**Measurement of Changes in Force Generation of Cardiomyocytes through the Down Regulation of Vinculin using CRIPR-Cas Gene Editing**

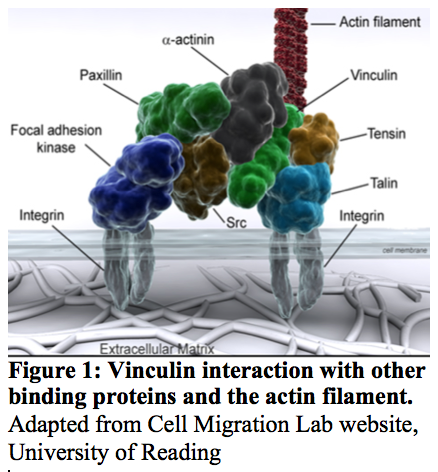
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1. **Introduction**

The study of mechanical forces on cells has recently become an increasingly interesting and growing field of research. The notion that cells are only stimulated by external stimuli which are then biochemically transmitted using diffusion mediated events to impact transcription and cell function has been remodeled. The new stance includes the fact that biochemical signaling pathways are also induced by mechanoreceptors at the cell surface. Cells have been accepted to be intrinsically mechanosensitive, with mechanical force effects being studied in cellular homeostasis, disease, and development. External forces such as fluid shear stress, stretch, pressure, and extra cellular membrane (ECM) contraction can all remodel and have structural effects on the ion channels, cadherin/integrin adhesions, and cytoskeletal structures of an organism’s cellular organization.

With the cytoskeleton (CSK) being a highly dynamic construct allowing for rapid reorganization in response to external/internal signaling and changes many have considered it the most vital cellular component for the study and understanding of force transmission. Cardiac cells (cardiomyocytes) are a prime subject for researchers to analyze and perform CSK experimentation on in order to better understand the regulators and moderators of mechanobiology. This is true because cardiomyocytes are constantly under mechanical stress through active stretching and self-generated mechanical force (Jacot 2009).

Cardiac proteins which mediate attachment of CSK to the ECM and other cells are key players in not only structure but also development, propagation, and survival of the vital organs which they constitute (Wolfgang 2008). Mutations to these proteins, a recently studied example being Titin, have been found to cause fatal cardiovascular conditions such as Dilated cardiomyopathy (DCM). DCM is characterized by left ventricular dilation and systolic dysfunction (Hinson 2015). The disease is associated with sudden cardiac death and in cases where autoimmune disease has been eliminated it is believed to be caused by genetic mutations (Jefferies 2010). One mutation that has been recorded to have a gene-phenotype relationship with DCM was found to be the cytoskeletal protein Vinculin (Olson 2002).

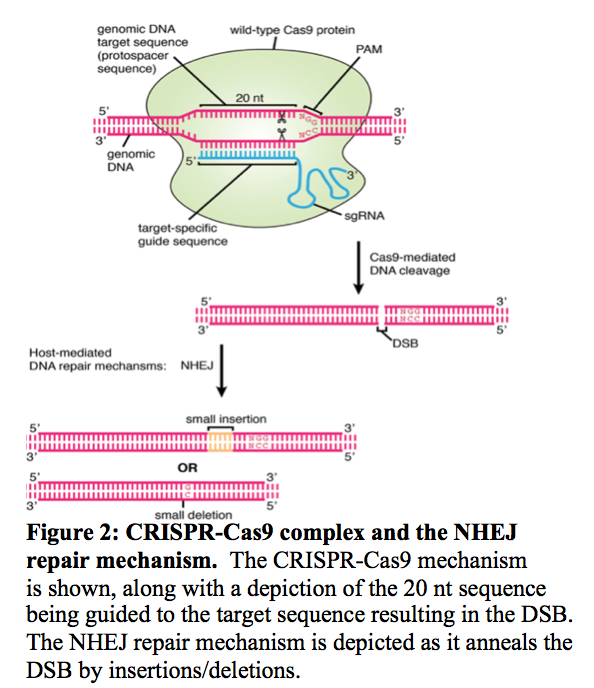
Vinculin is a ubiquitously expressed and highly conserved intracellular protein which is a crucial part of the cell adhesion complex. The 117- kDa protein is composed of eight four-helix bundles that are then organized into four tandem pairs (Bakolitsa 2004). These eight bundles make up for the globular head, a proline rich region (“strap”), and a tail domain which comprise the binding sites for protein interactions. The vinculin head (VH) contains talin, α-actinin, and α- and β- catenin binding sites. The tail (VH) consists of actin, paxilin, and phosphatidylinositol 4,5-biphosphate while in the strap vasodilator-stimulated phosphoprotein (VASP), actin-related protein 2/3 (Arp2/3), and vinexin binding sites dwell (Dumbauld 2013). Figure 1 above illustrates a few of these interactions in relation to the actin filament and integrins of the ECM. Activation of vinculin occurs through head-tail binding to proteins of the actin-based cytoskeleton aforementioned. Once activated it is recruited to focal adhesions and enhances integrin stimulation thus strengthening the binding of cells to ECM ligands and allowing for resistance from forces applied to the cell surface (Zemljic-Harpf 2007). Vinculin-deficient cells have been shown to have a reduced cortical CSK stiffness along with decreased cell adhesive strength (Dumbauld 2013). Although research has shown that vinculin plays an in important role in the vitality of cardiomyocytes, functional analysis of the protein deficiency has yet to be tested so that contractile performance and its possible ramifications could be analyzed.

