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DYS-HAC-iPS Cells: The Combination of Gene and Cell Therapy to Treat Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD), one of the most prevalent pediatric genetic disorders (1 in 3,500 newborns), is caused by point mutations or deletions in the gene encoding dystrophin, a major component of the cytoskeleton of muscular fibers. Disruption of dystrophin results in structural instability within cardiac and skeletal muscle and accelerates turnover of the myogenic stem cell pool, ultimately leading to death in afflicted individuals.[1] Gene transfer approaches to treat DMD are hampered by the very large size of the dystrophin locus (2.4 Mb) and the limited penetration into muscle by therapeutic viral vectors carrying the mini-dystrophin gene or exon-skipping constructs. Furthermore, obtaining autologous myogenic progenitors for use in DMD patients presents a particularly difficult challenge for the development of cell-based therapies.

Since the discovery of the dystrophin gene, several investigators have attempted to exploit adult stem cells and gene transfer as therapeutic approaches to DMD. In an important step toward that end, as described in this issue of *Molecular Therapy*, Kazuki *et al.* deftly combined two recent innovations so as to provide an unprecedented opportunity to overcome the obstacles facing DMD therapy[2]: the use of a human artificial chromosome (HAC) to express full-length dystrophin (DYS) and induced pluripotent stem (iPS) cells derived from a DMD patient's own fibroblasts to provide the autologous cellular resource (**Figure 1**).

Several vector systems have been developed to express defective genes for gene therapy, including retro-, lenti-, adeno-, and adeno-associated viruses.[3] The application of adenovirus has been successful, but the duration of gene expression is limited, whereas adeno-associated virus is characterized by a restricted DNA packaging capacity, such that it can carry only a mini-dystrophin gene or small exon-skipping constructs. Retro- and lentiviral vectors have the capacity to deliver a gene of greater length, but their use can lead to adverse events due either to the integration of vector constructs into endogenous chromosomes or to the induction of an immune response stimulated by viral gene expression. To overcome the limitations encountered when using such vectors and to allow expression of the complete dystrophin gene in an endogenous context, the Oshimura laboratory developed an HAC vector that carries the whole genomic locus of dystrophin (DYS-HAC).[4] In addition, a gene encoding green fluorescent protein was inserted into the vector to allow the presence of the latter to be monitored within cells, and a suicide gene encoding thymidine kinase was also introduced into the vector so as to allow negative selection against the transduced cells if necessary.

Forced expression of four transcription factors (Oct4, Sox2, Myc, and Klf4) dedifferentiates somatic cells and induces them to become iPS cells that possess the critical features of embryonic stem (ES) cells: self-renewal and pluripotency.[5,6] Regaining the potential to differentiate into all three germ layers of the body, these iPS cells provide a powerful resource for cell-based therapy for DMD patients who require cellular regeneration of their muscle.[7] Because iPS cells are derived from the patient's own cells, they do not induce immune rejection when transplanted back into patients—a huge obstacle for strategies that make use of allogeneic transplantation.

In their experiments, Kazuki *et al.* generated iPS cells from fibroblasts harvested from mdx mice—a murine model of DMD—by expressing only three reprogramming factors; they excluded the potentially oncogenic Myc. Using microcell-mediated chromosome transfer, the DYS-HAC vector was transferred to the mdx-iPS cells, where it restored dystrophin expression in the teratomas that grew from the cells, as well as in chimeric mice derived from such cells, demonstrating the potential of the combined therapy for DMD. Transferring DYS-HAC into human DMD-iPS cells proved more challenging because of the intrinsic difficulty of performing single-cell cloning of human ES and iPS cells. DMD-iPS cells were generated only when DYS-HAC was first transferred into the DMD fibroblasts before iPS cell generation. Subsequent *in vivo* differentiation of a teratoma confirmed the functional expression of dystrophin from the DYS-HAC-iPS cells.

