Correction of DYSF Mutation Using CRISPR and hiPSCs Technologies

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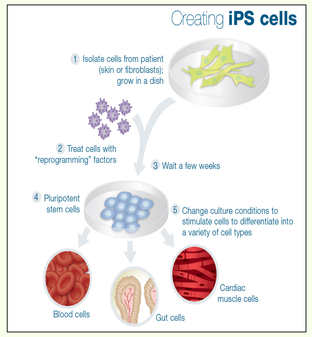
INTRODUCTION

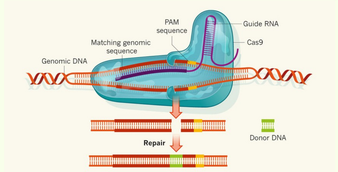
Dysferlin, also known as dystrophy-associated fer-1-like protein, is an important human protein involved in the repair of skeletal muscle. Mutations in the DYSF gene encoding the dysferlin protein cause degenerative muscle diseases that can also result in heart problems or failure.

The goal of our project is to correct the mutations in the DSYF gene specifically linked to a debilitating disease called limb girdle muscular dystrophy which is linked with cardiomyopathy. We utilized two major scientific technologies to carry out our experiments: hiPS-CMs reprogramming, and CRISPR.

Human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) have proved to be a valuable tool for modeling cardiovascular disease. Pioneered by Shinya Yamanaka in 2006, hiPSCs are a type of stem cell that can be obtained by transforming fully differentiated adult somatic cells into pluripotent stem cells through the introduction of four genes which encode specific transcription factors. This technique has allowed researchers to generate in vitro functional, beating cardiomyocyte models to study the effects of various disease. For our experiments, fibroblasts (a type of cell in connective tissues) were taken from patients with a mutated DYSF gene and reprogrammed into hiPS-CMs, which were then treated with CRISPR to correct the mutations.

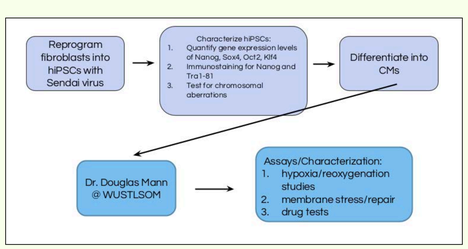


CRISPR, which stands for clustered regularly interspaced short palindromic repeats, is normally part of the bacterial immune response, as it allows bacteria to cut viral DNA into pieces, incorporate it into their own genome, and then when reinfected with the same virus -- use the viral fragments to guide the Cas9 (cutting) enzyme to disable virus. Recent research has harnessed the power of CRISPR to work as a genome-engineering and editing tool, which we have utilized in our experiments in order to correct the mutations in the DYSF gene.



MATERIALS & METHODS

1. Retrieve fibroblasts from 2 patient lines
2. Reprogram fibroblasts into pluripotent stem cells
3. Perform immunostaining to check for specific pluripotency markers
4. Perform qPCR (amplification and quantification of DNA) to test for gene expression of specific pluripotency genes
5. Analyze DNA sequences to check that cells retain original mutations
6. Cloning - introduce sgRNA (single guide RNA) into vector
7. Nucleofection - insert the vector into cells to test which sgRNA works best
8. Use most efficient sgRNA to correct mutation with CRISPR
9. Differentiate hiPSCs into cardiomyocytes to see if they exhibit normal phenotypes (done by collaborator)
10. Check karyotype of cells for abnormalities (ex tetraploidy)



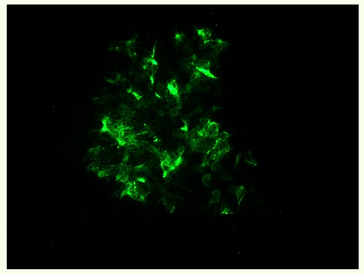
RESULTS

As the project is still incomplete, we have yet to obtain conclusive results. However, the following graphs and images display the various results that were achieved at different stages of the project.

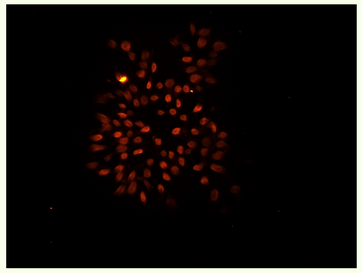
Tests for Pluripotency

We used immunostaining, an antibody-based method that is used to detect specific proteins in a sample, to determine whether our fibroblast samples were successfully transformed into pluripotent stem cells. We stained for various proteins that indicate pluripotency using the antibodies TRA-1-81, NANOG, and DAPI, and images indicating that the transformation was successful can be seen below.