Hinson et al (2015) performed this functional testing on another protein that was associated with DCM, titin. They speculated that mutations to the gene in induced pluripotent stem cell-derived cardiomyocytes would result in a decreased contractile force of the cell and lead to a pathogenic response. The researchers implemented pre-mature protein truncations to vital segments of the genome that coded for titin and grew the cells between two polydimethylsiloxane pillars through which functional studies of contractile performance could be measured. Their findings demonstrated that mutant titin protein in the cardiomyocytes resulted in sarcomere insufficiency, impaired response to mechanical stress, and weakened cell signaling stimulation.

Conceivably a mutation to vinculin in cardiomyocytes would induce a similar result, which is the purpose of experimentation suggested in this proposal. By using the CRISPR-Cas mechanism to generate a vinculin (Vcl) knockout genotype, cells expressing the phenotype in cardiomyocyte will be grown on polydimethylsiloxane pillars, through the methodology described in Dumbauld et al. Functional analysis will be used to show that mutations to the vinculin protein is a cause of DCM.

1. **Experiment**

The aim of this experiment is to provide functional analysis of cardiomyocytes which have had the expression of vinculin mutated using CRISPR-Cas gene editing. Analysis will be conducted by testing traction force measurement with a microfabricated postarray deflection device (mPAD). Vinculin (k.o.) cells will be compared to vinculin (w.t.) cells, if the cells were properly mutated to lack vinculin then I would expect the contractile force generation of knockout cardiomyocytes to be significantly less than that of wildtype cells.

II.A. Generation of a Vinculin Knock Out Cell Line using CRISPR-Cas

In order for cardiomyocytes of differing vinculin expression to be compared, gene specific editing must be performed. This could be accomplished through a number of different genomic engineering methods that have been established including Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) but the method chosen for this proposal was clustered regulatory interspaced short palindromic repeats/ CRISPR-associated (Cas) system (Gaj 2014). CRISPR has been employed to produce a surplus of gene editing functions that include gene knockout and knock in. In order to down regulate the vinculin expression of cardiomyocytes a knockout system will be performed in embryonic cells due to the postnatal nature of cardiomyocytes.

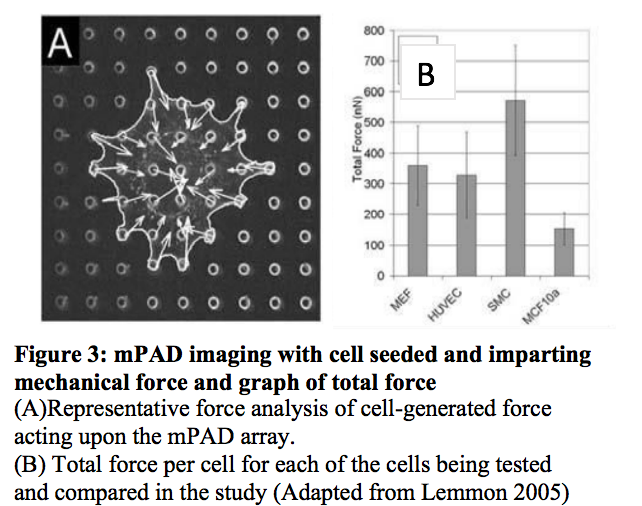
The method was employed by Chen et al (2016) in which general considerations and experimental protocols were described for creating genetically modified mice using the *Streptococcus pyogenes* (SP) CRISPR-Cas9 system (Chen 2016). A 20-nucleotide RNA sequence at the 5’ end of single guide RNA stipulates target specificity through a complementary sequence to a protospacer sequence located at the target site along with a Protospacer Adjacent Motif (PAM) directly adjacent to it. The system imparts a double stranded break (DSB) at a specific place in the genome as directed by the sgRNA in which the cell’s own non-homologous end joining (NHEJ) mechanism allows for nucleotides to be inserted/deleted in-between the broken ends.

