CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

IMPROVED PLASMID-BASED MUSCLE GENE THERAPY

A thesis submitted in partial fulfillment of the requirements
For the degree of Master of Science in Biology

By

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ABSTRACT

IMPROVED PLASMID-BASED MUSCLE GENE THERAPY

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Muscular dystrophies are a group of inherited disorders characterized by progressive muscle weakness, and degeneration. One approach to treatment would be the replacement of the deficient protein via gene therapy. For effective gene therapy, both efficiency of gene delivery and stable expression of the transferred gene are important factors. Our goal was to determine which of the following mammalian expression vectors would be more useful (more stable) for muscle gene therapy; pAcGFP1-C1 and pEPito. We were also interested in switching/substituting both vectors' CMV promoter/enhancer region with the muscle specific promoter, Desmin (DES), to increase their stability for muscle gene therapy. This was accomplished by transfecting C2C12 myotubes with the aforementioned vectors. Both vectors showed relatively continuous GFP expression. Myotubes transfected with pEPito continued to express GFP till day 8. Cells transfected with pACGFP1-C1 also showed continuous GFP expression till day 6. Our results show that both vectors are promising candidates for gene therapy in muscle cells as they maintained stable gene expression of the GFP reporter gene for at least 6 days. Further studies should be done in order to determine the maximum duration that the myotubes would be able to maintain the plasmids and show continuous expression. Future studies

could be done to assess stability of these vectors in mice which may lead to future gene therapy trials for muscle disorders and improving gene therapy strategies for other disorders.

CHAPTER1: INTRODUCTION

Muscular Dystrophies

Muscular dystrophies refer to a group of inherited disorders characterized by progressive muscle weakness, wasting and degeneration (Yuasa et al., 2002). Dystrophic muscle disease can occur at any age. Early or childhood onset muscular dystrophies may be associated with profound loss of muscle function, affecting ambulation, posture, cardiac and respiratory function. Late-onset muscular dystrophies or myopathies may be mild and associated with slight weakness and an inability to increase muscle mass (McNally and Pytel, 2007).

Muscular dystrophies encompass a range of disorders. The 9 major types of mucular dystrophies are: Duchenne (DMD), Myotonic, Becker (BMD), Limb-girdle, Facioscapulohumeral, Congenital, Oculopharyngeal, Distal, Myotonic, and Emery-Dreifuss (Theadom et al., 2014). Muscular dystrophies are classified by clinical manifestations, pattern of inheritance, muscle affected age of onset, and general progression (Figure 1) (Lovering et al., 2005).

The phenotype of muscular dystrophies arise from a diverse set of genetic pathways (McNally and Pytel, 2007). Many of these clinical phenotypes overlap, therefore these phenotypes cannot always be linked to a distinct gene (Mercuri and Muntoni, 2013). They are usually associated with proteins that have similar functions in different genes.

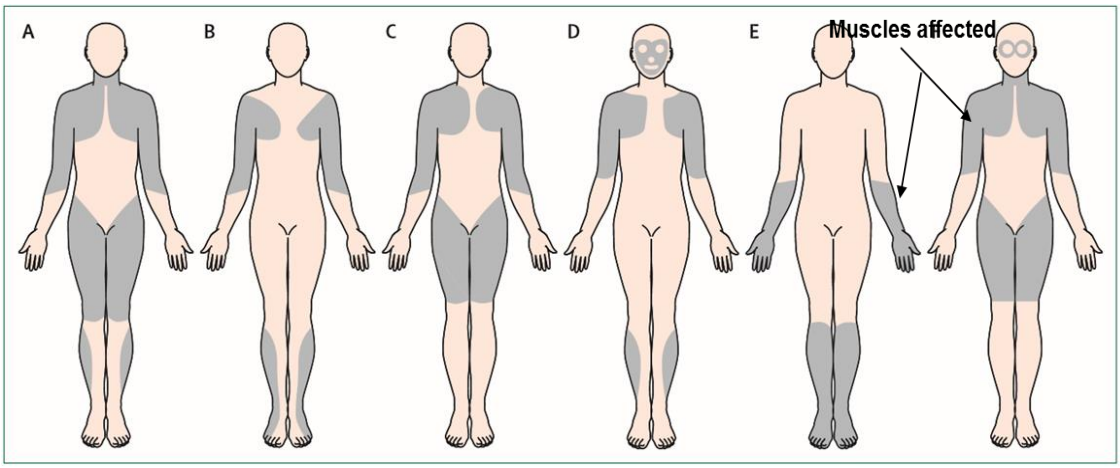

Figure 1: Distribution of muscle weakness in various dystrophies A: Duchenne and Becker muscular dystrophy. B: Emery-Dreifuss muscular dystrophy. C: Limb girdle muscular dystrophy. D: Facioscapulohumeral muscular dystrophy. E: Distal muscular dystrophy. F: Oculopharyngeal muscular dystrophy Shaded areas represent the affected muscles. (After: Mercuri and Muntoni et al., 2013).

Dystrophin

Dystrophin assembles the dystrophin–associated glycoprotein complex (DAGC or DGC). It is a critical protein that links the cytoskeleton (actin filaments and microtubules) to the extracellular matrix (ECM) (Figure 2) (McGreevy et al., 2015), providing physical strength to muscle fibers (Yuasa et al., 2002). The absence of dystrophin results in the instability of the DAGC, resulting in high damage susceptibility of the membranes, leading to necrosis and regeneration. This continuous cycle of muscle damage and regeneration causes progressive muscle mass loss. This muscle loss is replaced with connective tissue (Wang et al., 2009). Mutations in genes that affect the synthesis of any of the DAGC components, usually lead to muscle degeneration due the instability of the DAGC complex. Some of the disorders that lead to muscle dystrophy due to the instability of the DAGC complex, are: protein O-mannosyltransferase deficiency (POMT), Duchenne muscular dystrophy (DMD), limb-girdle muscular dystrophy (LGMD), congenital muscular

dystrophy (CMD), Emery-Dreifuss muscular dystrophy (EDMD), Walker-Warburg syndrome (WWS) and rippling muscle disease (RMD). Because muscle has such distinctive structural and regenerative properties, many of the genes implicated in these disorders target pathways unique to muscle, or more highly expressed in muscle, like DAGC (Figure 3) (McNally and Pytel, 2007).

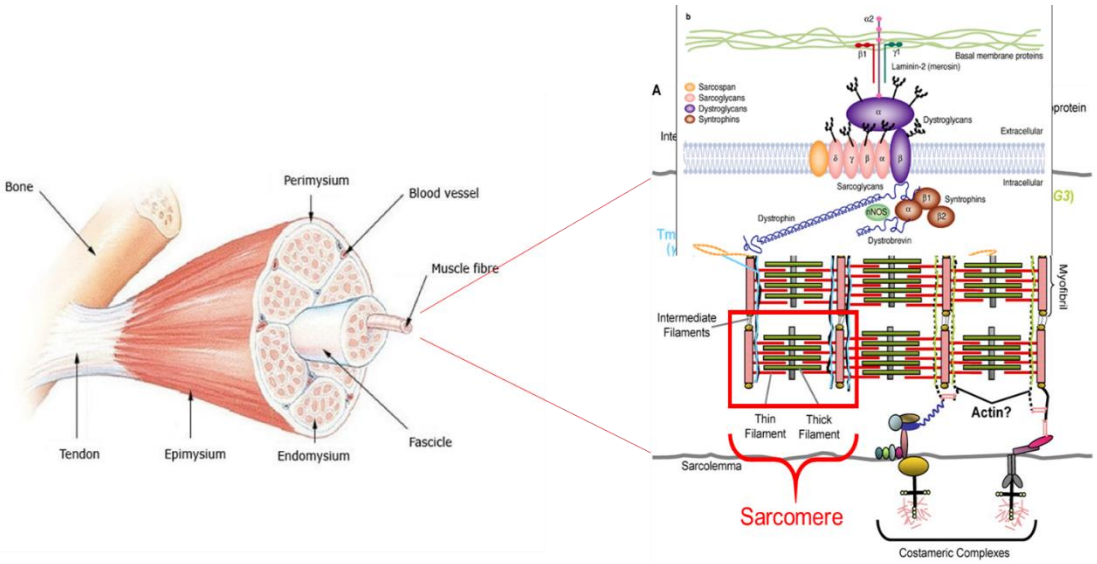

Figure 2. Role of Dystrophin in the muscle. Dystrophin links the extracellular matrix (ECM) to the actin filaments in the cytoskeleton. (Reproduced from: training.seer.cancer.gov) (After: Expert Reviews in Molecular Medicine© 2002 Cabridge University Press and Kee et al., 2004)

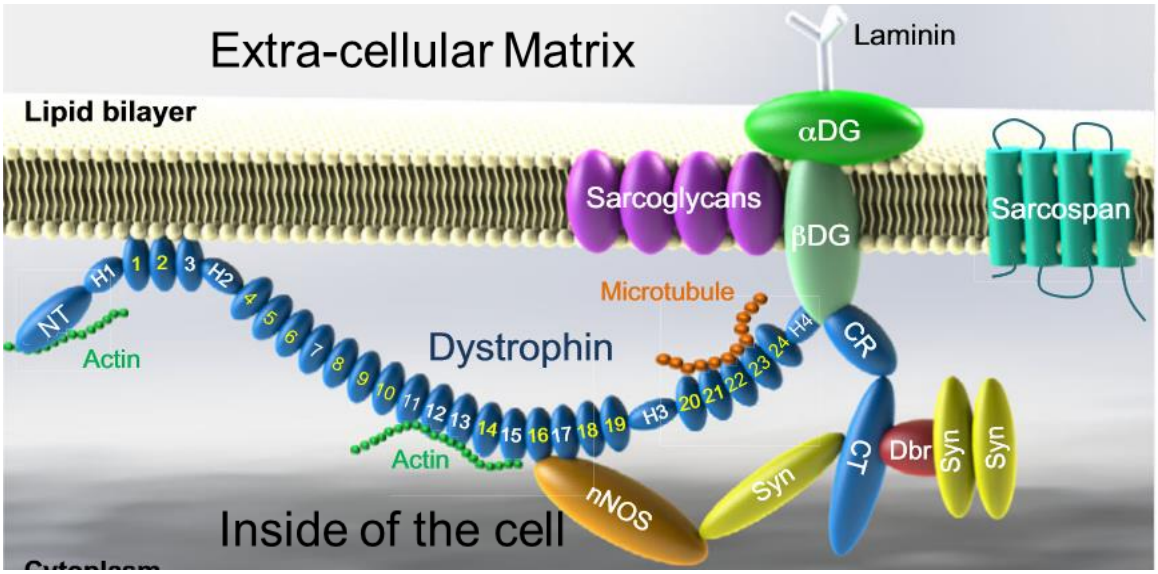

Figure 3. The Dystrophin-associated glycoprotein complex (DAGC). Dystrophin and other proteins that make up the complex are shown. (After: McGreevy et al., 2015)

Dystrophin Gene

Dystrophin, is one of the largest genes in the body. It is located on Xp21.3-p21.2. It consists of 79 exons and spans more than 2 million base pairs (Figure 4) (Hoffman et al. 1987, 1988; Koenig et al. 1988; Muntoni et al. 2003). Due to Dystrophin's large size, it is more prone to mutations. Frame shift mutations usually lead to no expression of dystrophin and result in DMD. In-frame deletions however lead to the production of a shorter protein which still carries some function, leading to BMD (Figures 5 & 6) (Hoffman and Kunkel, 1989; Monaco et al., 1988; McGreevy et al., 2015).

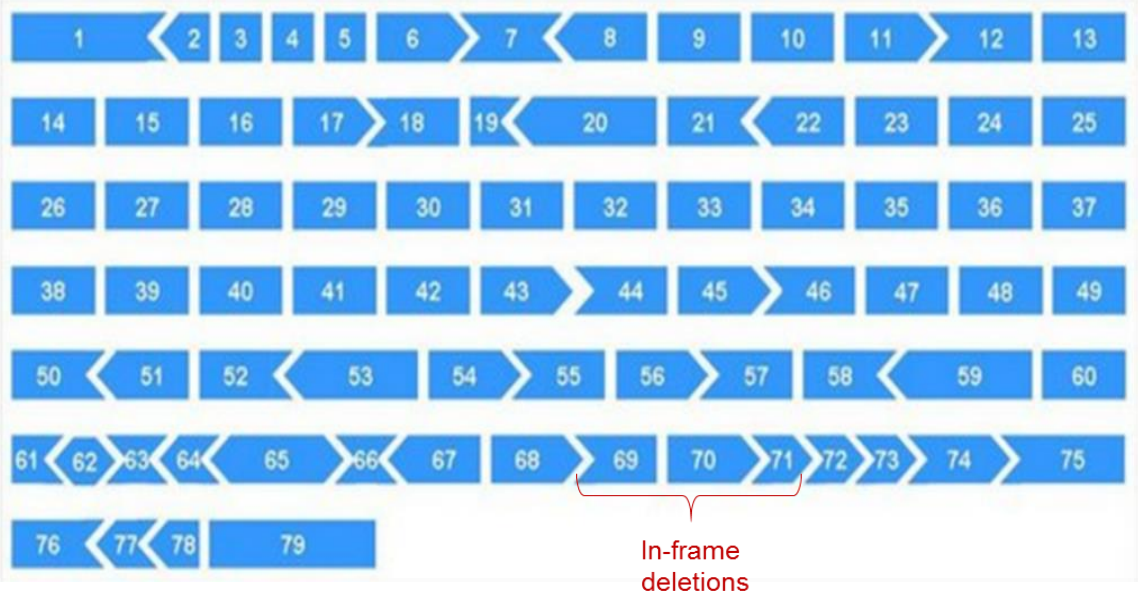

Figure 4. The Dystrophin gene. Dystrophin is one of the largest genes in the genome, consisting of 79 exons and spanning 2.3 million base pairs. (Reproduced from: http://mayopathy-researchcenter.blogspot.com/)

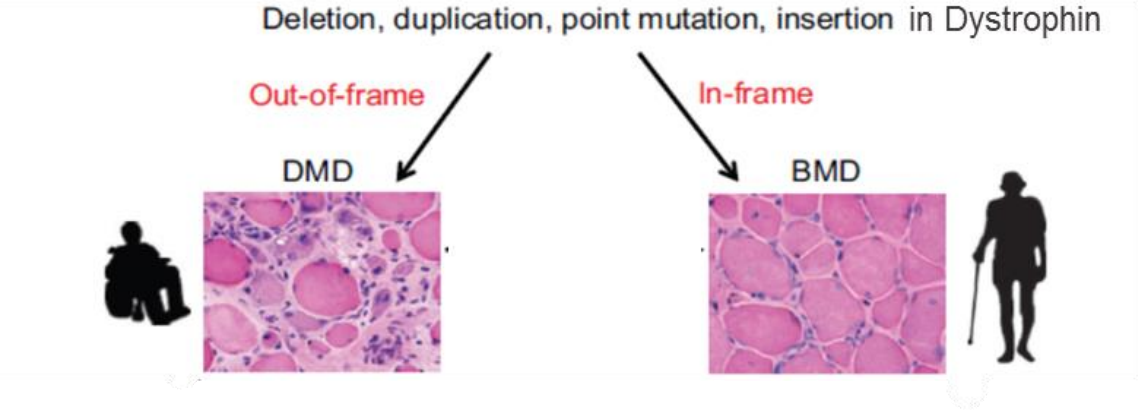

Figure 5. Mutations in the Dystrophin gene. In-frame mutations leading to Becker muscular dystrophy (BMD) with a less severe phenotype (right). Out-of-frame mutations leading to Duchenne muscular dystrophy (DMD). (After: McGreevy et al., 2015)