Thus, the laborious collaborative efforts of stem cell and gene therapy scientists have allowed the development of the materials necessary for DMD treatment. However, the cure for DMD or other genetic muscular dystrophic diseases is far from imminent. **Figure 1** depicts a popular model of pluripotent stem cell–based DMD cell therapy in a sequential manner. The derivation of a patient's specific iPS cells and the genetic rescue are performed *in vitro*, and they can currently be routinely achieved despite the need to improve on the reprogramming approach. The current methods used to derive iPS cells—using retro- or lentiviruses to overexpress reprogramming genes—raise the concern of potential tumorigenicity.[8] However, new approaches for reprogramming that avoid genetic alterations should be refined within the near future thanks to the pioneering

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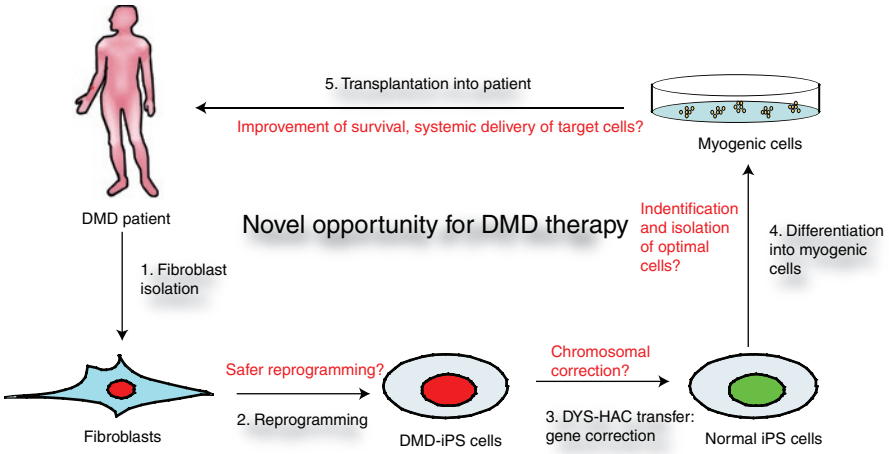

Figure 1 A novel approach to treating Duchenne muscular dystrophy (DMD) using a combination of gene and iPS cell-based therapy. The sequential completion of combinatorial DMD therapy is represented in the diagram. Fibroblasts are readily cultured from a DMD patient (1) and reprogrammed to generate iPS cells (2). Using a human artificial chromosome (HAC) expressing the whole chromosomal region of dystrophin (DYS), the genetic defect in DMD can be repaired in fibroblasts before reprogramming, or in iPS cells (3) as described by Kazuki *et al.*[2] Myogenic cells differentiated from corrected normal iPS cells (4) can then be transplanted back into the patient to cure the DMD (5). Shown in red are challenges to this therapeutic approach to DMD. Further optimization of reprogramming and genetic correction needs to be achieved. Laborious efforts will be directed at identifying and isolating the most efficient cells for transplantation and toward defining the optimal transplantation method.

efforts of a fairly large number of stem cell scientists. Indeed, nonintegrating viruses, multiple transient transfections, and protein transduction have all been used to produce iPS cells, albeit at a low efficiency.[9–12] Key issues that must yet be addressed are how to (i) maximize the utility of iPS cells through the identification and isolation of specific cell populations that can efficiently generate myogenic cells and repair damaged tissue and (ii) optimize the cellular delivery method to preserve the function of the transplanted cells.

Candidate myogenic cells can be selected from differentiated ES and iPS cells using methods developed to isolate adult stem cells of a particular muscular lineage,[13] such as satellite cells,[14] muscle-derived stem cells,[15] side-population cells,[16] bone marrow–derived stem cells,[17] mesoangioblasts,[18] pericytes,[19] and muscle-derived CD133$^+$ stem cells.[20] Established knowledge of cell surface markers used to prospectively isolate the above adult stem cells facilitates the identification of the desired cell types from iPS cells. For example, cells expressing markers of satellite cells (CD56), side-population cells (CD34), myoendothelial cells (CD34, CD114), pericytes (CD146, PDGFRβ), mesoangioblasts (NG2+), and CD133$^+$ cells can be isolated during iPS cell differentiation and then tested for their myogenic potential. However, because most of the cell surface markers are shared by adult stem cells of other lineages, isolating candidate cells with multiple markers will enhance the likelihood of obtaining a pure myogenic cell population. Barberi and colleagues' 2007 report exemplifies how to successfully isolate engraftable skeletal muscle from human ES cells in two steps, using a mesenchymal stem cell marker (CD73) and a myoblast marker (CD56).[21] The genetic approach based on the overexpression of known genes (e.g., *Pax3*) essential for myogenic commitment will be very useful in developing therapeutic approaches for DMD, because this method drives differentiating cells to become myogenic, thereby reducing the portion of uncommitted or nonmyogenic populations of cells.[22]