Utilizing the target regions considerations that were mentioned in Qin et al a 20-nucleotide sequence was chosen from the coding region of vinculin as it was sequenced by Weller et al in 1990. sgRNA was synthesized utilizing a T7 promoter, the 20-nucleotide sequence guide, and sgRNA scaffold, with reference to Chen 2016. The sgRNA was then incubated with purified Cas9 protein. Fertilized mouse zygotes were collected into which the sgRNA/Cas9 complex would be introduced. Chen et al applied an electroporation method (CRISPR-EZ) they found to be more effective in delivery of the complex into the zygotes than typical microinjection.

Once introduced with the complex embryos were then transferred into the oviduct of females where the transgenic modification could propagate and form a viable offspring. In the cell’s nucleus, the sgRNA guides the Cas9 to specified areas where it will then mediate DNA cleavage as a double break within the target DNA. The small nucleotide insertions that occur as a result of the non-homologous end joining pathway’s efficient and error-prone repair allows for gene mutations (insertions, deletions, or frameshifts) to occur and thus effect the genotypic and phenotypic outcome of the mouse (Chen 2016). Assuming that the inserted embryo was able to differentiate and mature into viable pups, genotypic and phenotypic analysis is then conducted upon labor to identify the affected mouse and ensure proper gene editing occurred (Chen 2016).

CRISPR-Cas 9 Vcl knockout does not appear in research but biological supply companies have provided the sgRNA specific plasmid for sale and use (SCBC). Once the gene has been implemented the functional analysis testing of the cardiac cells has to be studied, for which Lemmon et al (2005) have described a method to effectively accomplish the task (Lemmon 2005)

II.B. Functional Analysis by Traction Force Measurements

****Cardiomyocytes expressing the vinculin gene and those that previously had the protein knocked out will then be collected via a biopsy and seeded to the top surface of microfabricated postarray deflection devices. The mPADs will be constructed and the cardiomyocytes introduced as previously described (Chen). The cells will then be stained utilizing vinculin specific antibodies for immunofluorescent and direct staining. After being seeded on mPADs the cells are placed into a LIVECELL chamber to maintain temperature and CO2 levels (37˚C and 10% respectively). In order to calculate the direction and magnitude of tip deflection, images were collected and processed through Matlab code as it was previously written and described (Lemmon 2005).

Comparison of cell-generated force for the two cell lines will then be conducted. The representative force analysis for the vinculin wt and vinculin ko cardiomyocytes will show the total force (nN) per cell for each cell type. Figure 4 shows an example of the data that will be produced from the analysis along with immunofluorescence images of cells imparting mechanical forces on the top surface of a mPAD.

1. **Discussion**

If all goes well, the cardiomyocytes that were genetically modified to not express the protein will clearly exhibit less total force upon the mPAD array when compared to wildtype cardiac cells collected from the same species of mouse. The lack of expression will have been confirmed from genotypic and phenotypic analysis but the results of the traction force measurement will be able to convey the difference in force generation between the two cell lines. Such a finding would lead to results much like that found in Hinson et al (2015) in which it could be indicated that vinculin mutations cause DCM by disrupting linkage structure and adaptive remodeling (Hinson 2015).

Although these results are the ideal, possible issues may arise that do not support the predicted hypothesis. It is possible that cell remodeling and compensation for loss of the vinculin would occur and a significant difference in the force generation would not be measured. It is also possible that given the structure and lack of ECM in the mPAD array upon which the cells were seeded that vinculin did not have a significant effect on actin binding and overall mechanical strength of the cell. Another possibility is that the lack of vinculin is not the reason for decreased force generation but instead it is a structural issue of the cardiac cells. It was proposed to me by my mentor that gap junctions tend to be affected by altercations to cytoskeletal changes. Thus, when studying the calcium release of rhythmic heart beats the electropotential tendency of the cells are compromised and begin to release calcium in an un organized and sporadic manner, better known as Calcium sparks. It is a strong possibility that DSM is not caused solely by the loss of protein and its stabilizing/strengthening factor but also its ability to maintain structure among the lattice of cells it constitutes.

The knowledge assessed and tested by this proposal could possibly lead to further studies of cells which generate and conduct force. Potentially certain defects can be identified by their functional analysis of generated force or resistance to it. From that information and the incorporation of iPs driven cells, a non-invasive method of assessing heart health, muscle viability, and any number of factors could be tested for in order for people to be better informed about what is going on inside of their bodies.

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