Stem Cell Therapy for Duchenne Muscular Dystrophy

**Reporting Period:** Year 1

The goal of this project is to develop a new approach for therapy of Duchenne muscular dystrophy (DMD). In our strategy, we use skin cells from patients as the starting material and convert these cells into stem cells by adding "reprogramming" genes. We then add the therapeutic dystrophin gene to the stem cells to correct the mutation that causes DMD. The corrected stem cells are grown in a manner so that they will become muscle precursor cells. This process is called differentiation. The differentiated muscle precursor cells are injected into diseased muscles to restore healthy muscle fibers. The overall goal of the project is to demonstrate the entire strategy in a mouse model of DMD, using both mouse and human cells. The milestones for the first year of the project were 1A) to demonstrate the complete strategy for making stem cells from the mouse model and adding a correct dystrophin gene at a precise location in the chromosomes, and 1B) to demonstrate differentiation of mouse and human stem cells into muscle precursor cells that will later be used for engraftment. We achieved both of these milestones. Milestone 1A. Our project takes advantage of the recent discovery that ordinary skin cells can be "reprogrammed" into stem cells that are similar in their properties to embryonic cells. The reprogramming process is carried out by introducing four genes into skin cells that can change the pattern of expression of genes in the cells to that of embryonic cells. The reprogramming genes are usually introduced into cells by putting them into viruses that can incorporate, or integrate, themselves into the chromosomes. This process is effective, but leaves behind viruses embedded in the chromosomes, which can activate genes that cause cancer. My laboratory has developed a safer method for reprogramming, in which no viruses are used. Instead, we utilize an enzyme that can place a single copy of the reprogramming genes into a safe place in the chromosomes. In our method, the reprogramming genes are present on small circles of DNA that are easily made from bacteria grown in the laboratory. The circles of DNA, along with DNA that codes for the integration enzyme, are introduced into patient skin cells. The enzyme causes the reprogramming genes to incorporate into a chromosome at a single, safe location. After the cells are reprogrammed, the reprogramming genes, which are no longer needed, are precisely removed from the chromosomes by using another enzyme. These cells appear to be safe to use in the clinic. In addition, we developed a method to add the therapeutic dystrophin gene to the reprogrammed cells at a precise location. By adding a correct copy of the dystrophin gene, the stem cells now have the potential to make healthy muscle. These corrected stem cells were used to create muscle precursor cells in Milestone 1B. Milestone 1B. In these experiments, we demonstrated that cells reprogrammed and corrected by our methods can be grown in such a way that they differentiate into muscle precursor cells that have the capacity to become healthy muscle fibers. This process of differentiation takes place over a period of about two to three weeks, while the stem cells are grown in plastic dishes in an incubator. The cells are grown in culture medium that contains substances that allow the cells to differentiate from generalized stem cells into cells that are committed to produce muscle. We followed two procedures published in the literature for differentiation of the cells. We analyzed the cells at different time points to see if they had the characteristics of muscle precursor cells. First, we observed the cells under the microscope and saw that they fused together into long fibers, which is characteristic of muscle cells. Moreover, the fibers began to contract and twitch, which is typical of muscle fibers. To analyze the cells at the molecular level, we stained them with antibodies that recognize proteins that are made in muscle precursor cells. We were able to detect staining in some of the cells in the culture, indicating that they were becoming muscle precursor cells. This was demonstrated for both human and mouse stem cells. To measure what fraction of the cells had become muscle precursors, we mixed the culture containing differentiated mouse stem cells with an antibody that binds to the surface of muscle precursor cells. The cells were analyzed with an instrument that can measure how many cells in the culture bind the antibody. We found that 5 – 10% of the cells stained with the antibody. This result indicated that a significant fraction of the cells had become muscle precursors, with the potential to be engrafted. In the coming year, these corrected and differentiated mouse stem cells will be introduced into DMD mice to repair muscle damage. We will also apply our reprogramming and correction methods to human cells from DMD patients.

