not induce immune rejection as in heterologous transplantation.

Furthermore, patient's -derived iPSCs make it possible to model *in vitro* the etiology, and the pathophysiological progression, of different muscular dystrophies, to perform automated pre-clinical drugs screenings, and to set up in vitro protocols of gene editing before *in vivo* testing With patientspecific hiPSCs, this should be able to help in identifying new correlations between the established etiologic cause of each type of muscular dystrophy and the presence of genetic and epigenetic modifiers in the human genome, information which is crucial for design more efficacious pharmacological therapies.

Early attempts to achieve myogenic differentiation of the PSCs *in vitro* The first attempt to direct ESCs into a myogenic fate was carried out by inducing the formation of the embryoid body (EB*)* in vitro. The EBs are clusters of PSCs that can spontaneously differentiate into precursor cells of the three germ layers of the embryo. For example, mouse ESCs (mESCs) were allowed to aggregate in EBs, that in turn, were cultivated in hanging drops for two days, then in suspension for three days. After five days, the EBs were plated, with the myotubes detected four days later, showing the capability of the ESCs to acquire the myogenic fate almost spontaneously. However, a large proportion of cells in these mouse EBs (mEBs) differentiated into neuronal cells. Similar

results were obtained using human EBs (hEBs) cultured in growth medium supplemented with dexamethasone, insulin-transferrin-selenium (ITS), glutamine, and epidermal growth factor (EGF) the myogenic precursors induced differentiated properly only when transplanted in the regenerating muscle of a recipient mouse These early experiments demonstrated that to obtain homogenous and consistent muscle commitment and differentiation in vitro, it was necessary to identify the proper combination of pro-myogenic cues. A seminal advancement in this direction was achieved by the other groups, which, for the first time, reported the successful differentiation of hESCs into CD73+ mesenchymal progenitor cells, and then into myoblasts. In these studies, hESCs were cultured at low-density in serum free medium supplemented with ITS. Importantly, the progressive increase of serum concentration over the course of the cell culture increased the percentage of CD73+ cells, and allowed the mesenchymal precursors to progress toward different mesodermal fates, such as bone and muscle, through a transient endodermal/mesodermal stage. Eventually, this process resulted in the generation of muscle-committed cells, positive for the neural cell adhesion molecule (NCAM+), an established marker of human myogenic cells. Sorted NCAM+ cells generated myotubes in vitro, and colonized the regenerating muscle of recipient, immunocompromised mice. Subsequently, they were able to generate myogenic cells from mESCs, via the induction of an early mesodermal lineage, using serum-free, chemically-defined, culture media supplemented with the synchronized addition of Bmp4 and lithium chloride (LiCl). These pioneer studies set the foundation for step-wise systems for the myogenic commitment in vitro. hPSCs-derived myogenic cell transplantations As an ideal autologous cell source for therapy of muscular dystrophies, hiPSCs can be generated from patient's somatic cells, processed for genetic correction, differentiated in vitro, and then transplanted back into the patient. The step wise differentiation system has some advantages. For example, it is inherently transgene-free, thus avoiding mutagenic risks for the patient, and can be standardized according to good manufacturing practices. However, there are some limitations. One of the major issues of the step-wise differentiation systems is the generation in vitro of mixed cell populations, including terminally differentiated myotubes and other non-muscle cell types, such as neurons. Consequently, the presence of a potentially large percentage of contaminating, non-myogenic, cells in vitro strongly reduces the engraftment efficiency of the therapeutic cells in vivo, and results in low rates of satellite cell niche colonization and muscle regeneration. In comparison, the directly programmed iPAX7/iPAX3 hiPSC-derived myogenic progenitors, which comprise mostly PAX7+/PAX3+

cells, resulted in far better muscle engraftment. The differences in engraftment efficiency between the direct programming and step-wise differentiation protocols can be explained by the fact that the iPAX7/iPAX3 hiPSCs may represent a purer and more homogeneous myogenic population than the myogenic cells generated through the step-wise differentiation systems. In addition, iPAX7/iPAX3 hiPSCs can have a cellular status more similar to muscle progenitors than myoblasts. Evidence indicates that the Pax7+ myogenic progenitors, such as freshly isolated satellite cells, hold a better regeneration capacity than do the myoblasts, which allows the former cells to enter the muscle stem cell niche, a key prerequisite for long-term therapeutic effects.