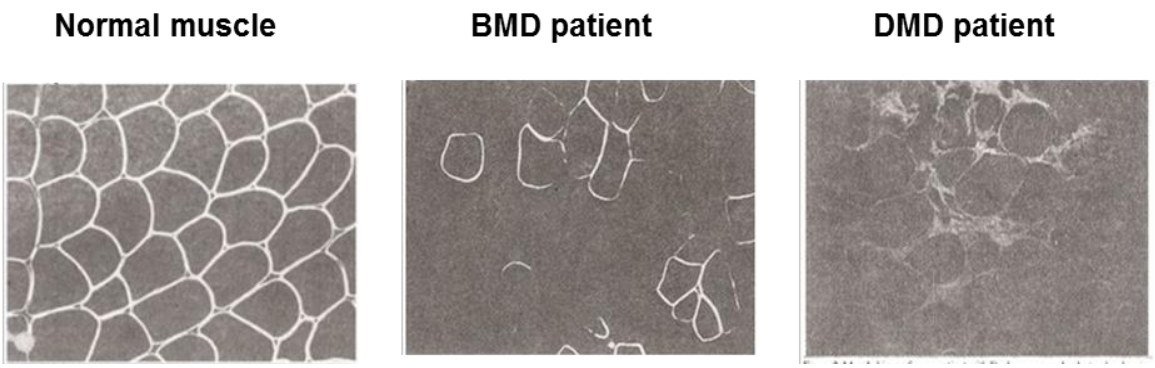

Figure 6. Immunohistochemical staining of the membrane with Dystrophin antibodies. Presence of dystrophin in the cellular membrane in normal muscle tissue, Becker and Duchenne individuals. (Adapted from: Lovering et al., 2005)

Duchenne and Becker Muscular Dystrophy

Amongst muscle dystrophies, Duchenne muscular dystrophy (DMD) is the most severe and common form of muscular dystrophy (Figure 7). It is x-linked and affects ~1 in 3500 newborn males. (Yuasa et al., 2002). Individuals affected with DMD can be diagnosed from elevated serum levels of muscle enzymes in the blood. Elevated serum levels of muscle enzyme, creatine kinase can also be used to detect carrier heterozygotes. Duchenne patients usually show muscle weakness by the age 5. Loss of independent ambulation, respiratory failure or cardiomyopathy is seen in their late teen years or twenties (Figure 8) (Muntoni et al. 2003). A milder form of DMD is Becker muscular dystrophy (BMD). Becker patients show slower progression and have less severe symptoms (Yuasa et al., 2002).

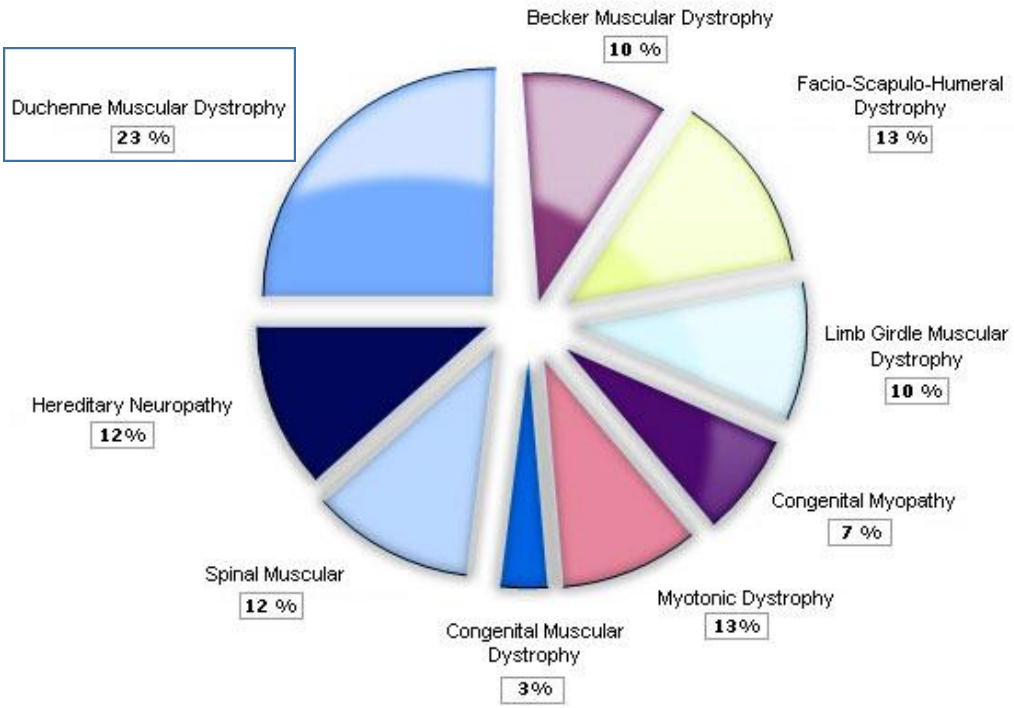

Figure 7. The prevalence of muscular dystrophies. Prevalence of 9 major types of muscular dystrophies. (Reproduced from: http://www.amsvans.com/blog/muscular-dystrophy)

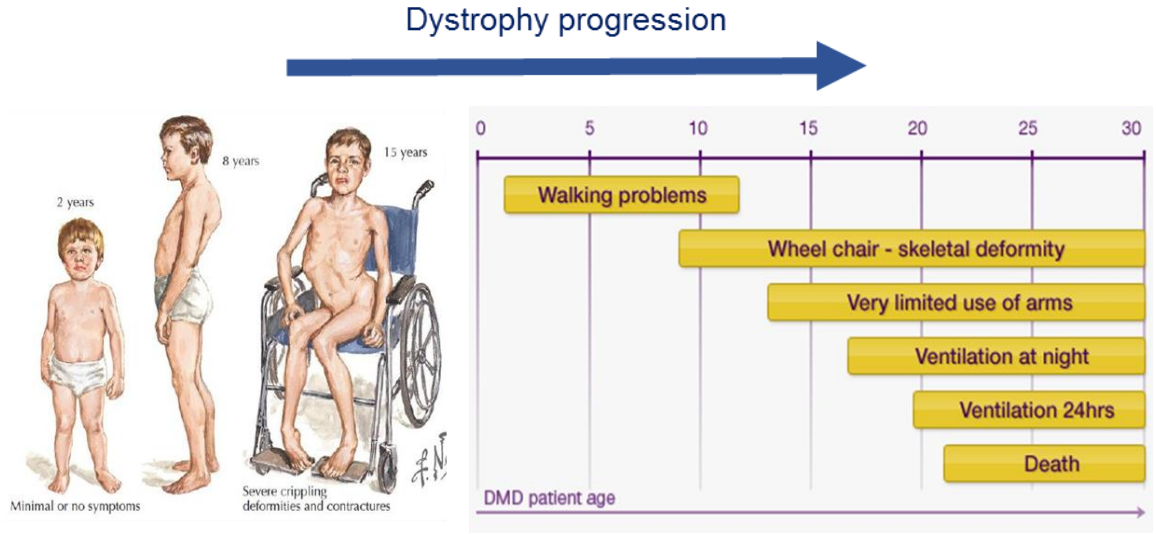

Figure 8. Timeline for DMD patients and DMD progression. (Reproduced from :http://prosensa.eu/patients-and-family/duchenne-muscular-dystrophy ; https://www.netterimages.com/images/vpv/)

Gene therapy for Muscular Dystrophies

Many of the muscular dystrophies are monogenic, and approaches to treatment are fairly straightforward; replacement of the faulty gene. An ideal treatment would be simply to replace the missing protein by gene therapy (Figure 9) (Lovering et al., 2005). Gene therapy has been successful in treating a few monogenic disorders such as severe combined immunodeficiency syndrome (SCID), Lipoprotein lipase deficiency (LPLD), Leber congenital amaurosis (LCA) and Hemophilia B (Wang et al., 2014).

Currently, there is no effective therapy to stop the deadly progression of muscle disorders, although several promising experimental strategies are currently under investigation. (Yuasa et al., 2002) For effective gene therapy both efficiency of gene delivery and stable expression of the transferred gene are important factors that need to be considered. Viral vectors are very efficient in transgene delivery to the host, however, because of their viral components they may stimulate an immune response. Also they may randomly integrate into the host's genome and cause mutagenesis and cancer. In order to overcome the safety issues associated with viral vectors, the development of alternative vectors is therefore vital for further progress in this field, in particular, vectors which remain episomal and are therefore less genotoxic. One unique class of vectors is based on scaffold matrix attachment regions (S/MARs) elements, which are maintained extra-chromosomally and replicate in vitro and in vivo. S/MAR based plasmids are mitotically stable during cell division because they allow attachment to the nuclear matrix, allowing proper segregation into daughter cells **(**Figure 12**)** (Argyros et al., 2011).

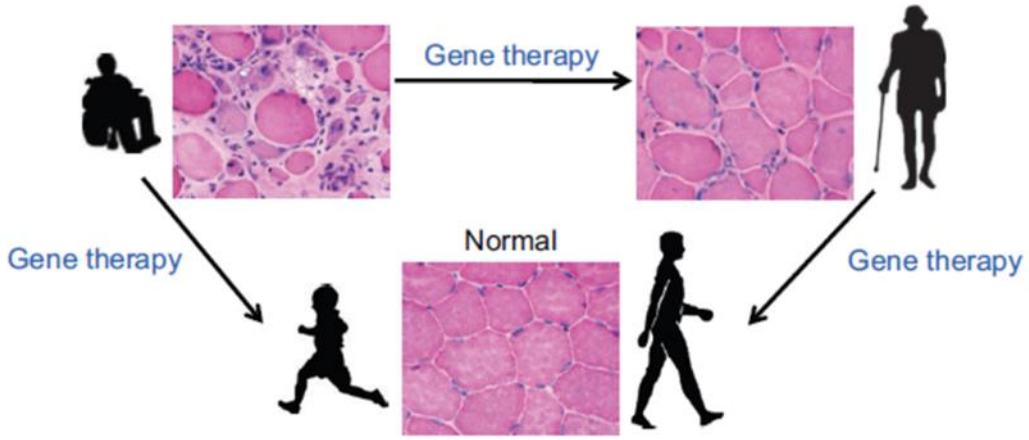

Figure 9. **Becker and Duchenne Dystrophy tissue comparison after gene therapy.** This figure shows cross sections of muscle fibers from individuals with Duchenne, Becker and wild type genotypes. In the case of Duchenne muscular dystrophy, abnormal increased arrangement of the myofibers in the striated appearance and extensive replacement of muscle fibers by adipose cells is seen. The top right picture shows a Becker muscular dystrophy with less severe phenotype of the disease. (After: McGreevy et al., 2015)

Purpose of Study

The purpose of this study was to improve plasmid-based gene therapy for muscular dystrophies. I was interested in focusing on different plasmids in order to determine which would be more efficient for gene therapy of muscle disorders. I decided to compare two mammalian expression vectors; pAcGFP1-C1 (Lacking an S/MAR element) (Figure 10) and pEPito (Figure 11) that is S/MAR-based (see Figure 12). I was also interested in comparing regulatory elements that may improve expression of cloned genes in cultured myofibers, because a major problem associated with non-viral vectors is their low stability due to gene silencing. I chose to study a muscle specific promoter, called Desmin (DES) and a ubiquitous chromatin opening element (UCOE).

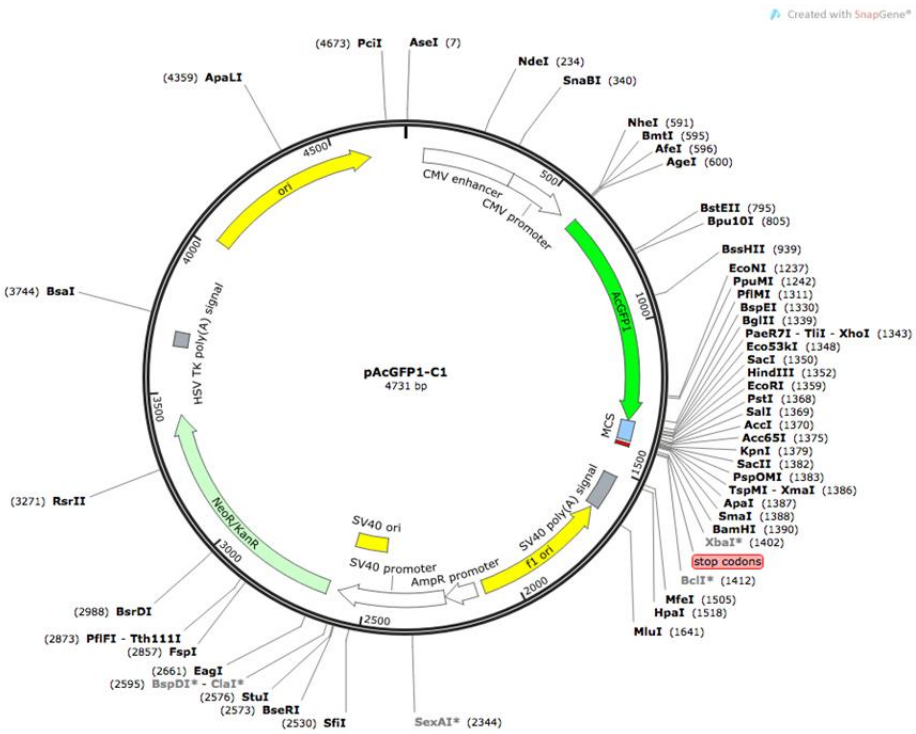

Figure 10. Map of pAcGFP1-C1. Map of the 4731 bp pAcGFP1-C1 CMV promoter, GFP reporter gene and kanamycin resistance marker. (www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17846)

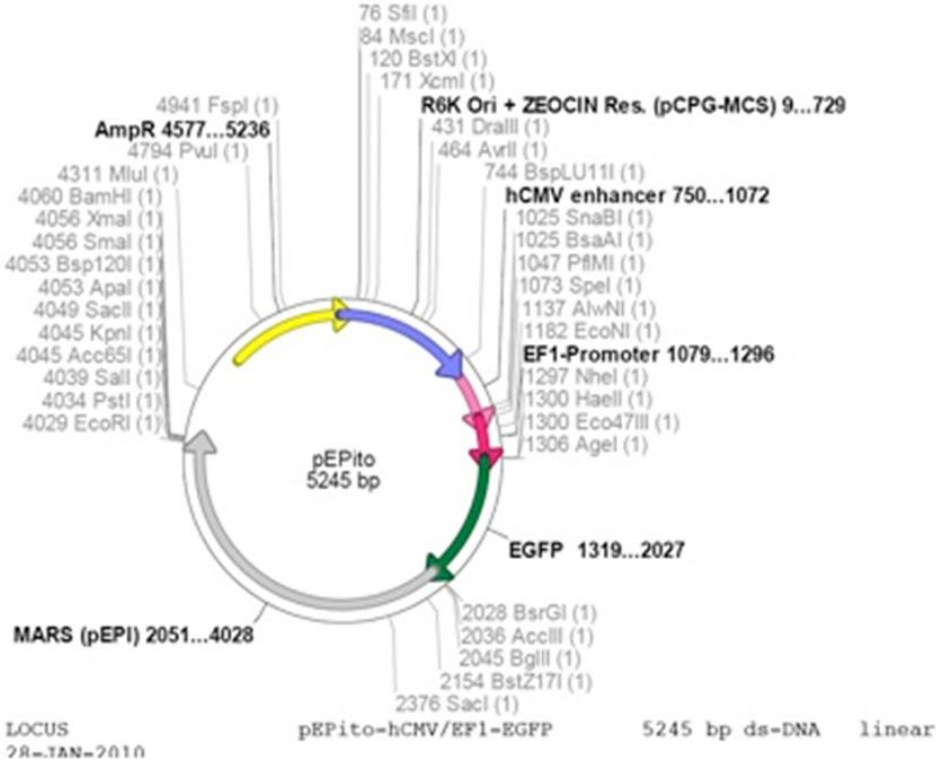

Figure 11: Map of pEPito. Map of the 5245 bp pEPIto vector. The CMV promoter, GFP reporter gene and ampicillin resistance marker (Plasmid Files pAcGFP1-C1, 2014).

