Production and purification of recombinant human laminin-111 for pre-clinical testing in a murine model of laminin-α2 deficient congenital muscular dystrophy

A thesis submitted in partial fulfillment of the requirements for the degree of

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May, 2017
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prepared under our supervision by

DERYAN ELAINA SMITH

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testing in a mouse model for laminin-α2 deficient congenital muscular dystrophy

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requirements for the degree of

BACHELOR OF SCIENCE IN NUTRITION

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Dean Burkin, Ph.D., Thesis Advisor

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Tamara Valentine, Ph. D., Director, Honors Program

May 2017
ABSTRACT

Laminin α2-deficient congenital muscular dystrophy (LAMA2-CMD) is a fatal genetic neuromuscular disease that currently has no cure. Patients with LAMA2-CMD suffer from muscle wasting, respiratory issues, and neurological effects until eventual death at a premature age due to loss of the adult isoforms of laminin, laminin 211/221, from the basal lamina. Previous research in the Burkin lab has shown effective treatment of the dy^W mouse model of LAMA2-CMD through successful substitution of these adult laminin isoforms using injections of the mouse embryonic laminin isoform, laminin-111. What has not been explored in these studies is whether the dy^W mouse model could also be successfully treated with a recombinant human form of laminin-111, as human patients would require treatment with the human isoform. To determine whether human laminin-111 could be used to treat the LAMA2-CMD mouse model, recombinant human laminin-111 (rhLAM-111) was produced in Chinese hamster ovary (CHO) cells for extraction and purification to be later used for pre-clinical testing in the dy^W mouse model. Our results show rhLAM-111 purification was unsuccessful, indicating heparin affinity chromatography, gel filtration, immobilized metal affinity chromatography (IMAC), and phosphocellulose columns were not efficient or optimized for successful purification and concentration of rhLAM-111. These results indicate the need for a different purification strategy in order to produce enough pure laminin for further research examining the effectiveness of rhLAM-111 in treating the dy^W mouse model of LAMA2-CMD.
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>AAV9</td>
<td>adeno-associated virus serotype 9</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR-associated protein 9</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CHO-S</td>
<td>Chinese hamster ovary suspension</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CS</td>
<td>conditioned media</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>diH₂O</td>
<td>deionized water</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOC</td>
<td>sodium deoxycholate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm (tumor)</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>hLAM-111</td>
<td>human laminin-111</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>HR</td>
<td>high resolution</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LAMA2</td>
<td>laminin α2</td>
</tr>
<tr>
<td>LAMA2-CMD</td>
<td>laminin α2 congenital muscular dystrophy</td>
</tr>
<tr>
<td>MA</td>
<td>methyladenine</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>mg/mL</td>
<td>milligrams per milliliter</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
</tr>
<tr>
<td>mS/cm</td>
<td>millisiemens per centimeter (unit of conductivity)</td>
</tr>
<tr>
<td>MT (buffer)</td>
<td>buffer containing MES and Tris</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride, salt</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NaPO₄</td>
<td>sodium phosphate</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyImaleimide</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffer saline containing Tween 20</td>
</tr>
<tr>
<td>PC</td>
<td>phosphocellulose</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>pH</td>
<td>per hydrogen</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>rhLAM-111</td>
<td>recombinant human laminin-111</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>SC</td>
<td>Santa Cruz (Biotechnology)</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>w/v</td>
<td>% weight of solution in total volume of solution</td>
</tr>
<tr>
<td>x g</td>
<td>times gravity</td>
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</table>
INTRODUCTION

Laminin α2 congenital muscular dystrophy (LAMA2-CMD) is a genetic neuromuscular disease which currently has no cure. Patients suffering from this disease exhibit progressive muscle weakness, vision problems, and premature death. Children with this autosomal recessive disease can suffer from these symptoms and become wheelchair-bound, requiring ventilator assistance to breathe. Eventually, those suffering from LAMA2-CMD will die prematurely between 10 and 20 years of age, from the progressive muscle degradation which leads to cardiopulmonary failure.