**Reporting Period:** Year 2

The goal of this project is to develop a new approach for therapy of Duchenne muscular dystrophy (DMD). In our strategy, we use skin cells from patients as the starting material and convert these cells into stem cells by adding "reprogramming" genes. We then add the therapeutic dystrophin gene to the stem cells to correct the mutation that causes DMD. The corrected stem cells are
plasmids based on Epstein-Barr virus are used to carry the reprogramming genes into human cells. These muscle proteins began to appear in the iPSC as they were undergoing the differentiation process. To make healthy muscle, these corrected stem cells were used to create muscle precursor cells in Milestone 1B. In the Milestone 1B experiments, we demonstrated that cells reprogrammed and corrected by our methods can be grown in such a way that they differentiate into muscle precursor cells that have the capacity to become healthy muscle fibers. This process of differentiation takes place over a period of several weeks, while the stem cells are grown in plastic dishes in an incubator. The cells are grown in culture fluids that contain substances that allow the cells to differentiate from generalized stem cells into cells that are committed to produce muscle. We analyzed the cells at different time points to see if they had the characteristics of muscle precursor cells. We observed the cells under the microscope and saw that they fused together into long fibers, which is characteristic of muscle cells. Moreover, the fibers began to contract and twitch, which is typical of muscle fibers. To analyze the cells at the molecular level, we stained them with antibodies that recognize proteins that are made in muscle precursor cells and also demonstrated that they contained messenger RNA that encoded muscle proteins. We also verified that the cells expressed the dystrophin gene that we inserted into them and produced normal dystrophin protein. To measure what fraction of the cells had become muscle precursors, we mixed the culture containing differentiated mouse stem cells with an antibody that binds to the surface of muscle precursor cells. The cells were analyzed with an instrument that can measure how many cells in the culture bind the antibody. We found that 20 - 50% of the cells stained with the antibody. This result indicated that a significant fraction of the cells had become muscle precursors cells, with the potential to be engrafted. In Milestone 2A, we introduced these corrected and differentiated mouse stem cells into DMD-model mice to repair muscle damage. We injected the cells into a leg muscle, and three weeks later, we detected engrafted cells by staining for dystrophin. In the coming year, we will carry out the final experiments, Milestone 3, in which human cells that have been reprogrammed and corrected are engrafted into disease model mice.

**Reporting Period:** Year 3 • NCE

This project has led to great progress in the development of a stem cell therapy for Duchenne muscular dystrophy. During the project period, we went from a conceptual strategy to making all parts of the strategy work, while at the same time discovering improvements in all aspects. The studies began with developing a new and potentially safer way to reprogram mouse cells. We started with skin cells from mdx disease model mice and introduced a plasmid, or circle of DNA, that encoded four genes that could reprogram the skin cells back into embryonic-like cells. We used an enzyme from bacteria called a “recombinase” to paste the reprogramming genes into a safe place in the mouse chromosomes. The next step was to use a second recombinase enzyme to place a correct copy of the dystrophin gene, the gene that is mutated in this form of muscular dystrophy, into a precise position next to the reprogramming genes. Once this was accomplished, we used a third recombinase to delete the portions of inserted DNA that were no longer needed, including the reprogramming genes. These steps left us with “induced pluripotent stem cells”, or iPSC, that were corrected for the disease-causing mutation. In the next step, we used methods to grow the iPSC that induced them to become muscle precursor cells. We measured these changes by monitoring several proteins that are typical of muscle cells. These muscle proteins began to appear in the iPSC as they were undergoing the differentiation process. Once the cells were differentiated, we injected them into the leg muscles of living mice that had muscular dystrophy. We showed that the cells we injected were able to engraft into the muscle, where they could repair and replace damaged muscle fibers. Having successfully carried out the complete stem cell strategy using mouse cells, we published our findings in a scientific journal and sought to develop a similar strategy using human cells. We found that the reprogramming strategy that we had used in mouse cells did not work well in human cells. Therefore, we turned to a reprogramming method that was recently reported by two labs, in which plasmids based on Epstein-Barr virus are used to carry the reprogramming genes into human cells. The long-lasting plasmids
provided a sufficient dose of the reprogramming genes, such that the human cells became iPSC. In order to supply a correct copy of the mutated gene, we developed a new method of genome engineering called DICE, for dual integrase cassette exchange. In this method, a short DNA sequence called a “landing pad” was positioned in a special place in the chromosomes called H11. This location has features that make it favorable as a spot to place introduced genes. The landing pad contains recognition sequences for two different recombinase enzymes. When a piece of DNA carrying the genes we want to insert is flanked by recognition sequences for the two enzymes, the landing pad is replaced by the gene we would like to insert. By using this method, we generated iPSC that had a new gene inserted precisely at the H11 location. The next step is to differentiate the cells into muscle precursor cells. The procedure that had worked in mouse cells was not effective for the human cells. We tried two new methods, and both generated human muscle precursor cells at good efficiency. We transplanted the differentiated muscle precursor cells into leg muscles of immune-deficient mice. The mice needed to be immune-deficient in order to accept grafts of human cells without rejecting the cells. We obtained evidence that the human cells successfully engrafted into the muscle. Until now, we had been introducing the stem cells by injecting them directly into a muscle with a needle. This procedure works well in the small muscles of a mouse, but would not work well in the much larger muscles of a human. Therefore, we also began developing a new stem cell delivery method in which the stem cells are introduced into an artery, where they can access muscle tissue by passing through the blood vessel wall and into the muscle tissue. We generated preliminary results suggesting that this arterial delivery system might be a successful means to distribute healthy stem cells to diseased muscles throughout the body. We intend to continue developing this stem cell strategy so that it can be used to help repair the muscles in patients with muscular dystrophy.