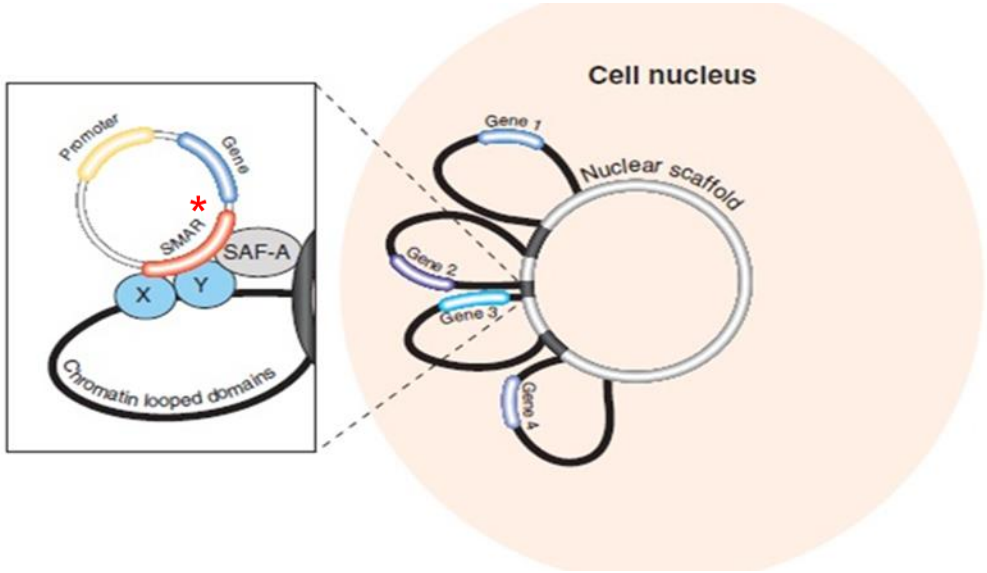

Figure 12. Scaffold Matrix Attachment Region (S/MAR). Interaction mechanism of S/MAR with the nuclear scaffold via the Scaffold Attachment Factor-A (SAF-A), which provides mitotic stability of the plasmid. (After: Argyros et al., 2014)

pEPito

PEPito is a member of the PEPI-1 family and an S/MAR-based vector The pEPito vector is derived from the pEPI-1 plasmid which is a non-viral episomal expression vector for mammalian cells. pEPito is a great candidate for gene therapy as it has high efficacy both in vitro and in vivo (Figure 13). However, some research has shown that it hasn't had an impressive performance in some mammalian cells (Haase et al., 2010).

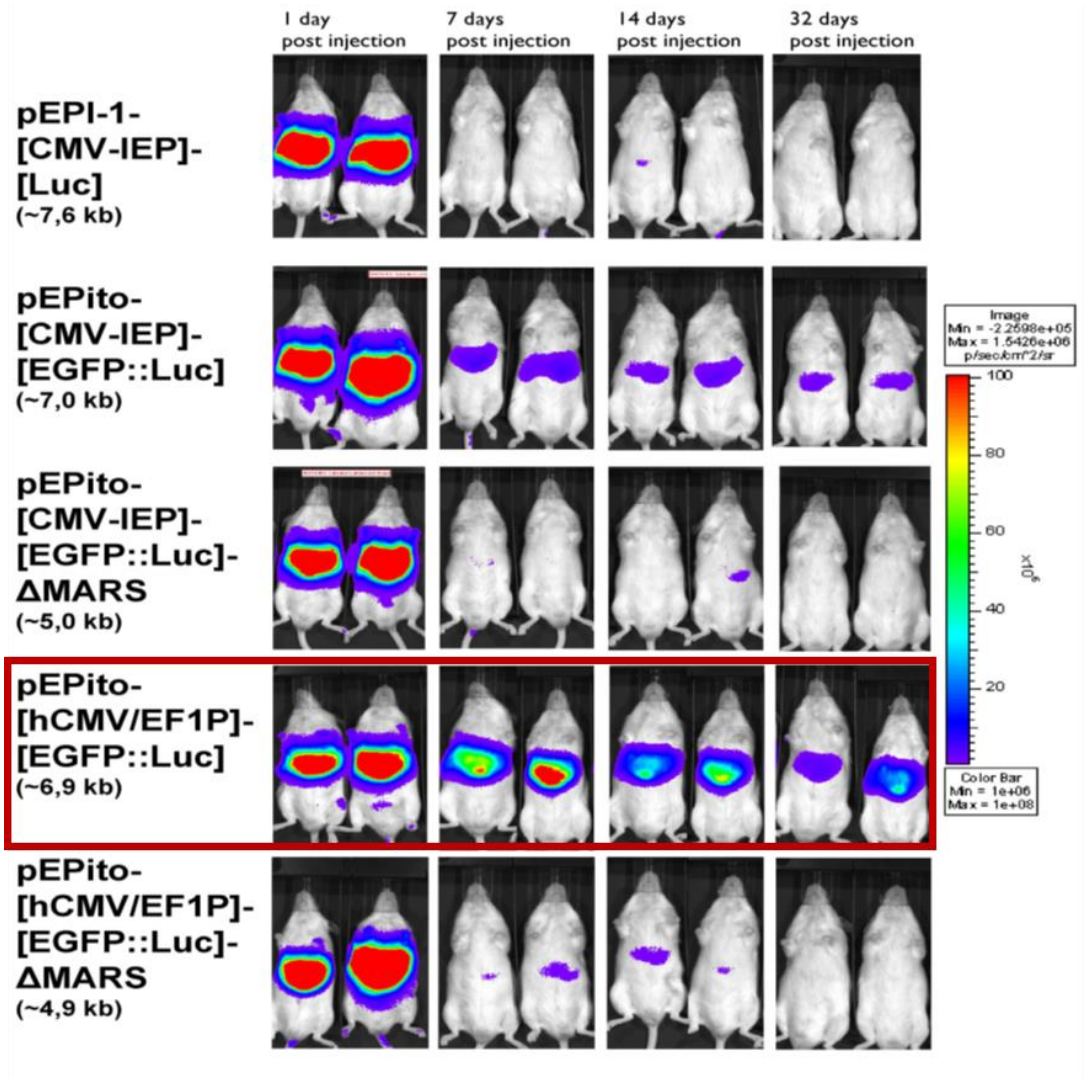

Figure 13. Expression comparison of pEPI-based vectors. pEPito shows higher efficiency compared to other members of the pEPI family when injected into mouse hind-muscles. (After: Haase et al., 2010)

Desmin Promoter

Previous studies have shown that a muscle specific promoter called Desmin (DES), had a more reproducible and tissue-specific transgene expression profile compared to other muscle-specific promoters like that of creatine kinase (CKM). Desmin has also been shown to be a superior promoter, because it increased the gene expression 6-8 times, compared to CKM (Haase et al., 2010). Hence, including a muscle-specific promoter like DES, would be an important step to the optimization of a vector for muscle gene therapy.

Ubiquitous Chromatin Element (UCOE)

The Ubiquitous Chromatin Element (UCOE) is derived from the human β-interferon gene cluster. UCOE is a1.5 Kb methylation-free CpG island sequence (Talbot et al., 2009) which is found upstream of house-keeping genes. The use of UCOE upstream of a promoter will most likely prolong gene expression and lead to more stable expression in muscle cells by preventing gene silencing.

Hypotheses

I hypothesize that the duration of expression of a reporter gene (GFP), in an S/MAR-based plasmid will be longer than in a plasmid lacking an S/MAR element in cultured muscle cells. Second, a plasmid with DES and UCOE, will express the GFP reporter gene, longer than a plasmid vector that does not include these elements in cultured muscle cells.

CHAPTER 2: MATERIALS AND METHODS

Materials

The Desmin promoter (cloned into the MA884 lentiviral vector) and the UCOE + Desmin promoter (cloned into the MA885), was a gift from Dr. Antoniou (King's College London School of Medicine). The pAcGFP1-C1 vector was purchased from Clontech (Mountain View, CA). The pEPIto mammalian expression vector was purchased from the Plasmid Factory, Bielefeld, Germany (lot #PF1164-100615, reference #TUC-15962). The One Shot® Dh5-alpha Chemically Competent Cells were purchased from Invitrogen (Carlsbad, CA). The Pure Link TM Quick plasmid Miniprep kit was purchased from Invitrogen (Grand Island, NY). The C2C12 Murine Myoblasts were a gift from Courtney Young at UCLA. Fetal Bovine Serum (FBS) and Donor Horse Serum was purchased from Atlanta Biologicals (Flowery Branch, GA). DMEM (1.0 g/L Glucose, w/o L-Glutamine, Product code: 12-707F, Lot# 0000515663) and DMEM (4.5 g/L Glucose w/o L-Glutamine, Product code: 12-614Q, Lot# 0000478723) was purchased from Lonza (Walkersville, MD). Opti-MEM® Reduced Serum Media (GIBCO), 0.25% Trypsin-EDTA (GIBCO) Lipofectamine® LTX and Plus™ Reagent (Cat no.A12621) were purchased from Invitrogen (Grand Island, NY). Forward and reverse primers were purchased from Integrated DNA Technology (IDT) (Coralville, Iowa). dNTP (10 mM), LongAmp® Taq DNA Polymerase (M0323S) and T4 DNA Ligase, were purchased from New England Bio Labs (Ipswich, MA). MgCl2 (25 mM) and Shrimp Alkaline Phosphatase (SAP) were purchased from Roche. DreamTaq PCR Master Mix (2X) containing DreamTaq DNA polymerase, 2X DreamTaq buffer, dNTP 0.4 mM each, and 4 mM MgCl2 was purchased

from Thermo Fisher Scientific. The Leica Inverted Microscope (DMI4000 B) was kindly provided by Dr. Kelber (California State University, Northridge).

Isolation of the Desmin Promoter

The Desmin Promoter was isolated from the MA884 lentiviral vector. Specific forward and reverse primers were designed to contain Nhe1 and BspH1 restriction sites at their 5' ends, compatible with the (digested) pAcGFP1-C1 and pEPito vectors (Table 1). PCR was then performed to amplify the ~1.8Kb Desmin promoter from the MA884 lentiviral vector.

Fragment	**Sequence**	**Length**	**Melting Temperature**	**GC%**
Forward primer	5' TAACT**TCATGA**CTCGAGGGC TGGATAAGAA 3'	30bp	60.3 °C	43.3 %
Reverse primer	5' TAATA**GCTAGC**GCCGGCCGG AGAG 3'	24bp	64.3 °C	62.5 %

Table 1: Desmin forward and reverse primer sequences.

The PCR reagents were added in a PCR tube in the following order: 8.7 ul of Nano-pure water, 1 ul of 25 mM MgCl2, 12.5 ul of DreamTaq PCR Master Mix (2X), 1 ul of Forward Primer (5 pmoles/ ul), 1 ul of Reverse Primer (5 pmoles/ ul) and 0.8 ul of pMA884

Vector (35 ng/ul) were added to each PCR tube see (Table 2), and placed into the thermocycler with specific PCR conditions seen in Table 3.

Reagents	**Volume (ul)**
Nano-pure H_2O	8.7 ul
25 mM MgCl2	1 ul
DreamTaq PCR Master Mix (2X)	12.5 ul
Forward Primer (5 pmoles)	1 ul
Reverse Primer (5 pmoles)	1 ul
pMA884 Vector (35 ng/ul)	1 ul
Total	25 ul

Table 2: PCR reaction mixture for amplifying Desmin.

There was also a negative control that included all of the PCR reagents above, except for the template DNA (MA884 vector). 1 ul of nano-pure water was used instead. After confirmation of successful amplification of the Desmin promoter, a restriction digestion with BspH1 and Nhe1 was performed to produce compatible overhangs. These overhangs were compatible with the vectors.

Steps	Temperature (°C)	Time	Cycle
Initial Denaturation	95	5 Minutes	1
Denaturation	95	35 Seconds	40
Annealing	57	35 Seconds	
Extension	72	95 Seconds	
Final Extension	72	8 Minutes	1

Table 3: PCR conditions for amplifying Desmin.

0.8% Agarose Gel Electrophoresis

After the first PCR reaction was completed, 2 ul of 1 Kb Plus Ladder (Thermo Fisher) was mixed with 2 ul of 6X loading dye (Fermentas) and was loaded (lane 1 Fig.14a. and lane 4 of Fig. 14b.). 10 ul of the sample was mixed with 2 ul of 6X loading dye (Fermentas) and was loaded into lane 4 and 5. Electrophoresis of a 0.8% agarose gel was ran for one hour and 20 minutes at 90 volts. The 1 kb Plus DNA Ladder (Thermo Fisher) was used as a reference to estimate the size of electrophoresis bands. The gel was stained with ethidium bromide (EtBr) and imaged under UV light.

Digestion of Desmin

The amplified Desmin was digested with Nhe1 and BspH1 restriction enzymes. 2.5 ul of ethanol precipitated Desmin amplicon (~700ng/ul) and 2.5 ul of 10X CutSmart Buffer® (NEB) were added to 17.8 ul of nano-pure water in a 0.2 ul Eppendorf tube. 1.1 ul of BspH1 (10,000 units/ml) (NEB) and 1.1 ul of Nhe1 (10,000 units/ml) were added to

the same tube. The total reaction volume was 25 ul (Table 4). The reaction was mixed and centrifuged. Then the reaction was allowed to proceed at 37°C for 17 hours in the thermocycler.

Reagents	Volume (ul)
Nano-pure water	17.8 ul
Desmin amplicon (~700 ng/ul)	2.5 ul
10X CutSmart buffer (NEB)	2.5 ul
10,000 units/ml BspH1 (NEB)	1.1 ul
10,000 units/ml Nhe1 (NEB)	1.1 ul
Total	25 ul

Table 4. Digestion reaction of Desmin.

Isolation of the Desmin + UCOE Sequence

The Desmin + UCOE sequence was isolated from the MA885 lentiviral vector. Specific forward and reverse primers were designed to contain Nhe1 and BspH1 restriction sites at their 5' ends, compatible with the (digested) pAcGFP1-C1 and pEPito vectors (Table 5). PCR was then performed to amplify the ~3.3 Kb Desmin + UCOE sequence from the MA885 vector.