Laminin is a heterotrimer glycoprotein in the extracellular matrix, made up of an α chain, β chain, and γ chain. The protein contains globular and rod-like regions, and is important in the structure of basement membranes, cell signaling, and cell adhesion. LAMA2-CMD is the result of a gene mutation in the laminin α2 (LAMA2) gene. The mutation of the LAMA2 gene causes loss of the extracellular laminin-α2 protein, which is required to form laminin isoforms laminin-211 and laminin-221. These laminin isoforms are required for anchoring muscle cells to the basal lamina. Without this anchoring, muscle cells are damaged during contraction and fail to properly regenerate after damage. Research from the Burkin laboratory suggests that the mouse embryonic isoform of laminin, mouse laminin-111, can substitute for laminin-211 in the dy^W mouse model of LAMA2-CMD, as well as the mdx mouse model of Duchenne muscular dystrophy, and thus may serve as a therapeutic treatment for human LAMA2-CMD patients (Rooney, J.E., Knapp, J.R., Hodges, B.L., Wuebbles, R.D., & Burkin, D.J., 2012; Rooney, J.E., Gurpur, P.B., & Burkin, D.J., 2009). However, it is unknown if human laminin-111 (hLAM-111) can produce the same preventative effects as mouse laminin-111 in mouse models for
LAMA2-CMD. This research will determine if rhLAM-111 will prevent muscle pathology and improve muscle function in the dy\textsuperscript{w} mouse model of LAMA2-CMD. I will test the hypothesis that human laminin-111 produced and purified in our lab will prevent the pathological development of LAMA2-CMD by functionally substituting for laminin-211. This question will be answered by analyzing histological and muscle function parameters.

The dy\textsuperscript{w} mice will be treated with hLAM-111 and will have their tissue and muscle function analyzed to determine the effectiveness of the treatment. Before treatment, hLAM-111 must be produced in our laboratory using Chinese hamster ovary (CHO) cells. Effectiveness of production and the purity of the hLAM-111 protein will be determined using tests such as western blots, affinity chromatography, gel filtration, and immobilized metal ion affinity chromatography (IMAC). Once the hLAM-111 is effectively produced and purified, it will be used for treatment. During treatment, mice affected with LAMA2-CMD will be monitored in their cages with laser-locomotion activity monitors to determine the overall activity and movement of the mice. They will also be tested for grip strength. After treatment, the mice will be humanely euthanized, and various muscle tissues will be collected and used to conduct multiple analytical tests, including western blots, immunofluorescence, histological measures of disease pathology, confocal microcopy, and protein and DNA assays.

The expected results from this research include successful production of hLAM-111 and effective treatment of dy\textsuperscript{w} mice. The analytical tests used to determine the purity of the produced hLAM-111 are expected to indicate if the laminin produced from the CHO cells was able to be purified to relative homogeneity through affinity
chromatography and gel filtration chromatography. We will determine the identity of laminin using antibodies in a Western blot analysis, and will determine the purity of the laminin preparation by polyacrylamide gel electrophoresis. In addition, muscle strength tests such as locomotion monitoring and grip strength will indicate if the treatment prevented muscle pathology in the dy<sup>W</sup> mice. The histological analysis of the mouse tissues will indicate effective distribution of laminin in muscle tissue, adequate muscle fiber regeneration, reduced apoptosis and fibrosis, and myofiber membrane integrity. Locomotion activity and grip strength tests will determine muscle strength in the treated mice. These results would provide the field of muscular dystrophy research with methods for producing hLAM-111 and expand research on possible cures for LAMA2-CMD to include hLAM-111 rather than previously used mouse laminin-111, which can not be used in humans.

The significance of this research lies in its potential to contribute a great deal to the field of pharmacology, as it would move laminin treatments further along the path toward human trials. If laminin continues to be successful in treating dy<sup>W</sup> mice in our lab and other pharmacological labs, large animal trials could begin using laminin. Following large animal trials, clinical trials will be initiated, and if successful, laminin could become a therapeutic treatment for muscular dystrophy within the next decade.