Stem Cell Therapy for Duchenne Muscular Dystrophy

**Grant Type:** Early Translational II

**Grant Number:** TR2-01756

**Project Objective:** Demonstrate proof of concept for hiPSC derived satellite cell therapy for Duchenne muscular dystrophy (using the PIs phiC32 integrase iPSC generation and correction technologies at safe harbor site).

**Investigator:**

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<tr>
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<th>Michele Calos</th>
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**Disease Focus:** Muscular Dystrophy, Pediatrics, Skeletal/Smooth Muscle disorders

**Human Stem Cell Use:** iPSC Cell

**Cell Line Generation:** iPSC Cell

**Award Value:** $2,267,261

**Status:** Closed

**Application Title:** Stem Cell Therapy for Duchenne Muscular Dystrophy
Public Abstract: Duchenne muscular dystrophy (DMD) is the most common and serious form of muscular dystrophy. One out of every 3,500 boys is born with the disorder, and it is invariably fatal. Until recently, there was little hope that the widespread muscle degeneration that accompanies this disease could be combated.

However, stem cell therapy now offers that hope. Like other degenerative disorders, DMD is the result of loss of cells that are needed for correct functioning of the body. In the case of DMD, a vital muscle protein is mutated, and its absence leads to progressive degeneration of essentially all the muscles in the body.

To begin to approach a therapy for this condition, we must provide a new supply of stem cells that carry the missing protein that is lacking in DMD. These cells must be delivered to the body in such a way that they will engraft in the muscles and produce new, healthy muscle tissue on an ongoing basis.

We now possess methods whereby we can generate stem cells that can become muscle cells out of adult cells from skin or fat by a process known as “reprogramming”. Reprogramming is the addition of genes to a cell that can dial the cell back to becoming a stem cell. By reprogramming adult cells, together with addition to them of a correct copy of the gene that is missing in DMD, we can potentially create stem cells that have the ability to create new, healthy muscle cells in the body of a DMD patient. This is essentially the strategy that we are developing in this proposal.

We start with mice that have a mutation in the same gene that is affected in DMD, so they have a disease similar to DMD. We reprogram some of their adult cells, add the correct gene, and grow the cells in incubators in a manner that will produce muscle stem cells. The muscle stem cells can be identified and purified by using an instrument that detects characteristic proteins that muscles make.

The corrected muscle stem cells are transplanted into mice with DMD, and the ability of the cells to generate healthy new muscle tissue is evaluated. Using the mouse results as a guide, a similar strategy will then be pursued with human cells, utilizing cells from patients with DMD. The cells will be reprogrammed, the correct gene added, and the cells grown into muscle stem cells. The ability of these cells to make healthy muscle will be tested by injection into mice with DMD that are immune-deficient, so they will accept a graft of human cells.

In order to make this process into something that could be used in the clinic, we will develop standard procedures for making and testing the cells, to ensure that they are effective and safe. In this way, this project could lead to a new stem cell therapy that could improve the clinical condition of DMD patients. If we have success with DMD, similar methods could be used to treat other degenerative disorders, and perhaps even some of the degeneration that occurs during normal aging.
Statement of Benefit to California:
The proposed research could lead to a stem cell therapy for Duchenne muscular dystrophy (DMD). This outcome would deliver a variety of benefits to the state of California.

First, there would be a profound personal impact on patients and their families if the current inevitable decline of DMD patients could be halted or reversed. This would bring great happiness and satisfaction to the thousands of Californians affected directly or indirectly by DMD.

Progress toward a cure for DMD is also likely to accelerate the development of treatments for other degenerative disorders. The most obvious targets would be other forms of muscular dystrophy and neuromuscular disorders. However, the impact would likely also stimulate medical progress on a variety of conditions in which a stem cell therapy could be beneficial. These conditions may even extend to some of the normal processes of aging, which can be traced to depletion of stem cells.

An effective stem cell therapy for DMD would also bring economic benefits to the state. Currently, there is a huge burden of costs associated with the care of patients with long-term degenerative disorders like DMD, which afflict thousands of patients statewide. If the clinical condition of these patients could be improved, the cost of maintenance would be reduced, saving billions in medical costs. Many of these patients would be more able to contribute to the workforce and pay taxes.

Another benefit is the effect of novel, cutting-edge technologies developed in California on the business economy of the state. Such technologies can have a profound effect on the competitiveness of California through the formation of new manufacturing and health care delivery facilities that would employ California citizens and bring new sources of revenue to the state.

Therefore, this project has the potential to bring health and economic benefits to California that are highly desirable for the state.

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