Fragment	Sequence	Length	Melting Temperature	GC %
Forward primer	5'TAACT**TCATGA**GAGACGCCGT GGCC 3'	25 bp	60.3 °C	43.3 %
Reverse primer	5'TAATA**GCTAGC**GCCGGCCGG AGAG 3'	24 bp	62.6 °C	56 %

Table 5. : Desmin + UCOE primer sequences.

PCR was performed multiple times using different Taq polymerases including, Standard Taq DNA Polymerase (1/50), DreamTaq (Thermo Fisher Scientific), Platinum Taq High Fidelity (Invitrogen) , Q5 High Fidelity DNA Polymerase (NEB) and LongAmp polymerase (NEB). LongAmp Taq DNA Polymerase was the only polymerase that resulted in amplification which I will describe here. Also a magnesium titration was done in order to determine the optimal magnesium concentration for the primers and producing the proper amplicon.

The PCR reagents were added in an Eppendorf tube in the following order: 8.25, 7.25 and 6.25 ul of nano-pure water (adjusted according to MgCl2 concentration respectively), 0, 1 and 2 ul of 25 mM MgCl2 (titration for 2, 3 and 4 mM of MgCl2. The amount of water was adjusted accordingly to reach a 20 ul total reaction volume), 5 ul of LongAmp buffer (5X), 0.75 ul of 10 mM dNTP, 2 ul of Forward Primer (5 pmoles/ul), 2 ul of Reverse Primer (5 pmoles/ ul) and 0.5 ul of MA885 Vector (35 ng/ul) were added to each Eppendorf tube (Table 6), and placed into the thermocycler with specific PCR conditions (Table 7).

There was also a negative control that included all of the PCR reagents above, except the template DNA (MA885 vector). 1 ul of nano-pure water was used instead.

Reagents	**Volume (ul)**
Nano-pure H_2O	8.25, 7.25 & 6.25 ul
25 mM MgCl2	0, 1 & 2 ul (2, 3 and 4 mM total MgCl2 conc.)
LongAmp Buffer (5X)	5 ul
dNTP (10 mM)	0.75 ul
Forward Primer (5 pmoles)	2 ul
Reverse Primer (5 pmoles)	2 ul
MA885 Vector (20-30 ng/ul)	1 ul
LongAmp Taq Polymerase (NEB)	1 ul
Total	20 ul

Table 6: PCR reaction mixture for amplifying the Desmin + UCOE sequence.

Steps	Temperature (°C)	Time	Cycle
Initial Denaturation	94	2 minutes	1
Denaturation	94	30 seconds	40
Annealing	58 & 60	60 seconds	
Extension	68	3.5 minutes	
Final Extension	68	12 minutes	1

Table 7: PCR conditions for amplifying Desmin + UCOE.

0.8% Agarose Gel Electrophoresis

After the PCR reactions were completed, 2 ul of 1 Kb Plus Ladder (Thermo Fisher) was mixed with 2 ul of 6X loading dye (Fermentas) and was loaded (lane 1 Fig.15). 10 ul of contents of each PCR tube were mixed with 2 ul of 6X loading dye (Fermentas) and were loaded into lanes 3-8. Lane 2 included the negative control with no template and lane 9 included 9 ul of MA885 template mixed with 2 ul 6X loading dye (Fermentas). A 0.8% Agarose gel was electrophoresed for one hour and 30 minutes at 85 volts. The 1 kb Plus DNA Ladder (Thermo Fisher) was used as a reference to estimate the size of electrophoresis bands. The gel was stained with ethidium bromide (EtBr) and imaged under UV light (see figure 15).

Digestion and DNA gel extraction of the pAcGFP1-C1 Vector

The pAcGFP1-C1 vector was digested with Pci1 and Nhe1 restriction enzymes. 12 ul of pAcGFP1-C1 (115 ng/ul), and 5 ul of 10X buffer 2.1 were added to 30.2 ul water in a 0.2 ul Eppendorf tube. 1.4 ul of; Pci1 (10,000 units/mL) (NEB) and 1.4 ul of Nhe1

(10,000 units/mL) (NEB) were added to the same tube. The total reaction volume was 50 ul (Table 8). The reaction was mixed and centrifuged. Then the reaction was allowed to proceed at 37°C for 17 hours in a thermocycler.

1 ul of SAP was added after the reaction was completed to remove the 5' phosphate groups. The reaction was incubated in a thermocycler at 37°C for 1 hour and denatured at 65°C for 20 minutes to deactivate the SAP. 2.5 ul of 1 kb Plus Ladder was loaded (Figure 16, lane 1 and 2). 3 ul of the uncut vector was mixed with 2 ul of 6X loading dye (Fermentas) and was loaded into lane 3 (Figure 16b). 15 ul of the sample was mixed with 2 ul of 6X loading dye (Fermentas) and placed into lane 4 (Figure 16a). Samples were electrophoresed for 1.5 hours at 85 volts on a 0.8% agarose gel, in order to confirm that the pAcGFP1-C1 vector was completely digested. The gel was stained in EtBr for 7 minutes, destained in water, and recorded using a gel doc system.

Reagents	**Volume (ul)**
Nano-pure water	30.2 ul
pAcGFP1-C1 vector (115 ng/ul)	12 ul
10X buffer 2.1 (NEB)	5 ul
10,000 units/mL Pci1 (NEB)	1.4 ul
10,000 units/mL Nhe1 (NEB)	1.4 ul
Total	50 ul

Table 8: The digestion reaction of pAcGFP1-C1.

After the gel was electrophoresed, a slice of the gel that contains the 4100 bp pAcGFP1-C1 backbone, was placed in an Eppendorf tube, spun down and DNA gel extraction was performed with a QIAquick Gel Extraction Kit (Qiagen) (as in http://www.indiana.edu/~lchenlab/protocol_files/agarose_gel_extraction.pdf). The gel-purified pAcGFP1-C1 was eluted in TE and stored at -20° C for future ligation.

Double Digestion of the pEPito Vector

The pEPito vector was digested with Pci1 and Nhe1 restriction enzymes. 2.4 ul of pEPito (100 ug/ul), and 5 ul of 10X buffer 2.1 were added to 39.6 ul nano-pure water in a 0.2 ul Eppendorf tube. 1.5 ul of, Pci1 (10,000 units/mL) (NEB) and 1.5 ul of Nhe1 (10,000 units/mL) (NEB) were added to the same tube. The total reaction volume was 50 ul (Table 9). The reaction was mixed, centrifuged and was allowed to proceed at 37°C for 17 hours in the thermocycler.

1 ul of SAP was added after the reaction was completed to remove the 5' phosphate groups that prevent the vector from reattaching to the digested nucleotides. The reaction was incubated in a thermocycler at 37°C for 1 hour and denatured at 85°C for 20 minutes to deactivate the SAP. 2.5 ul of 1 kb Plus Ladder was loaded in lane 1 (Figure 17). 2 ul of the uncut vector was mixed with 2 ul of 6x loading dye (Fermentas) and was loaded into lane 4. 20 ul of the sample was mixed with 3 ul of 6X loading dye (Fermentas) and placed into lanes 2 and 3. Samples were electrophoresed for 1.5 hours at 85 volts on a 0.8% agarose gel, in order to confirm that the pEPito vector was completely digested. The gel was stained in EtBr for 7 minutes, destained in water, and recorded using a gel doc system.

Reagents	Volume (ul)
Nano-pure water	39.6 ul
pEPito vector (100 ng/ul)	2.4 ul
10X buffer 2.1 (NEB)	5 ul
10,000 units/mL Pci1 (NEB)	1.5 ul
10,000 units/mL Nhe1 (NEB)	1.5 ul
Total	50 ul

Table 9: pEPito digestion reaction.

pEPito Transformation and Purification

Transformation of pEPito was performed with multiple E-coli hosts in order to obtain more copies of the plasmid. Transformation was performed with DH5-α competent cells, which did not result in any colonies. Transformation with K12 SM10 (λ pir) competent cells was also performed (as in https://www.addgene.org/plasmid-protocols/bacterial-transformation/), which also did not result in any colonies. Transformation of pEPito with HM 101 and DB3.1 competent cells were also attempted (Addgene protocols, bacterial transformation), which resulted in some colonies in Luria-Bertani (LB) agar plates with 100 ug/mL ampicillin (Figure 18).

The colonies that resulted from the transformation of DB3.1 with 40 ng of pEPito, were grown overnight at 37°C and shaken at 250 RPM. Plasmid contained in candidate transformants was isolated and purified using Pure Link ™ Quick plasmid Miniprep kit

(Invitrogen). The overnight LB-cultures were transferred into a microcentrifuge tube and were centrifuged at 14000 RPM for 1 minute. The supernatant was removed without disturbing the pellet. 250 ul of resuspension buffer with RNase was added to the cell pellet, and the pellet was re-suspended. Then, 250 ul of lysis buffer was added to the cell pellet and was gently mixed by inverting the capped tube 7 times. The tube was incubated for 5 minutes at room temperature. 350 ul of precipitation buffer was added to the tube and was mixed immediately by inverting the tube. Then, the lysate was centrifuged at 14000 RPM for 10 minutes. Afterwards, the supernatant was loaded into a spin column in a 2 ml wash tube, and was centrifuged for 1 minute at 12000 RPM. The flow through was discarded, and the column was placed back into the wash tube. 500 ul of wash buffer with ethanol was added to the column, and the precipitate was incubated for 1 minute at room temperature. Then, the column was centrifuged for 1 minute at 12000 RPM. The flow through was discarded, and the column was placed back into the wash tube. 700 ul of wash buffer with ethanol were added to the column and was centrifuged at 14000 RPM for 1 minute. The flow through was discarded, and the column was placed into the wash tube. The column was centrifuged for 3 minutes at 14000 RPM. The wash tube was discarded with the flow-through. The spin column was placed in a clean 1.5 ml recovery tube and 50 ul of pre-warmed TE Buffer were added to the center of the column. The column was incubated for 5 minutes at room temperature and then centrifuged at 14000 RPM for 2 minutes. The column was discarded and the DNA concentration was measured using the Nano-Drop 2000 Spectrophotometer (Thermo Scientific). The purified plasmids were then loaded in to a 0.8% agarose gel and electrophoresed to determine the size of the transformed plasmid DNA (Figure 19).

Sub-Cloning of Desmin into the pAcGFP1-C1 Backbone

After the Desmin promoter was isolated from MA884, digested and the pAcGFP1-C1 CMV promoter was excised, was linearized, ligation was performed to assemble the construct. The ligation was performed in the following order: 0.3 ul of nuclease free water, 14 ul of the digested pAcGFP1-C1 vector (5.6 ng/ul), 2.7 ul of the digested Desmin promoter (87 ng/ul), 2 ul of 10X DNA Ligase Buffer (NEB), and 1 ul of T4 DNA Ligase (NEB) were added into the Eppendorf tube. Therefore, the total ligation reaction was 20 ul. The 0.2 ml Eppendorf tube was vortexed and centrifuged gently. In the ligation process, there was a negative control which contained nano-pure water instead of the DES promoter. The tubes were then incubated in a thermocycler at 16°C overnight.

Reagents	**Volume (ul)**
Nuclease-free water	0.3 ul
Digested pAcGFP1-C1 (5.6 ng/ul)	14 ul
Insert: digested Desmin promoter (87ng/ul)	2.7 ul
10X DNA Ligase Buffer (NEB)	2 ul
T4 DNA Ligase (NEB)	1 ul
Total	20 ul

Table 10. Ligation reaction for pAcGFP1-DES-C1.

Transformation of pAcGFP1-C1

Transformation was performed for confirmation of a successful ligation. Transformation was done by pipetting 100 ul of MAX Efficiency® DH5α™ Competent Cells (Invitrogen) into 15 mL falcon tube that was on ice. 5 ul of the ligation reaction was immediately added and the mixture was incubated on ice for 30 minutes. The tube was heat-shocked for 30 seconds at 42°C, and the mixture was put onto ice for two minutes. 250 ul of pre-warmed Super Optimal Broth (S.O.C.) was added to the 15 ml falcon tube. The mixture was set to shake at 37°C at 225 RPM for 60 minutes. The negative control tube contained the same components, but the ligation reaction was not added. 350 ul of the transformation contents were plated on Luria-Bertani (LB) agar plates with 50 ug/mL Kanamycin. The plates incubated overnight at 37°C (Figure 20).

Isolation and Purification of pAcGFP1-DES-C1

11 colonies were chosen and were shaken overnight at 37°C and 250 RPM. The construct was isolated and purified using Pure Link ™ Quick plasmid Miniprep kit (Invitrogen). The overnight LB-cultures were transferred into an Eppendorf tube and were centrifuged at 3000 RPM for 5 minutes. The supernatant was removed without disturbing the pellet. 250 ul of Resuspension Buffer with RNase was added to the cell pellet, and the pellet was resuspended. Then, 250 ul of Lysis Buffer was added to the cell pellet and was gently mixed by inverting the capped tube 7 times. The tube was incubated for 5 minutes at room temperature. Three hundred and fifty ul of Precipitation Buffer was added to the tube and was mixed immediately by inverting the tube. Then, the lysate was centrifuged at 14000 RPM for 10 minutes. After that, the supernatant was loaded into a Spin Column in

a 2 ml Wash Tube, and was centrifuged for 1 minute at 12000 RPM. The flow through was discarded, and the column was placed back into the Wash Tube. Five hundred ul of Wash Buffer with ethanol was added to the column, and the sample was incubated for 1 minute at room temperature. Then, the column was centrifuged for 1 minute at 12000 RPM. The flow through was discarded, and the column was placed back into the Wash Tube. 700 ul of Wash Buffer with ethanol was added to the column and was centrifuged at 14000 RPM for 1 minute. The flow through was discarded, and the column was placed back into the Wash Tube. The column was centrifuged for 1 minute at 12000 RPM. The Wash Tube was discarded with the flow-through. The Spin Column was placed in a clean 1.5 ml recovery tube and 75 ul of preheated TE Buffer was added to the center of the column. The column was incubated for 5 minutes at room temperature and then centrifuged at 12000 RPM for 2 minutes. The column was discarded and the DNA concentration was measured using the Nano-Drop 2000 Spectrophotometer (Thermo Scientific). The purified pAcGFP1-DES-C1 was stored at -20 °C.

Digestion and Confirmation of pAcGFP1-DES-C1

PAcGFP1-DES-C1 was digested with Age1 to confirm it included the Desmin insert. The restriction reaction reagents were added in the following order: 19.5 ul of Nano-pure water, 2 ul of pAcGFP1-DES-C1 (800 ng/ul), 2.5 ul of 10X Buffer 1.1 (NEB), and 1 ul of Age1 (NEB) were added to the Eppendorf tube. The total reaction volume was 25 ul and was mixed by vortexing, and collected by centrifugation. The digest was allowed to proceed 37°C overnight. One ul of Shrimp Alkaline Phosphatase (SAP) was added after the reaction was complete to remove the 5’ phosphate groups in order to protect the vector from re-circularizing. The reaction was incubated in a thermocycler at 37°C for 1 hour and

denatured at 80°C for 20 minutes to inactivate the SAP. 2.5 ul of 1 kb Plus Ladder was mixed with 2 ul of 6X loading dye (Fermentas) and was loaded (Figure 22a, lane 1). 3 ul of the digested pAcGFP1-DES-C1 was mixed with 2 ul of 6X loading dye (Fermentas) and was loaded in lanes 2-9. The 0.8% agarose gel was run for 1.5 hours at 85 volts to confirm that the pAcGFP1-DES-C1 was completely digested. The agarose gel was stained with EtBr, rinsed in water, and recorded with the gel doc system.

