Abstract of proposed student project.

Overall Goals of the Research: The overall goal of the project is to develop a CRISPR gene therapy-based approach for the treatment of inherited Dilated Cardiomyopathy (DCM) secondary to titin mutations. Our short-term objective is to demonstrate the utility of homology-independent targeted integration (HITI) in a titin-deficient Doberman Pincher (DP) model that develops a dilated cardiomyopathy very similar to human disease[1, 2]. The anticipated impact of the project includes (1) demonstrating gene therapy in a large animal model of titinopathy; (2) developing gene editing strategies for a broad range of disorders caused by titin mutations in humans and other genes. This project focuses on a naturally occurring genetic disease in a canine model with cardiac physiology and pathophysiology more like that observed in the human heart as compared to small experimental animals. CRISPR/Cas9 is emerging as an effective method for correcting genetic defects. This aim is carrying out a step wise evaluation of HITI/MITI based CRISPR editing [3]of the DCM associated mutation in titin canine fibroblasts established from affected Doberman pinschers.

Specific Aim 1. Screen and test efficacy of sgRNA probes in A72 and Doberman Pinscher fibroblast cells. *Objectives*: Assess efficacy of the sgRNAs and saCas9 in cutting both primary fibroblasts and A72 cells. *We hypothesize that* sgRNAs will cut with greater than >50% efficacy in both the 5' and 3' ends of the titin gene when transfected together, rather than as individual sgRNAs. Rationale: This project builds upon our work testing individual sgRNAs for cutting efficacy, which range 10-20%. Based on previous studies, we reason the editing efficiency will increase when both sgRNAs are co-transfected into cells.

Approach: We have been conducting experiments to evaluate transfection efficiency in canine fibroblasts of different sgRNAs. Our workflow is appropriate for high throughput screening of CRISPR plasmids for efficiency. We have assessed CRISPR editing in "Ricky" cells and A72 canine cells based upon the HITI approach by transfecting sgRNAs targeting the 5' and 3' end of Titin in our "Ricky" cells. The editing observed for the two tested sgRNAs using SaCas9 in the 3' region may be sufficient for going forward with HITI studies with additional analysis but corresponding 5' partner sgRNAs must be identified before we can attempt two site HITI. We plan to conduct studies using synthetic sgRNA/RNP complex of candidate sgRNA introduced by nucleofection into cells. The student will test additional candidate saCas9 targeting sites in the 5' region which we manually selected. To date, the RNP based transfections using synthetic sgRNA in cells has provided the most robust cell editing and we are using this approach in subsequent analysis. After transfection of the A72 cells, cells will be harvested, genomic DNA purified, and the targeted sgRNA region amplified for further analysis. Sanger sequencing will be conducted, and data queried by the Synthego software program to determine efficacy of editing in the targeted Titin region (ICE analysis). We have already complexed the guide RNA candidates from successful screenings with their respective Cas9 enzyme proteins to form Ribonucleoproteins (RNP). We introduced gRNA programmed Cas9 RNPs into A72 canine fibroblast cell lines using designated nucleofection programs. We will determine editing by the T7 Endonuclease protocol, Sanger sequencing, and ICE/TIDE analysis.

The significance for Veterinary Medicine and the student. CRISPR/Cas9 gene editing methodology is growing in animal and human medicine. The FVSP student will learn the basics of cell culture and how to conduct cell transfections. These assays will follow standard protocols optimized in our laboratory. We have developed a small project which has a testable hypothesis and one that will move our larger project forward. The student will test different combinations of sgRNAs to identify which combination yields the highest cutting efficacy for Titin. We currently have a post-doctoral fellow, Dr. Noble Noble who is well versed in CRISPR methods, and the veterinary student will collaborate with our team and other FVSP students to learn about the potential of gene editing in small animal medicine. The student will learn cell culture, DNA extraction, gel electrophoresis, and DNA sequencing. We encourage trainees to present their research at the UF Genetics Institute symposium in the Fall. Students will develop their own posters and present to the wider UF community, representing our college in research on campus which is very important.