Indeed, both satellite cells and iPax7/iPax3 PSCs-derived myogenic cells show comparable engraftment rates in mouse recipient muscles, and contribution to serial cycles of muscle regeneration. In addition, it is now clear that the human myogenic progenitor cells derived from step-wise differentiation cultures are more similar to the muscle progenitors of the early fetal stages than they are to the adult satellite cells, as shown by assays of in vitro differentiation and transcriptomic analysis. Moreover, recent results show that the iPax7/iPax3 PSCs-derived myogenic progenitors increase their myogenic potential after the transplantation in the muscle of immunocompromised mice, and, once in the satellite cell niche, they show a molecular signature comparable to that of adult satellite cells. The above evidence indicates that the muscle environment in vivo instructs the PSCsderived myogenic cells to progress from a fetal/perinatal-like status into an adult-like myogenic status. Nevertheless, the molecular basis of this maturing process is still unknown. Recently, several groups have started to identify new surface markers characterizing the human muscle precursors, to improve the engraftment rates of the hiPSCs – derived myogenic precursors, with the goal of standardizing the in vitro procedures for clinical applications. The studies reported better engraftment results using cells isolated with markers than non isolated cells. So that to develop clinically applicable hiPSCs-based therapies, researchers have focused on deriving cells that have high potency in terms of regenerating and self-renew, i.e. cells that have similar features to those of adult satellite **(figure 1)**. Cells Based on this, progenitor cells can acquire a higher clinical potential. As discussed earlier, hiPSCs -derived myogenic progenitors have a molecular profile that is similar to fetal-stage myoblasts. Therefore, one of the most critical experiments to do is the induction, in vitro, of the progression of these cells toward more mature myogenic stages. It is important to consider whether the hiPSCs-derived myogenic

cells could be delivered systemically. In this respect, Gerli et al. recently demonstrated that modulating NOTCH and PDGF pathways can endow satellite cells with the ability to migrate trans -endothelially. Based on this finding, it is possible to predict that a proper combination of modulatory growth factors and cytokines in vitro can instruct the hiPSCs-derived myogenic cells to reach all the muscles of the body via the bloodstream.[7]

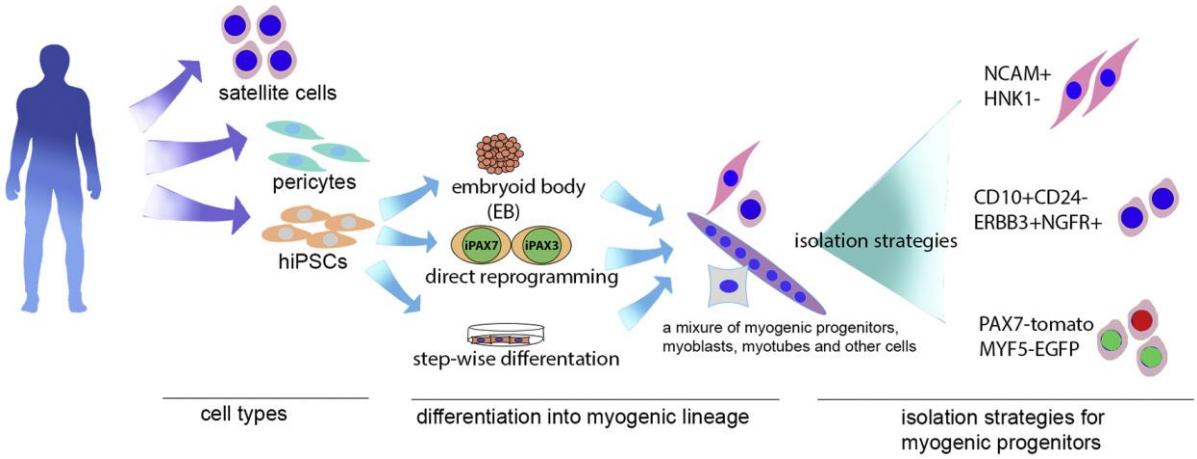

Figure 1

Conclusion

In conclusion stem cells due to their advantageous regeneration capability can give us a hope for cell transplantation therapy. hiPSCs that can be derived from patients open the path for autologous therapy. With this rapid development of serum free lineage specification protocols expandable myogenic progenitor cells can be differentiated from hiPSCs. This population of cells has similar characteristics to stem cells and has superior muscle regeneration capability compared with myoblasts. In combination with gene editing techniques, hiPSC-derived myogenic progenitor cells hold potential as an efficacious therapeutic avenue for MDs.

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