**LITERATURE REVIEW**

*Laminin and Laminin-α2 Deficient Congenital Muscular Dystrophy*

Laminin is a heterotrimer glycoprotein, composed of alpha (α), beta (β), and gamma (γ) polypeptide chains linked by disulfide bonds between its amino acid side chains (Durbeej, 2010). This protein acts as an adhesion molecule in the extracellular
matrix (ECM), and is important in the structure of basement membranes (Durbeej, 2010). The discovery of laminin in its first isoform occurred in 1979 (Timpl et al., 1979), and there are now 15 known isoforms of the ECM protein (Gawlik & Durbeej, 2011). The structure of laminin contains both globular regions and rod-like regions (Gawlik & Durbeej, 2011), and specific regions of the protein bind to myofibers to anchor them to the basement membrane (Burkin, Wallace, Nicol, Kaufman, & Kaufman, 2001). Of the three chains of laminin, the alpha chain possesses the most variety, with 5 different chains (Gawlik & Durbeej, 2011). One of the alpha laminin chains is laminin-α2, or LAMA2, which serves a variety of functions in basal lamina. Laminin-α2 binds the cellular receptors dystroglycan and α7β1 integrin in order to achieve adhesion of myofibers, assemble basement membranes, and allow signaling between cells (Gawlik & Durbeej, 2011). As it is the only alpha chain present in the extrasynaptic basement membrane in nerve tissue, laminin-α2 is mainly found in the neuromuscular system and adult skeletal muscle (Durbeej, 2010).

The proteins of the ECM are all products of different genes, and mutations of these genes cause a set of diseases known as muscular dystrophy. There are twelve forms of congenital muscular dystrophy known, and they are categorized based on which classes of proteins are affected by mutations (Peat et al., 2008). Laminin-α2 is the protein product of the LAMA2 gene, located on human chromosome 6q2 (Fig. 1) (Naom et al., 1997). The laminin-α2 protein is required for formation of laminin-211 and laminin-221, the
major laminin isoforms of the extracellular matrix of cardiac and skeletal muscle (Sasaki, Giltay, U. Talts, Timpl, & J. Talts, 2002; Van Ry, Minogue, Hodges, & Burkin, 2014). The α2 chain was originally called merosin (Ehrig, Leivo, Argraves, Ruoslahti, & Engvall, 1991), and therefore the loss of this protein in the ECM was originally characterized as merosin-deficient congenital muscular dystrophy type 1A, when researchers noticed the patients in their study all lacked the α2 chain (Tome et al., 1994). One year later, it was discovered that MDC1A, now called LAMA2-CMD, is caused by a LAMA2 mutation, resulting in the loss of laminin 211/221 from the basement membrane (Helbling-Leclerc et al., 1995; Naom et al., 1997), illustrated in Figure 2.

While an earlier study showed that this autosomal recessive disease composes only a small percentage of congenital muscular dystrophy types (Peat, et. al, 2008), a newer study suggests it is the most common type of congenital muscular dystrophy in the Western hemisphere, as there are over 90 different mutations in the LAMA2 gene (Turner, Mein, Sharpe, & Love, 2015). With the abundance of mutations in the gene, there is variation in the phenotypic presentation of the disease from moderate to severe (Naom et al., 1997).

LAMA2-CMD is an autosomal recessive disorder, meaning children with affected or carrier parents are susceptible to the early onset of this devastating disease (Campbell, 1995). According to Naom et al. (1997), the disease onset in humans is usually within the
first 6 months of life, and eventually results in premature death. Effects of LAMA2-CMD include hypotonia, joint contractures, decreased eye movement, kyphosis, scoliosis, respiratory failure, and feeding difficulties (Doe et al., 2011; Naom et al., 1997). In addition, because the α2 chain is the only chain expressed in neuromuscular cells such as Schwann cells, loss of this protein in LAMA2-CMD can also lead to peripheral neuropathy and seizures (Doe et al., 2011).

On the cellular level, another effect of LAMA2-CMD is decreased myofiber regeneration (Van Ry et al. 2014). In order to achieve regeneration of muscle tissue, myofiber stem cells called satellite cells must be activated by damage to eventually proliferate into myofibers. Located between the basal lamina and the myofiber, these satellite cells are activated by damage when the laminin isoforms signal their receptors. In the absence of laminin-α2, the ability to signal its receptor α7β1 integrin is lost and the satellite cells cannot be properly activated to proliferate (Van Ry et al. 2014).

Laminin-α2 is crucial for muscle fibers in skeletal and cardiac muscle to adhere to the basement membrane, and loss of this chain causes the myotubes without laminin-α2 to become unstable and undergo apoptosis (Doe et al., 2011). This sarcolemmal instability also causes the muscle cells to detach from the extracellular matrix during contraction (Campbell, 1995). Autophagy of cells increases in LAMA2-CMD, as bulk proteins and cellular organelles are digested by the cell’s own lysosomal hydrolases (Carmignac et al., 2011). The laminin-α2 chain deficiency has also been shown to cause micro-RNA deregulation in both skeletal muscle and blood plasma (Holmberg et al., 2014), further contributing to muscle pathology through unregulated post-transcriptional gene
expression. Other cellular-level pathology of LAMA2-CMD includes fibrotic infiltration and inflammation of cells, leading to progressive muscle tissue loss (Van Ry et al., 2014).