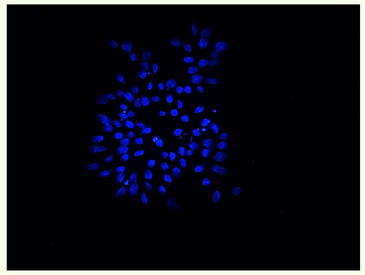
TRA-1-81



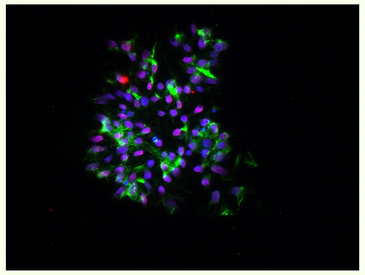
NANOG



DAPI (stains nuclei of the cells)

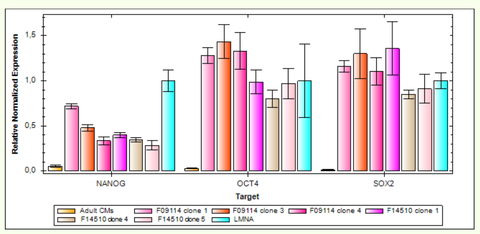


Merged pictures

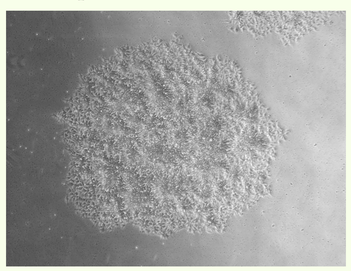


Results of qPCR for Pluripotency Markers

This graph displays how much gene expression there was for the proteins NANOG, OCT4, and SOX2, which are present in pluripotent stem cells, and were used to confirm pluripotency. The LMNA gene (cyan) was our positive control because it is known to indicate pluripotency in stem cells. The Adult CMs (goldenrod) were our negative control, because they were known to not be stem cells.

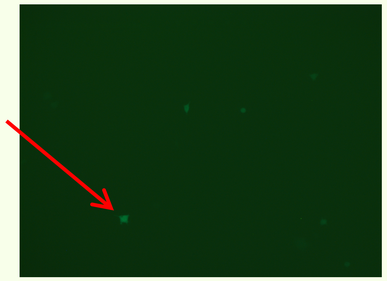


Photograph of Induced Pluripotent Stem Cells



Results of Nucleofection

The following image displays GFP positive cells after nucleofection with vector for sgRNA testing, indicating that the nucleofection was successful.



SUMMARY / CONCLUSIONS

Summary

Pluripotency - As we observed from the immunostaining and qPCR, the reprogrammed cells properly expressed the genes and markers specific to pluripotency, indicating that they had been successfully converted into hiPSCs.

hiPSCs DNA Sequencing - We amplified, extracted, and sequenced the DNA from the reprogrammed cells before initiating the CRISPR procedure, confirming that they retained the original DYSF gene mutations found in the fibroblasts.

Nucleofection - We introduced a vector containing the Cas9, sgRNA, and a GFP (green fluorescent protein) expression cassette into the hiPSCs with an electrical shock. We then identified and isolated the cells which glowed green (see image), indicating that they successfully took up the entire vector.

Current Status

We have developed 3 sgRNAs for use in the CRISPR/Cas9 editing of the DYSF gene, and are currently testing their relative cutting efficiencies to determine which sgRNA will be most successful at guiding Cas9 and the template DNA to the correct site.

Future Steps

After selecting the most effective sgRNA, we will proceed to correct the mutations with CRISPR. We will then differentiate the hiPSC lines (original mutation, corrected mutation) into cardiomyocytes and compare them to other healthy cardiomyocytes to observe differences in their phenotypes and determine if the treatment of the mutation was successful. We will also check the karyotype of the treated cells for any abnormalities, such as polyploidy.

ACKNOWLEDGEMENTS / REFERENCES

We'd like to thank our Principal Investigator Dr. Sean Wu, along with our amazing mentors Elda Dzilic and Alice Shieh for all of their guidance and support. We'd also like to thank Professor Jennifer Doudna for all the work she has done with CRISPR and for all her contributions to the scientific community.