The pAcGFP1-DES-C1 was also double digested with Nhe1, BahH1 and Eag1 restriction enzymes, in three tubes with each pair of enzymes. The restriction reagents were added in the following order: 17.5 ul of nano-pure water, 3 ul of pAcGFP1-DES-C1 (275 ng/ul), 2.5 ul of 10X buffer 2.1 (NEB), 1 ul of BamHI (NEB) and 1ul of Nhe1 (NEB) were added to microcentrifuge tube 1. The same reagents were added to tube 2, except Eag1 (NEB) was substituted for Nhe1. The reagents of tube 3, were the same as above with 1ul of Nhe1 and 1 ul of Eag1. The total reaction volume was 25 ul and was mixed by vortexing, and collected by centrifugation. The digest was allowed to proceed at 37°C overnight. 2.5 ul of 1 kb Plus Ladder were mixed with 2 ul of 6X loading dye (Fermentas) and were loaded in to a 0.8% agarose gel (Figure 22b, lane 1). 10 ul of the double digested pAcGFP1-DES-C1 was mixed with 2 ul of 6X loading dye (Fermentas) and was loaded in lanes 2-4 (Figure 22b.). The gel was run for 1.5 hours at 85 volts to confirm that the pAcGFP1-DES-C1 was completely digested. The agarose gel was stained with EtBr, rinsed in water, and recorded with the gel doc system.

Muscle Cell Culture

C2C12 Murine myoblasts were cultured with growth media and were incubated at 37°C in 5% CO_2. This growth media contained DMEM (with 4.5 g/L Glucose) (Lonza), 2mM L-glutamine, 1% penicillin-streptomycin (Invitrogen) and 20% Fetal Bovine Serum (FBS) (Atlanta Biologicals) and no sodium pyruvate. The C2C12 cells arrived in a vial. After being thawed, they were transferred to a T75 flask with growth media and were incubated at 37°C in 5% CO_2 for one day to relax the cells. The following day, the cells were observed under an inverted microscope to determine the degree of the confluency. The spent medium was aspirated from cells when they reached 90 % confluence. The cells were washed with 3 ml of pre-warmed 0.25% Trypsin-EDTA (GIBCO) to clean the cells from FBS. Immediately after adding Trypsin-EDTA, it was aspirated. 4 mL of pre-warmed Trypsin-EDTA was added to the flask, and was incubated at 37°C in 5% CO2 for 10 minutes. The bottom of the flask was carefully tapped. The adherent cells started to detach from the bottom surface. The flask was also observed under an inverted microscope to confirm that the cells had detached from the flask. 8 ml of growth media was added to the flask to deactivate the Trypsin-EDTA. The contents of the flask were transferred into a 15 ml falcon tube and centrifuged at 1200 RPM for 5 minutes. The pellet was resuspended in 5 ml of fresh growth media. Then, 1 ml the cells were added to two T75 flasks that each contained 19 ml growth medium. The flask were mixed in a figure 8 motion and were incubated at 37°C in 5% CO_2. The two T75 flasks were visualized under an inverted microscope to determine the degree of the confluence. The growth media was replaced every other day with fresh growth media to feed the cells. The cells were passaged 4 times as mentioned above. After the fourth passage, one of the T75 flasks that was 90% confluent

was trypsinized as described above. 10 ml of growth media was added to the pellet of cells and the pellet was resuspended thoroughly. 20 ul of the cells were transferred into a microcentrifuge tube and 20 ul of trypan blue was added to the cells and mixed carefully and 20 ul were transferred to a hemocytometer and were counted. A total of $4x10^5$ cells were plated in each well of a 6 well-plate for differentiation and later transfection. The plated cells were visualized after one day, using an inverted microscope. After the cells reached 100% confluence, the growth media was then aspirated and differentiation media was substituted. The differentiation media included all the growth media reagents, however the 20% Fetal Bovine Serum (FBS) was replaced with 2% Horse Serum (Atlanta Biologicals) and DMEM with 1 g/L glucose was used instead of 4 g/L. Lowering the serum concentration triggers the cells to differentiate by allowing the myoblasts to attach to one another, allowing multinucleated myotubes to form quickly. The 6 well plates were incubated at 37°C in 5% CO2 and viewed under the inverted microscope to observe differentiation. The differentiation media was replaced every day. Differentiation was noticeable under the inverted microscopes at day 2 post-differentiation (Figure 26). Myotubes were formed at day 3 post-differentiation (Figure 27).

Transient Transfection

Transient transfection was performed, at day 2 and day 3 post differentiation, using Lipofectamine® LTX and Plus™ Reagent and Opti-MEM® I Reduced Serum to transfect the myotubes with pAcGFP1-DES-C1, pAcGFP1-C1 and pEPito. The myotubes were grown in 4 plates that contained 6 wells, as there were 3 groups that the cells were transfected with, triplicates for each group, plus a negative control. For the transfection of each 6-well plate, transfection reagents were added to the tube in the following order: 15

ug of DNA (for each of the constructs) was diluted in 750 ul of Opti-MEM® I Reduced Serum along with 15 ul of Plus Reagent and were mixed very carefully. In a new tube, 60 ul of Lipofectamine® LTX was diluted in 750 ul of Opti-MEM® I Reduced Serum and were carefully mixed. The diluted DNA (of each construct) and Lipofectamine® LTX and Plus™ Reagent, were mixed together and were incubated at room temperature for 5 minutes. After the incubation period, the diluted DNA was added to diluted Lipofectamine® LTX Reagent. Then, the growth medium in the 6 wells was aspirated and replaced with 4 ml of differentiation medium without antibiotics. 250 ul of the DNA-lipid complex was added each well except the negative control's well and was mixed carefully by rocking the plates. Then, the cells were incubated at 37°C in 5% CO_2 and imaged at 3 different time points.

Fluorescence Microscopy

Fluorescence Microscopy was performed using the Leica automated invert scope. The cells that were transfected on day 3 of differentiation, were transfected with the following constructs; pEPito, pAcGFP1-C1 and pAcGFP1-DES-C1. A negative control was also used. The cells that were transfected on day 2 of differentiation were only transfected with pAcGFP1-C1, along with a negative control. The cells that were transfected on day 3 of differentiation were imaged at 3 different time points: day 2 post transfection (PT), day 3 PT and day 8 PT. Cells that were transfected on day 2 of differentiation were imaged at day 2 PT, day 3 PT and day 6 PT. Both phase contrast and fluorescent micrographs were captured and recorded for all the groups.

CHAPTER 3: RESULTS

Amplifying the Desmin Promoter from the MA884 Vector

The Desmin promoter was amplified by PCR from the MA884 vector, using forward and reverse primers with BspH1 and Nhe1 restriction sites at the 5' ends. Electrophoresis of a 0.8% agarose gel shows the 1800 bp long Desmin amplicon (Figure 14). Figure 14a, lane 1 and figure 14b, lane 4, show the successful separation of the 1 kb Plus Ladder, used for determination of sizes of bands in neighboring lanes. The other lanes, represent an 1800 bp band of Desmin- amplified and from the MA884 construct.

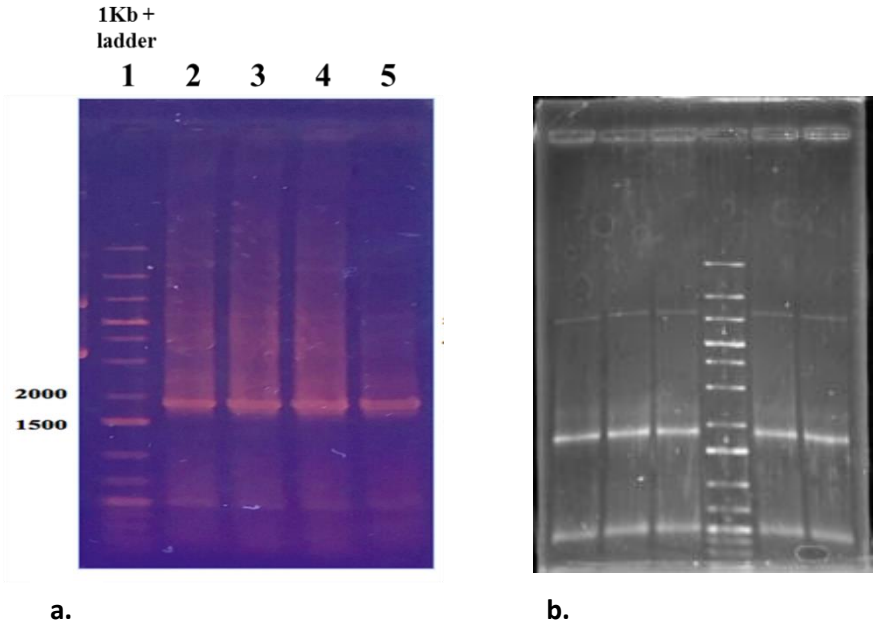

Figure 14. The successful amplification of Desmin. Figure **a.** Successful amplification of the cloned 1.8 Kb Desmin promoter by PCR in lanes 2-5. Lane 1 shows the successful separation of the 1Kb Plus ladder. Figure **b.** Lanes 1-3 and 5-6 show successful amplification of Desmin, while lane 4 shows separation of the 1Kb Plus ladder.

Amplifying the Desmin and UCOE Sequence from the MA885 Vector

Desmin + UCOE amplification was attempted by PCR, using forward and reverse primers with BspH1 and Nhe1 restriction sites at the 5' ends, from the MA885 vector. 0.8% agarose shows some amplification for the 3300 bp long Desmin and UCOE amplicon in lanes 3 and 5 (Figure 15). Figure 15, lane 1 show the successful separation of the 1 kb Plus Ladder, used for determination of sizes of bands in neighboring lanes. The amplification of the successfully amplified DES+UCOE in lanes 3 and 5 were not sufficient for ligation purposes.

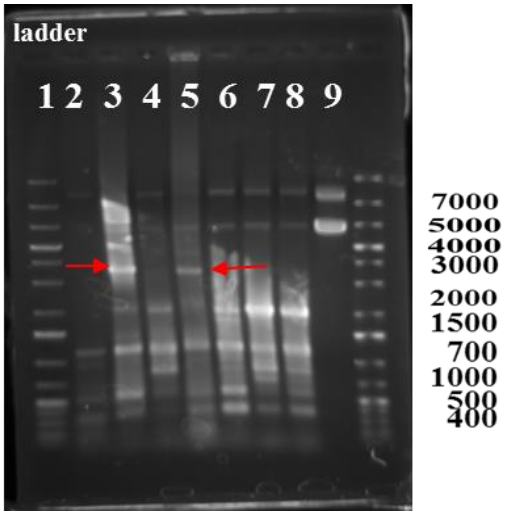

Figure 15. Amplification of the 3.3 Kb DES+UCOE amplicon.

Digestion of the pAcGFP1-C1 Vector

The pAcGFP1-C1 vector was cut with Pci1 and Nhe1 restriction enzymes to allow for CMV promoter excision and to produce compatible overhangs with the DES insert for ligation. Gel electrophoresis confirmed that digestion was complete, as the sizes of the 2 bands of DNA corresponded to the correct size on the ladder (Figure 16a). Lanes 1 and 2 represent the 1 kb Plus Ladder. Lane 4 represents the band of the linearized pAcGFP1-C1

vector backbone at 4100 bp and its excised CMV promoter at 649 bp. Figure 16b shows the uncut pAcGFP1-C1 vector in lane 3. The size of the uncut 4731 bp vector corresponds with the 4000 bp band of the ladder due to the plasmid's conformation (either nicked or supercoiled), causing it to run further on the gel and appearing to be smaller.

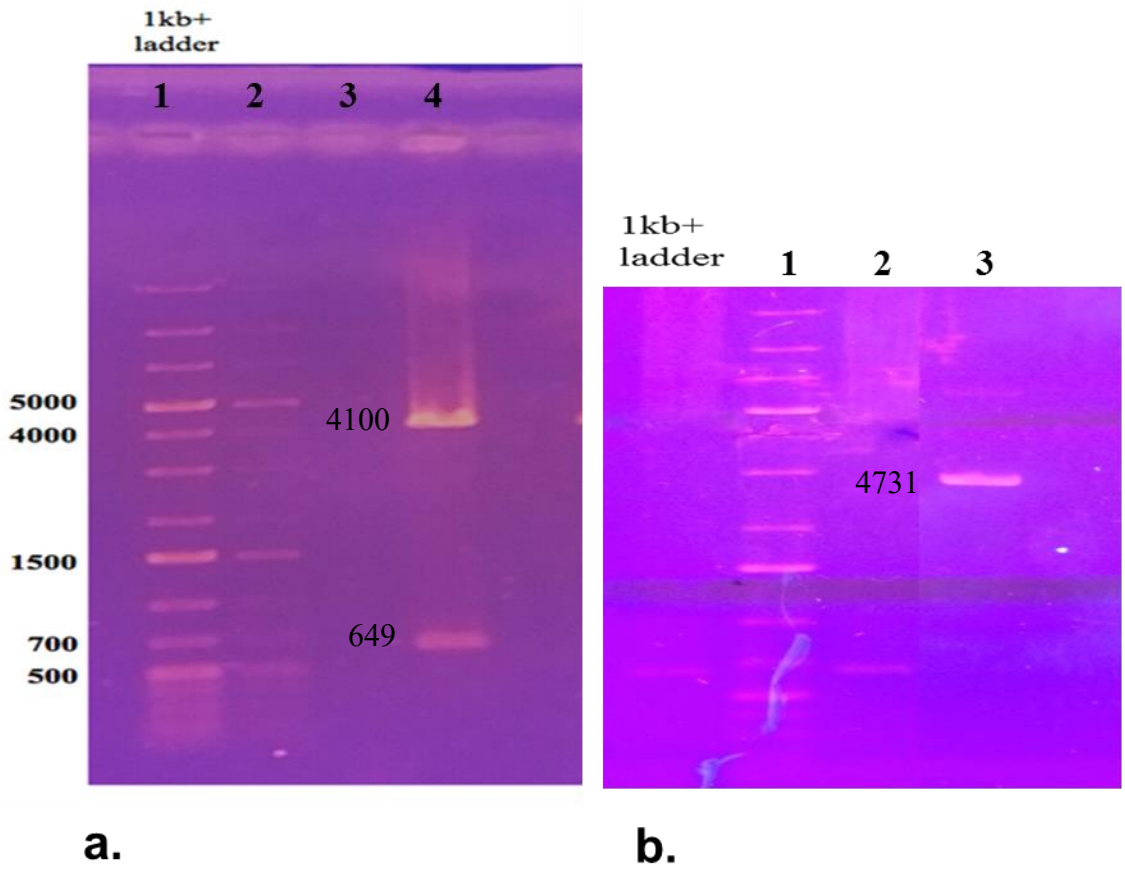

Figure 16: **a.** Lane 1 and 2 represent the separation of 1 kb Plus Ladder. Lane 4 shows the digested pAcGFP1-C1 backbone and excised 649 bp CMV promoter. **b.** Lane 3 shows the band of the 4731 bp uncut plasmid.