In addition to the difficulties associated with isolating the optimal cell types that have myogenic potential for transplantation, the iPS cell–based DMD therapy will confront the same barriers that hinder the successful application of adult stem cell–based approaches[23,24]: poor survival, limited self-renewal, and limited homing and migration after transplantation. Recent reports of myogenic functional recovery after the intra-arterial injection of mesoangioblasts and pericytes underscore the therapeutic potential of systemic delivery of myogenic cells.[18,19] Continuous efforts to optimize the use of adult stem cells in treating muscular dystrophy will be essential to the future application of myogenic cells derived from iPS cells. In addition, a completely new cell population distinct from known adult stem cells may yet be isolated from pluripotent stem cells, providing a population more closely resembling early embryonic multipotent cells with the capacity to be systematically transplanted.[25] Meanwhile, we should maintain our search for novel approaches to DMD therapy. Toward this end, Kazuki and colleagues have pioneered a unique therapeutic approach to the treatment of DMD by harnessing gene correction within pluripotent ES or ES-like iPS cells.

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miR-122 Continues to Blaze the Trail for MicroRNA Therapeutics

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As with the development of therapeutics based on RNA interference and traditional messenger RNA (mRNA) targeting with antisense, it is a liver-specific target RNA—microRNA-122 (miR-122)—that has emerged as the lead candidate for a microRNA therapeutic that could have the first meaningful clinical impact. miR-122 is by far the most abundant microRNA in the liver, and possibly in the entire human microRNA repertoire.[1,2] This early realization has spawned several investigations of the function of this tissue-specific microRNA. These show it to be important for liver cell identity,[2–4] lipid metabolism,[3,5] and, perhaps curiously, hepatitis C virus (HCV) replication.[6] Given the sheer abundance of this microRNA and the multitude of mRNAs that are derepressed following its inhibition,[3] it is possible that more functions will emerge. Because the liver is the organ that is most amenable to the delivery of oligonucleotides following systemic administration, antisense-targeting experiments in both mice and nonhuman primates contributed quite early and very significantly to the functional definition of miR-122, especially its role in maintaining cholesterol levels[3,5,7] and in HCV replication in a chimpanzee model.[8] As a result of such rapid progress, clinical trials are now under way to investigate the use of miR-122 as an antisense target for the treatment of chronic HCV infection. The phase I safety trials are sponsored by Santaris Pharma and involve healthy adult volunteers.

Setting the stage for targeting miR-122 in chronic HCV infection

It is the fact that this microRNA promotes rather than inhibits the function of a noncellular target RNA that has rendered miR-122 an attractive therapeutic target.[6] Through a combination of microRNA inhibition with simple 2′-*O*-methylated antisense molecules and elegant genetic studies, Jopling and colleagues determined that miR-122 engages two highly conserved sites in the 5′ noncoding region (NCR) of HCV in a seed-dependent manner, so as to facilitate the accumulation of HCV RNA.[6] A reduction of approximately 1 log in genotype I HCV RNA was observed in both Huh-7 replicon and transient transfection models when miR-122 was sequestered in this fashion.

It was around the same time that the feasibility of miR-122 inhibition *in vivo* was first demonstrated. Administration of relatively large amounts (three doses of 80 mg/kg on each of 3 consecutive days) of cholesterol-conjugated, 2′-*O*-methylated antisense oligonucleotides to C57BL/6J mice led to functional miR-122 inhibition within a week, as demonstrated by an approximate 40% reduction in total cholesterol and concomitant increased expression of predicted target genes, such as *Aldoa*.[3] A study published the following year[5] showed similar effects with a phosphorothioate 2′-*O*-methoxyethyl anti-microRNA, which produced largely comparable results when administered over a more extended period of time (4 weeks).

In 2008, Elmén and colleagues demonstrated the feasibility of functional miR-122 sequestration in nonhuman primates.[7] The authors employed an unconjugated, 15-nucleotide phosphorothioate DNA-LNA (locked nucleic acid) mixmer, SPC3649, that was shorter than the previous miR-122 antisense compounds, which were complementary to the entire length of miR-122. This modification was supposed to facilitate cellular uptake, and the use of high-affinity LNAs was expected to compensate for the shortened base-pair complementarity. Indeed, Elmén *et al.* show in their initial exploratory Huh-7 tissue culture studies that binding affinities correlate with miR-122 functional inhibition (via luciferase reporter and HCV replication assays) and that SPC3649 was more potent than the cholesterol-conjugated 2′-*O*-methyl antagomirs in a head-to-head comparison in C57BL/6J mice. These attributes translated into lowering total cholesterol in African green monkeys by 30–40% following three intravenous injections of 10 mg/kg over 5 days. The effect was dose-dependent, maximal at 1–2 weeks after administration of three doses of 10 mg/kg, and the reduction

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