Digestion and Transformation of the pEPito Vector

The pEPito vector was cut with Pci1 and Nhe1 restriction enzymes to allow for CMV promoter excision, and for producing compatible overhangs for ligation. Gel electrophoresis confirmed that digestion was complete (Figure 17). Lane 1 represents the 1 kb Plus Ladder. Lanes 2 and 3 represent the 4800 bp pEPito backbone and its excised 553 bp CMV promoter. Lane 4 shows the uncut pEPito vector.

The Desmin insert was ligated with the digested pEPito vector using T4 DNA ligase and transformed into DH5- α competent cells. However, transformation into DH5- α and K12 SM10 (λ pir) strains never yielded any colonies. Transformation of the original pEPito vector in these strains was also not possible. Amplification of pEPito was also attempted in HM101 and DB3.1 E-coli strains, which resulted in some colonies on LB-agar plates containing ampicillin (Figure 18). These colonies were grown overnight and plasmid was purified, and their DNA was loaded onto an agarose gel to be electrophoresed. None of the colonies were of the expected size, and instead they were smaller than that which was predicted for pEPito (Figure 19).

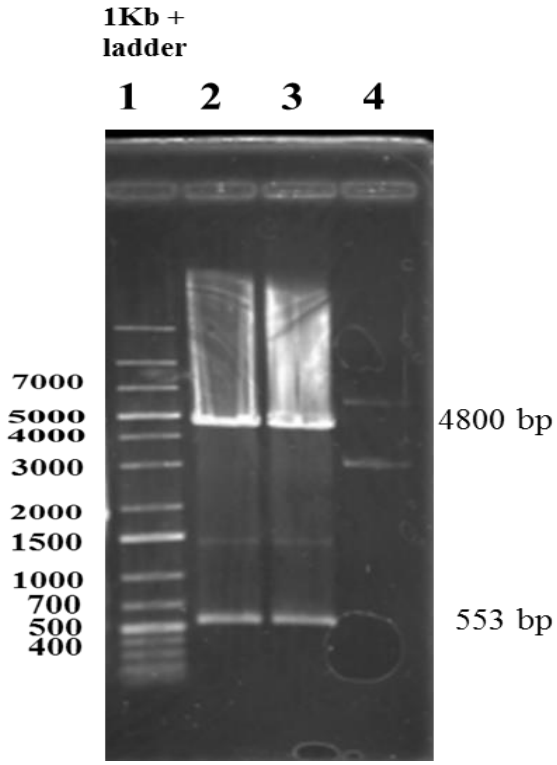

Figure 17. Digestion of pEPito. Lane 1 shows the 1 Kb Plus ladder. Lanes 2 and 3 show the successful digestion of the pEPito vector. The lower band represents the excised 533 bp CMV promoter and the larger band represents the 4800 bp pEPito backbone. Lane 4 shows the uncut pEPito plasmid.

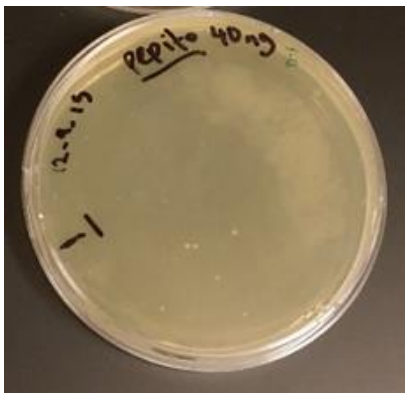

Figure 18. Colonies resulting from the transformation of DB3.1 E-coli competent cells on LB-agar ampicillin plates with pEPito.

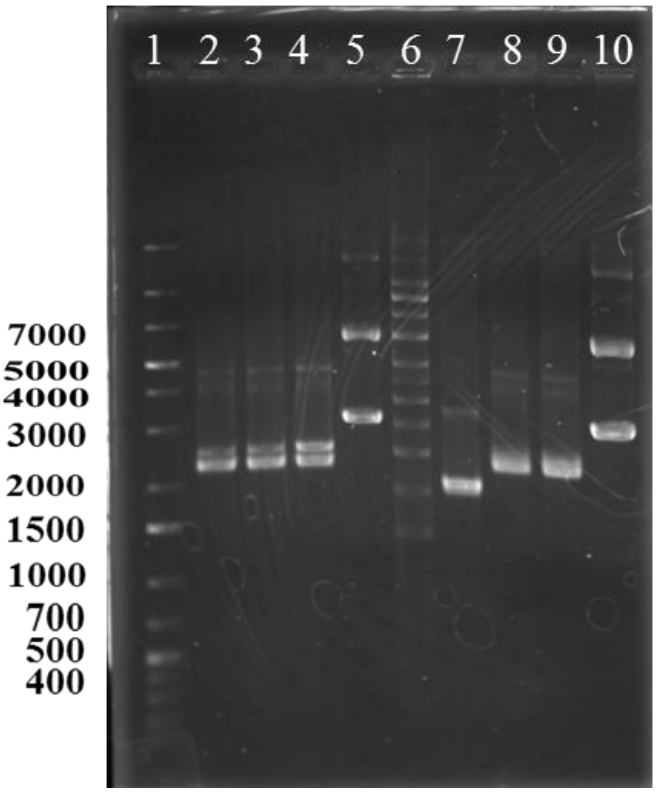

Figure 19. 0.8% agarose gel shows DNA constructs resulting from DNA purification of pEPito transformants in DB3.1 and HM101 strains. Lanes 5 and 10 show the uncut pEPito plasmid. DNA of the transformants are all smaller than pEPito, indicating that they are different in structure and size.

Ligation of Desmin with the pAcGFP1-C1 Backbone and Transformation

The Desmin promoter insert and the digested pAcGFP1-C1 vector were ligated using T4 DNA Ligase (NEB). Transformed cells were screened for insertion of the DES promoter into the pAcGFP1-C1 vector. The LB-agar/kanamycin plate shows many colonies as the result of this transformation (Figure 20).

Figure 20: A photograph of an LB agar plate with kanamycin, with 350 ul of the transformed cells that produced colonies of pAcGFP1-DES-C1.

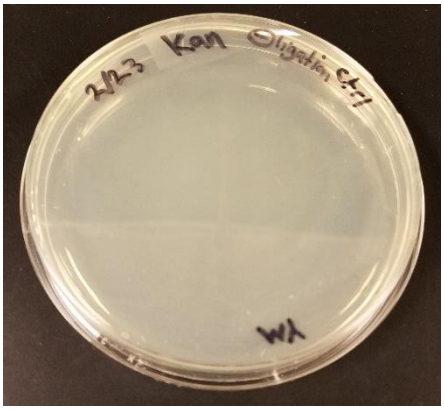

Figure 21: An LB agar plate with Kanamycin from the control transformation of pAcGFP1-C1 with no insert, shows no colonies.

Purification and Confirmation of the DES in the pAcGFP1-C1 vector

11 colonies were grown overnight in LB/kanamycin media and were purified using the Pure Link TM Quick plasmid Miniprep kit (Invitrogen). The DNA from the candidate colonies were digested with AgeI, to show that the pAcGFP1-C1 carried the IDS insert. A 0.8% agarose gel confirmed that the size of the pAcGFP1-DES-C1 candidates (6000 bp) matched the expected construct. (Figure 22a). Lane 1 shows the 1 kb Plus Ladder, which

was used to estimate the size of bands in the neighboring lanes. Lanes 2-9 show the digested linearized pAcGFP1-DES-C1. Lanes 3 and 7 show a slightly larger construct which does not correspond to the expected pAcGFP1-C1 size and is different in structure. The pAcGFP1-C1 constructs were also double digested and electrophoresed on a 0.8% agarose gel (Figure 22b). The construct was digested with the following enzymes; Nhe1 and BamH1 (lane 2, figure 22b), BamH1 and Eag1 (lane 3, figure 22b) and Nhe1 and Eag1 (lane 4, figure 22b). The BamH1 and Eag1 restriction enzymes were on the elderly side and did not share compatible buffers, which resulted in the appearance of faint bands on the agarose gel. The 0.8% agarose gel confirmed that the pAcGFP1-C1 carried the DES promoter.

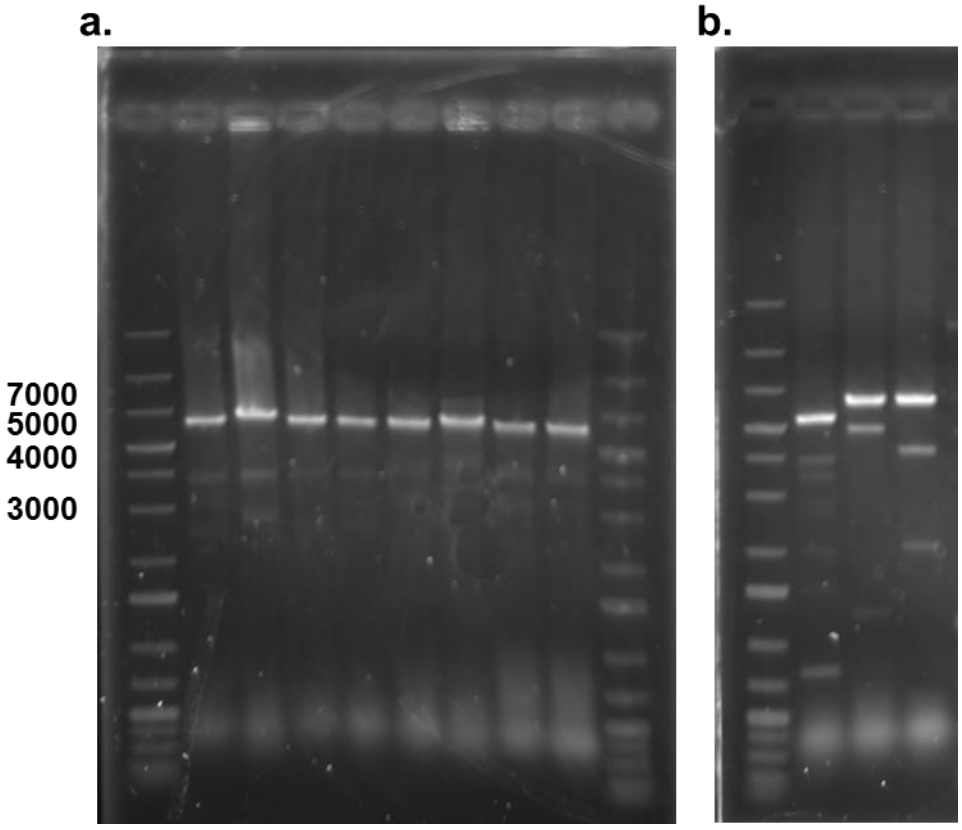

Figure 22: Screening Colonies using restriction digestion and 0.8% agarose. **a.** Plasmid purified ligates of pAcGFP1-DES-C1. Lanes 2, 4,5,6,8 & 9 contain the correct insert at 6000 bp. **b.** Double digestion of pAcGFP1-DES-C1 constructs for confirmation of successful ligation.

Culturing Myotubes from C2C12 Myoblasts

C2C12 Murine myoblast cell culture was positively accomplished. The cells were fed every other day until they reached 90% confluency. After a few passages, the myoblasts were cultured into 6 well-plates. When the myoblasts in the 6 well plates reached 100% confluency (see figure 25), the growth media was replaced with differentiation media which contained 2% horse serum (Atlanta Biologicals) instead of 20% FBS. The cells were maintained in differentiation medium throughout the rest of the project and was replaced with fresh media every day to maintain healthy cells. Formation of myotubes at an early stage started to appear on day 2 post-differentiation (PD) (see figure 26). The myotubes continued to elongate further into myotubes on day 3 post differentiation (see figure 27). The process of complete myotube formation is shown in figures 29 and 30.

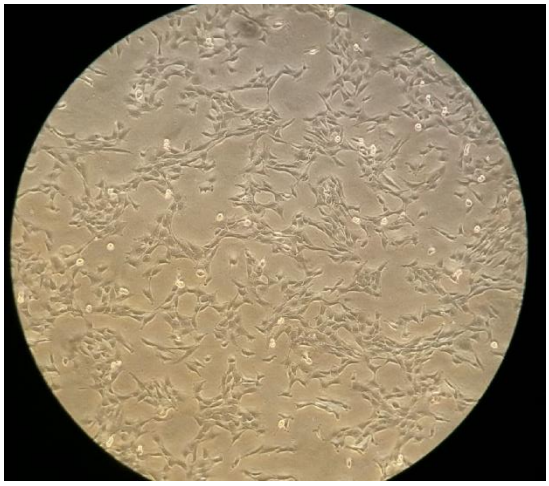

Figure 23: 40% Confluent myoblast cells under an inverted microscope using 100X objective. This figure shows that myoblast cells were positively cultured without any contamination.

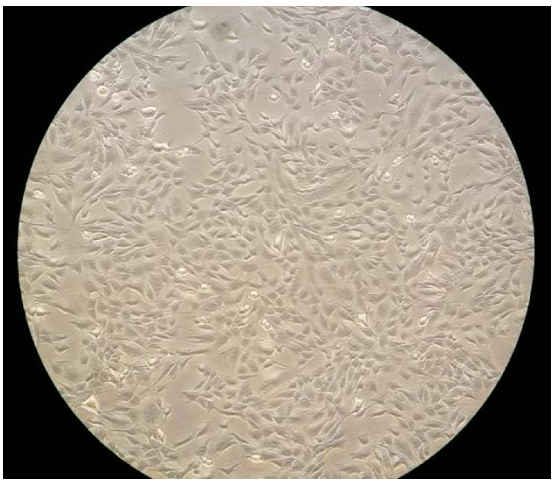

Figure 24: 60% confluent myoblasts imaged with an inverted scope at 100X.

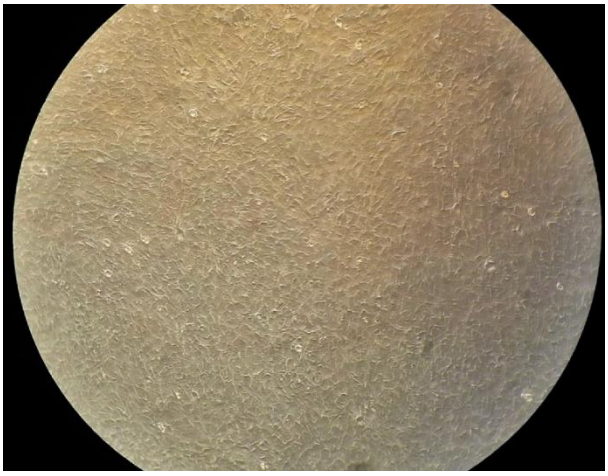

Figure 25: 100% confluent myoblasts, ready for differentiation (100X).

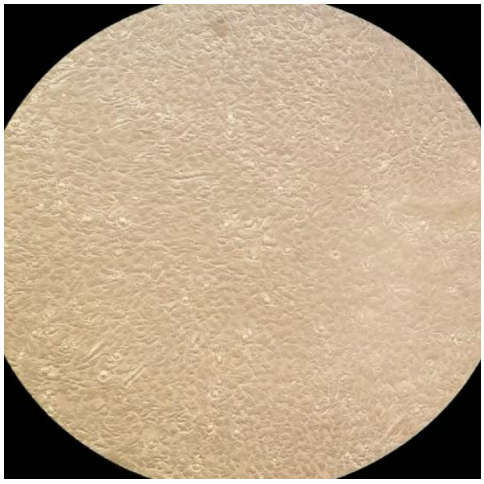

Figure 26: C2C12s at day 2 post differentiation. Myoblast are slowly starting to attach to one another to form multinucleated myotubes.

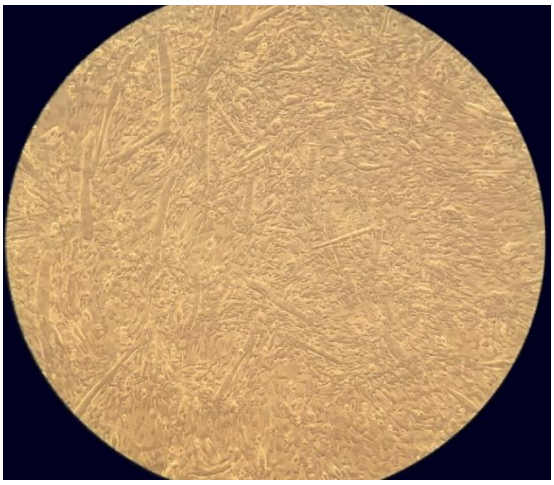

Figure 27: C2C12s at day 3 post differentiation. The formation of long tubule muscle cells (myotubes) is completely visible under the inverted scope.

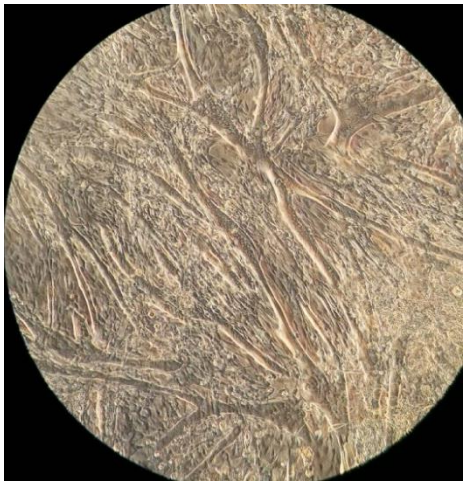

Figure 28: Myotubes at day 7 post differentiation 100X).

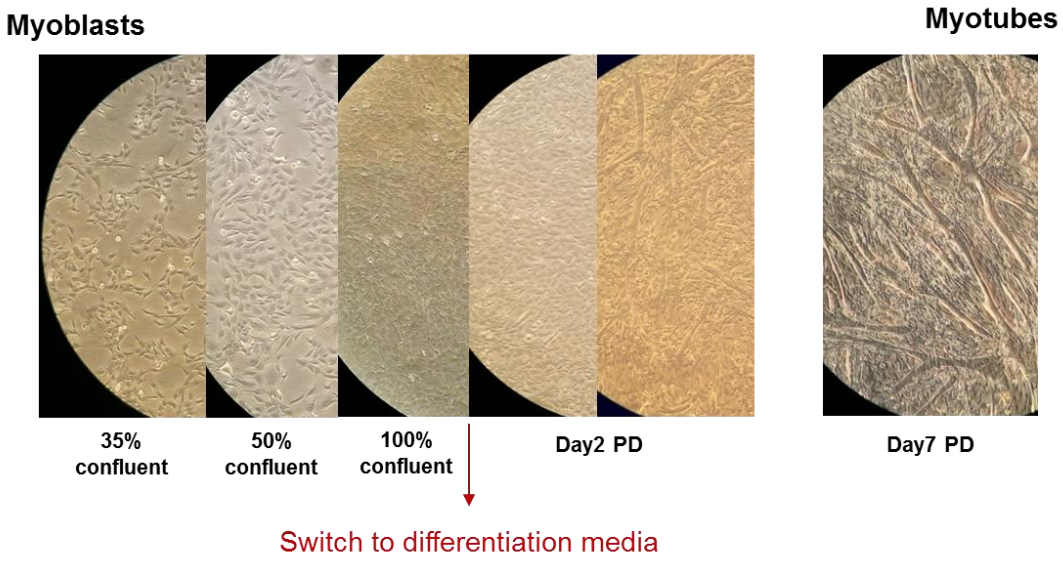

Figure 29: Stages of myoblast to myotube differentiation in C2C12 cells.

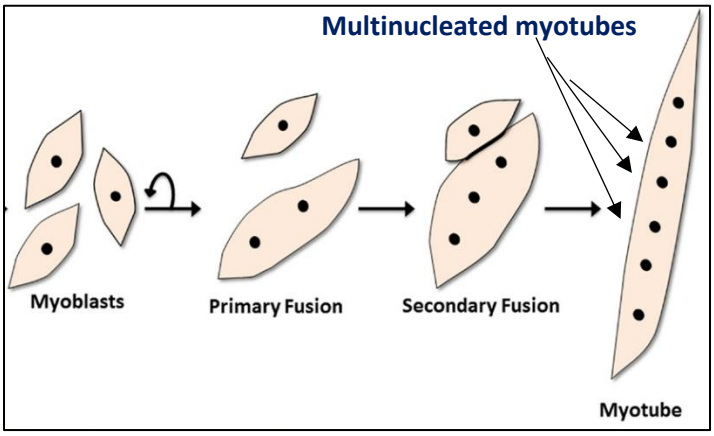

Figure 30. Myoblast differentiation. Myoblasts fusing to form multinucleated myofibers (Adapted from Enwere et al., 2014)

Transient Transfection of Myotubes at Day 3 Post Differentiation

Transient Transfection of myotubes was performed at day 3 of differentiation with 3 sets of constructs; 3 wells were transfected with pAcGFP1-DES-C1, 3 wells with pAcGFP1-C1 and 3 wells with pEPito. A negative control was also used, where no

constructs were used for transfection of the myotubes. Images of the 4 groups were taken with the DMI4000 B Inverted Microscope (Leica) at 2, 3 and 8 days post-transfection. Both phase contrast and fluorescent micrographs were taken from the 4 groups of myotubes at these 3 time points (see Figure 31).

Transient Transfection of Myotubes at Day 2 Post Differentiation

Transient transfection of myotubes was performed for a second time at day 2 post-differentiation with the pAcGFP1-C1 vector. A negative control was also used, which no constructs were used for transfection of the myotubes. Images of the myotubes transfected with pAcGFP1-C1 were recorded with the DMI4000 B Inverted Microscope (Leica) at 2, 3 and 6 days post transfection. Further imaging, past day 6 was not possible due to the layer of muscle cells detaching and lifting from the plates.

Fluorescence Microscope Images of Transfected Myotubes

After transfection, the plates containing the transfected myotubes were observed under the Leica DMI4000 B Inverted Microscope at 3 different time points. The Green Fluorescent Protein (GFP) filter was used to detect the GFP reporter gene expression since both pAcGFP1- C1 and pEPito plasmids carry a Green Fluorescent Protein sequence (AcGFP1 and eGFP respectively) (Figures 10 & 11).

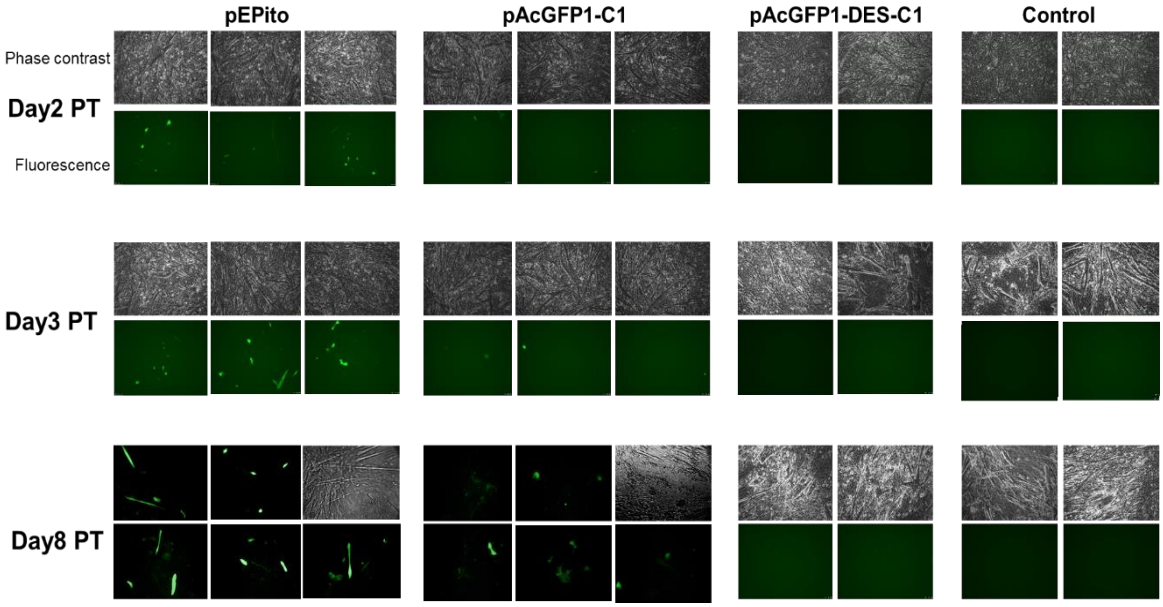

Figure 31. Transfection of myotubes on day 3 post differentiation. Phase contrast and fluorescent micrographs are displayed for cells transfected with pEPito, pAcGFP1-C1, pAcGFP1-DES-C1 and negative controls at days 2, 3 and 8 post transfection. The upper rows show the phase contrast micrographs and the lower rows display the fluorescent images. GFP expression is seen in cells transfected with pEPito and pAcGFP1-C1 vectors.

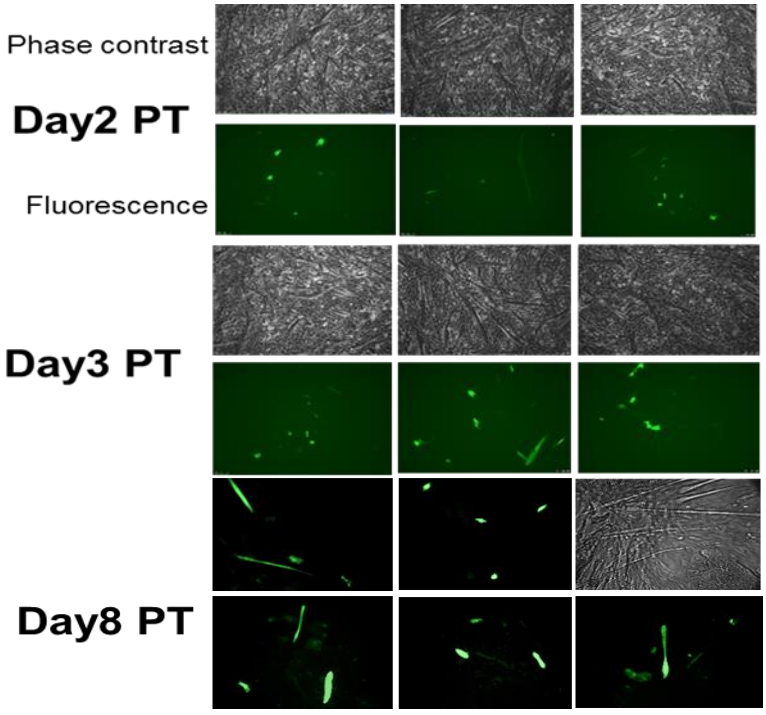

Figure 32: Phase contrast and fluorescent micrographs for myotubes transfected with pEPito at 3 different time points. GFP expression appears to increase overtime showing promise for the vector's stability in muscle cells.

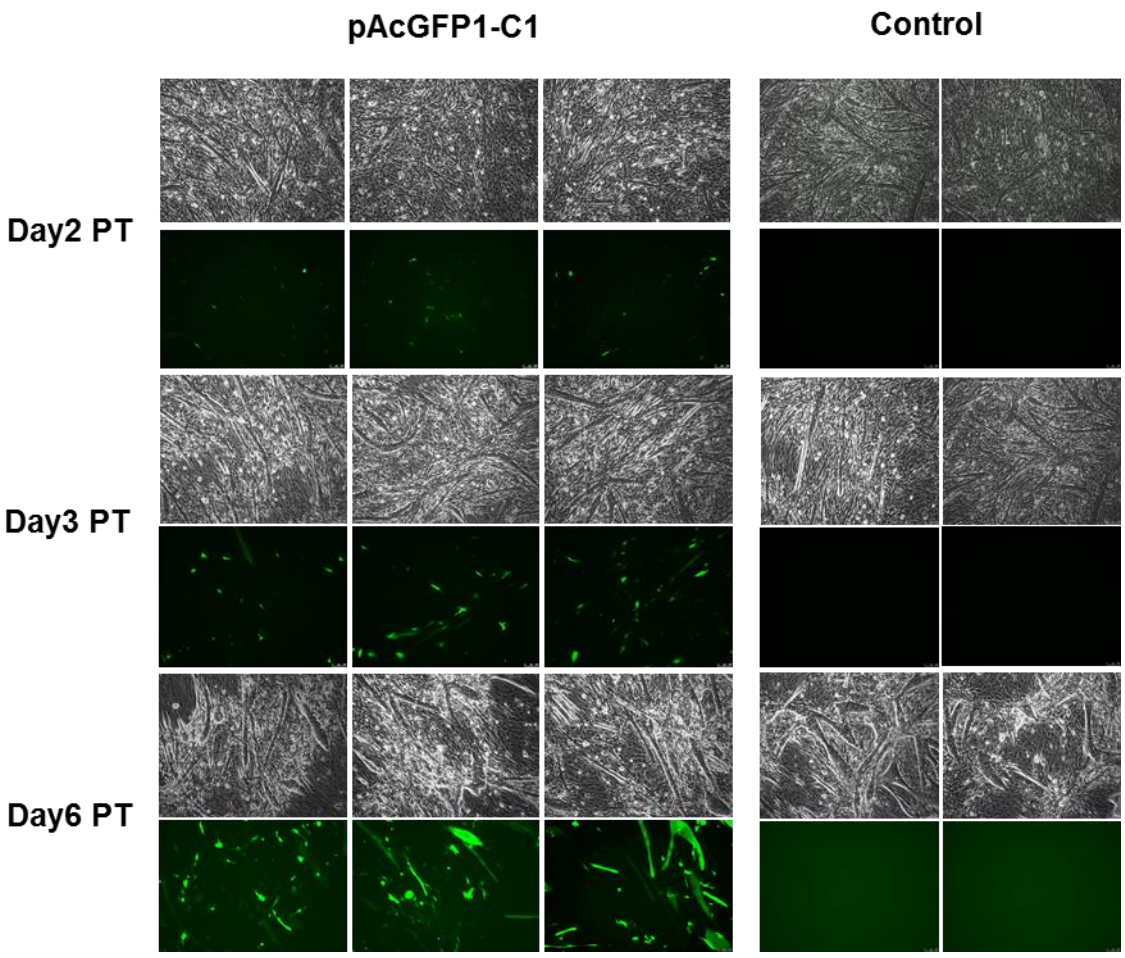

Figure 33. Micrographs of myotubes imaged at days 2, 3 and 6 post transfection with the pAcGFP1-C1 vector along with a negative control shown on the right, with no expression as expected. GFP expression at day 2 post-differentiation shows higher expression compared to cells transfected at day 3 post-differentiation.

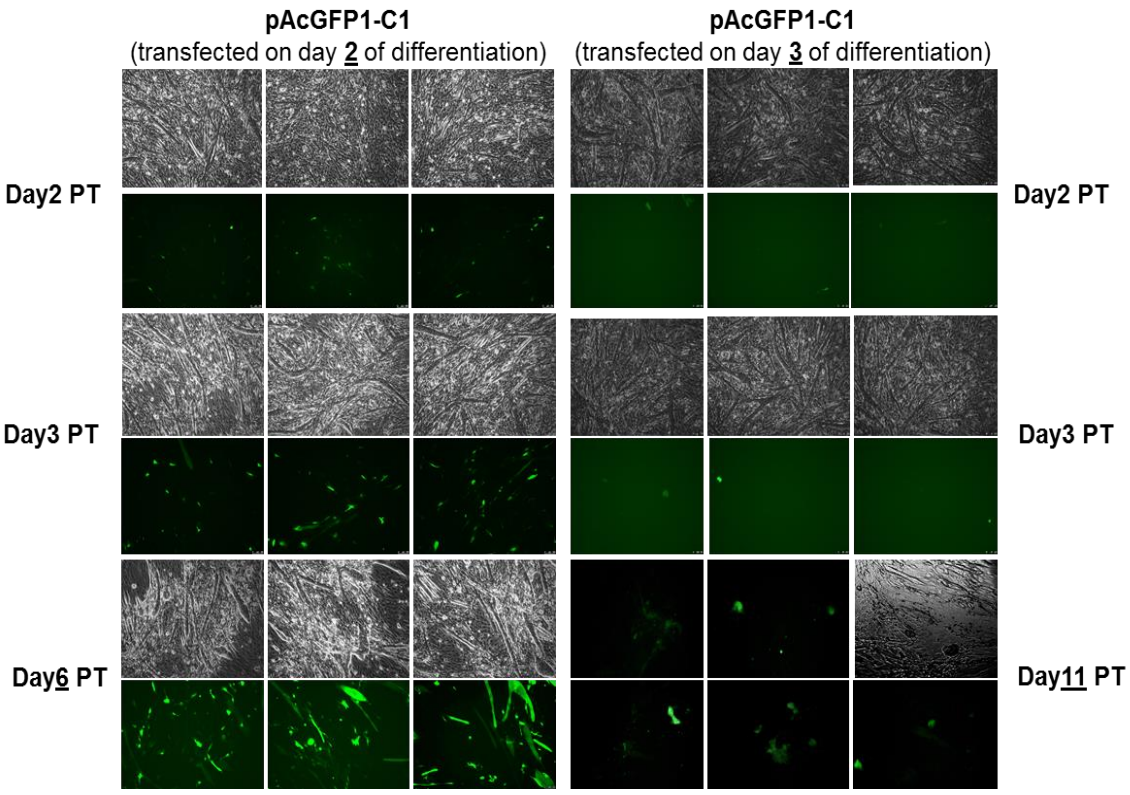

Figure 34. Comparing GFP expression myotubes transfected with pAcGFP1-C1 at day 2 PD (left) versus day 3 PD (right). GFP expression is significantly higher when the myotubes are transfected with pAcGFP1-C1 on day 2 of differentiation. GFP expression in both sets show an increase over time.

CHAPTER 4: DISCUSSION

Summary of Findings

The main purpose of this study was to determine which type of plasmid-based vectors would be most promising for gene therapy of muscle disorders. For this purpose two types of mammalian expression vectors were studied: pEPito, an S/MAR based vector and pAcGFP1-C1 vector that lacks an S/MAR sequence. Stability is a major issue associated with non-viral vectors. In order to address this issue elements that may contribute to drive the length of time for expression of plasmid-based vectors were considered. Working with pEPito was difficult, because it has a proprietary poison sequence that interferes with replication. For this reason, my work was much more challenging than expected.

Cultured myotubes transfected with both constructs, pEPito and pAcGFP1-C1 show stable and continuous gene expression of the GFP reporter gene. Expression levels for both vectors increased over time. Myotubes that were transfected sooner in their differentiation process (day 2 post-differentiation versus day 3 post-differentiation) with pAcGFP1-C1 show significantly higher expression than when transfected into the myotubes at day 3 post differentiation. When comparing expression levels of pAcGFP1-C1 with pEPito, pEPito shows higher transfection efficiency of the myotubes along with higher expression levels. This might indicate that pEPito is a better candidate for transfecting advanced myotubes, therefore more promising for transfection in muscle cells in vivo.

It was not possible to grow the pEPito vector in multiple strains of E-coli including DH5- α, HM 101, DB3.1 and K12 SM10 (λ pir) strains. This problem was due to the unique

sequences pEPito contains which leads to specific host requirements. With the issue of pEPito replication being solved, future studies can be done using pEPito.

pEPito Host Requirements

pEPito contains an R6K origin of replication (ori) which is necessary for plasmid amplification**.** For plasmids with an R6K origin of replication, a λ pir strain is required for the stable maintenance of the plasmid at 200-250 copies per cell.

pEPito also contains a ccdB suicide gene, that produces the ccdB protein which is toxic to the cells and causes cell death. In order to clone pEPito, the host must have a particular mutation in its Gyrase gene (gyrA462). The ccdB gene produces the ccdB protein, which binds to DNA gyrase in the host cell. DNA gyrase is a type of topoisomerase II (enzyme), which reduces the stress of DNA strands when they are being unwound with DNA helicase. However, the ccdB protein binds to a specific site in the host's DNA gyrase which causes the DNA gyrase to generate a double-stranded break within the DNA, killing the host (Figure 35). The gyrA462 mutation prevents the binding of the ccdB protein to gyrase and therefore the protein is no longer toxic to the cells. Note that DH5- α also has a mutation in its gyrase gene (gyrA96) however this mutation only provides resistance to nalidixic acid for the host and does not prevent the ccdb protein from binding to DNA gyrase.

Therefore pEPito requires a specific host that contains both λ pir and gyrA462 mutations. The only host that allows the replication of pEPito, is DB3.1 λ pir (House, et al., 2004). These pEPito host requirements were never provided by the Plasmid Factory (Germany), from which the pEPito plasmid was purchased. Upon referring to literature

reviews, we were able to find out about the DB3.1 λ pir strain. We were able to obtain the needed strain only at the end of my research. Fortunately, we were able to obtain the strain from the investigator who had reported its development in 2004.

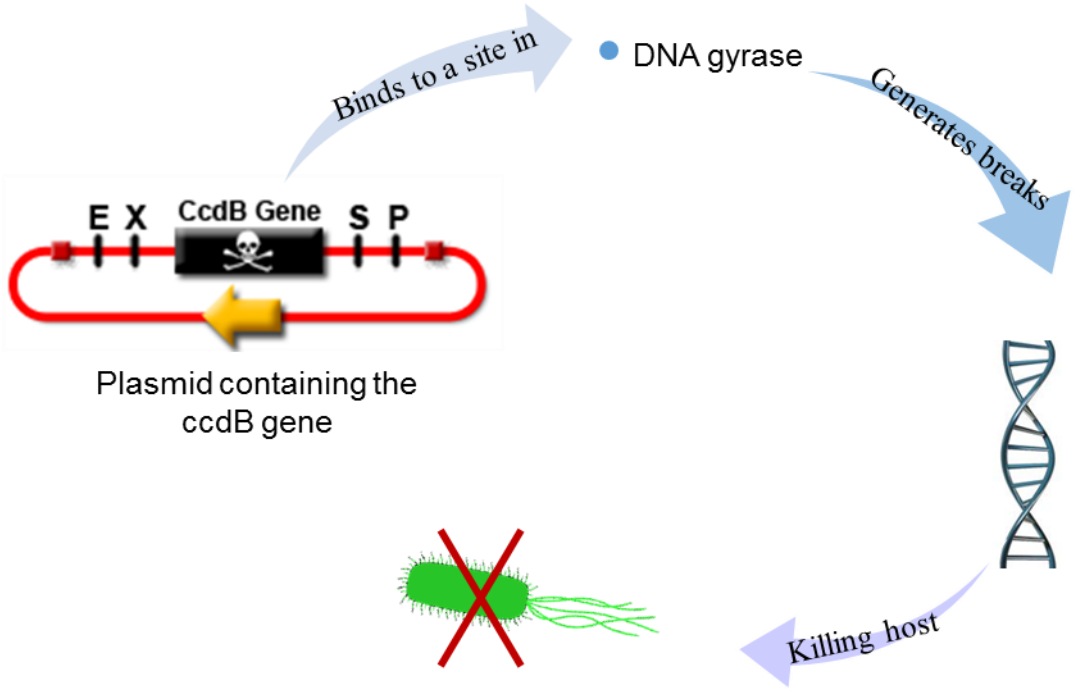

Figure 35. CcdB gene mechanism of action. (Reproduced from: http://parts.igem.org/Part:BBa_P1010)

Limitations

As explained above, the work was hampered considerably by the lack of a suitable host strain for the growth of pEPito and its derivatives. The work was further make difficult by the lack of detailed information on the desmin and UCOE clones. Cloning of the desmin and UCOE segments into my vectors was inhibited by the low yield in PCR of these

fragments. It was difficult to study the length of expression for technical reasons, in that cells began to detach from the surface of the culture dish during the experiments.

Significance of Study

Viral vectors are very robust and show high levels of gene expression along with continuous expression for gene therapy, however they could randomly integrate into the host's genome, leading to insertion mutagenesis and causing cancer by interrupting oncogenes and housekeeping genes. Plasmids on the other hand, do not have the safety issues associated with viral vectors, however they have relatively low stability and low expression levels. It is hoped that it would be possible to improve the efficiency and stability issues for plasmid-based vectors in muscle cells. This study helps identify which set of non-viral vectors would be better candidates for improving/promoting longer expression durations in muscle cells. The results of this study are very promising and set the foundation for future studies.

Future Directions

Future studies may be made quantitative by using constant exposure times for microscopy. It may be possible to improve the efficiency of PCR of desmin and UCOE by planning longer primers and by "tweaking" PCR conditions and times. This will allow higher yield in the cloning experiments. It will also be possible to assess expression quantitatively, rather than qualitatively, by keeping florescent auto exposures constant. It would also be important to extent the period of observing expression of the reporter gene.

If results of gene expression of a reporter gene after transfection in vitro in cultured cells are effective, this experiment should be tried in vivo in experimental animals. If that is successful, the construct can be used for preliminary gene therapy in humans.

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APPENDICES

APPEDIX A: 1.7 kb Desmin promoter Sequence

ctcgagg gctggataag aatatctgga actcccccat ctatttcaga agcttgtctc ttggatgaaa attagacact taatgggaaa gggctttgaa aagagtgcag taacaaagcc ccctttacaa tttacccggc acattcacac ccatcctgag gccaaagcca caggctgtga ggtctcactg tctcagcttc ctgagctata aaatgggaat gatgctagtc tctacctcct agggttggag aattgggggt catgggtgtg aagtgctcag cagcttggcc cacactaggt ggtcagtaca tgtaaggtat tattgttgct acatacatta gtagggcctg ggcctcttta aacctttata gggtagcatg gcaaggctaa ccatcctcac tttatatctg acaagctggg gctcagagag gacgtgcctg agctggggct cagacaagga cacacctact agtaacccct ccagctggtg atggcaggtc tagggtagga ccagtgactg gctcctaatc gagcactcta ttttcaggtt tgcattgcaa aagggtcagtccaagagggacctggagagccaagtggaggtgtagagcacggccagtacc catggagaat ggtggatgtc cttaggggtt agcaagtgcc gtgtgctaag gaggggggctt tggaggttgg caggccctct gtggggctcc atttttgtgg gggtgggggc tggagcatta taggggtgtgg gaagtgattg gggctgtcac cctagccttc cttatctgac gcccacccat gcctcctcag gtacccccct gcccccacag ctcctctcct gtgccttgtt tcccagccat gcgttctcct ctataaatac ccgctctggt atttggggtt ggcagctgtt gctgccaggg agatggttgg gttgacatgc ggctcctgac aaaacacaaa cccctggtgt gtgtgggcgt gggtggtgtg agtaggggga tgaatcaggg agggggcggg ggacccaggg ggcaggagcc acacaaagtc tgtgcggggg tgggagcgcacatagcaatt ggaaactgaa agcttatcag accctttctg gaaatcagcc cactgtttat aaacttgagg ccccaccctc gacagtaccg gggaggaaga gggcctgcac tagtccagag ggaaactgag gcttcagggcc agctcgccca tagacatac tggcagcttt ggccaggatc cctccgcctg ccaggctctc cctgccctcc cttcctgcct agagacccccc accctcaagc ctggctggtc tttgcctgag acccaaacct cttcgacttc aagagaatat ttaggaacaa ggtggtttag ggcctttcct gggaacaggc cttgaccctt taagaaatga cccaaagtct ctccttgacc aaaaagggga ccctcaaact aaagggaagcctctcttctgctgtctcccctgaccccactccccccccaccccaggacgaggagataaccaggg ctgaaagaggccgctgggggctgcagacatgcttgctgcctgccctggcgaaggattggtaggcttgccgtcac aggacccccgctggctgactcaggggcgcaggctcttgcgggggagctggcctcccgcccccacggccacg ggcccttttcctggcaggacagcgggatcttgcagctgtcaggggaggggaggcgggggctgatgtcaggagg gatacaaatagtgccgacggctgggggccctgtctcccctcgccgcatccactctccggccggccgcc

APPENDIX B: 1.5kb A2UCOE Sequence

CBX3 – HNRPA2B1 Core UCOE fragment Esp3I (BsmBI) fragment as described in Williams S et al., 2005

cgtctccggccctccgcgcctacagctcaagccacatccgaaggggagggagccgggagctgcgcgcgg
ggccgccgggggagggtggcaccgcccacgccgggcggccacgaagggcggggcagcgggcgcgc
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ctctcatctcgctcggctgcgggaaatcgggctgaagc**gactgagtc**cgcgatggaggtaacgggtttgaaat
caatgagttattgaaaagggcatggcgaggccgttggcgcctcagtggaagtcggccagccgcctccgtggga
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gctttcacatggcggacgctgccccgctggggtgaaagtggggcgcggaggcgggaattcttattccctttctaa
agcacgctgcttcgggggccacgg**cgtctc**

APPENDIX C: Tissue Culture Protocol

1. Turn on the heater for the water bath and warm your complete medium, PBS-0.53 mM EDTA (Versene), and Trypsin-EDTA at 37C (do not allow Trypsin to be inactivated by exceeding 37C). Approximate time = 30 minutes.

2. Spray your gloves and incubator door handle with 70% EtOH before opening the incubator.

3. Tighten the screw top before you remove the flask from incubator. Place in a Styrofoam cooler that has been wiped with 70% EtOH.

4. Observe cells under a cleaned inverted microscope (use gloves, Kimwipes, and 70% EtOH). Start with 10x objective, open iris diaphragm, and use 10x/20x phase slider:

a. View for confluency

b. Contaminants

c. Cell shape

d. Amount of floating/unadhering cells (round)

e. Take pictures from the center, lower left and upper-right corners of the flasks.

Cell Culture - Passaging and Freezing Information:

http://www.youtube.com/watch?v=gaG15lM1t5A