One of the mouse models for LAMA2-CMD is the dy W-/- mouse (Fig. 3), although there are multiple mutations in the mouse models for laminin-α2 loss (Guo et al., 2003). The dy W-/- mice express a minimal amount of a truncated, or shortened, α2, and develop signs of the disease within a few weeks of life—parallel to the early onset of severe symptoms in human LAMA2-CMD patients. Much like the human disease resulting in premature death, the disease in the dy W-/- mice is lethal at 10-15 weeks of age (Gawlik & Durbeej, 2011).

Previous studies exploring mechanisms of the disease and treatment possibilities for laminin-α2 chain deficient muscular dystrophy have examined a variety of methods using this animal model and the models of other types of muscular dystrophy. Some studies have focused on transgenic experiments with LAMA2-CMD in the mouse model (Bentzinger, Barzaghi, Lin, & Ruegg, 2005; Doe et al., 2011; Gawlik, Miyagoe-Suzuki, Ekblom, Takeda, & Durbeej, 2004; Kuang et al., 1998). They have included α1, α2, mini-agrin, and α.β1 integrin expression or overexpression in transgenic species to compensate for α2 chain loss in LAMA2-CMD.
Transgenic Experiments & Small Molecule Therapies

Early in the research surrounding LAMA2-CMD, CMD mouse models with an α2 transgene were generated to transgenically produce the α2 chain in which they would normally be deficient (Kuang et al., 1998). This transgene was expressed by a muscle specific promoter and the α2 chain was successfully synthesized and localized in muscle, improving the health and lifespan of the mice. However, the transgene did not compensate for the loss of α2 in non-muscle tissues, and the mice continued to exhibit some symptoms of the disease. While the methods of this study were able to successfully prolong life and reduce muscle pathology in the transgenic mice, effects of the disease in nervous tissue and the thyroid were still present, as the transgene did not compensate for the loss of α2 in these tissues.

Because the α1 chain is structurally similar to α2 and can contribute to myofibril regeneration (Gawlik et al., 2004), expression of this chain was also explored in early research. Laminin-α1 is not usually expressed in adult muscle, therefore mice deficient in α2 can be rescued with a laminin α1 transgene to express the laminin-α1 chain, much like the method of the 1998 Kuang et al. study. These mouse models have been shown to have greater life spans, increased activity, and overall better health than α2-deficient mice, indicating that the expression of the α1 chain can successfully reduce the muscle pathology of this disease (Gawlik et al., 2004). However, much like the Kuang et al. study, the α1 transgene has been unable to compensate for loss of α2 in nervous tissue. Mice with the α1 transgene continue to exhibit hind leg paralysis and peripheral neuropathy. In addition, central nervous system defects remained, as well as defects in the thymus, inner ear, and tooth.
Other transgenic methods have utilized proteins that are not highly homologous to laminin-α2, but rather are other ECM and basal lamina proteins, or are involved in apoptotic pathways. Miniaturized agrin transgenic mice have been shown to have decreased muscle degeneration and increased regeneration when bred with the dy^{3K}/dy^{3K} and dy^{W} mouse models, which completely lack laminin-α2 expression (Bentzinger et al., 2005). Mini-agrin possesses a high binding affinity for laminins, and has been shown to link certain laminin isoforms to dystroglycan, stabilizing the ECM. Mice with the mini-agrin transgene also exhibit greater amounts of the α5 chain, which contributes to restructuring of basement membrane by causing this isoform to polymerize and form laminin networks (Bentzinger et al., 2005). With these improvements in structure and stability, mini-agrin transgenic mice have demonstrated improved muscle pathology and increased lifespan. However, transgenic dy^{3K}/dy^{3K} mice, which do not have α2, exhibited smaller body size and earlier death in comparison to the transgenic dy^{W} mice, which still express a minimal amount of the truncated α2. These results continued the trend of partially successful transgenic methods and certain untreated features of CMD.

Inhibition of the apoptotic pathway in cells affected by CMD has been shown to improve the pathogenesis of the disease (Girgenrath, Dominov, Kostek, & Miller, 2004). Muscle pathology, lifespan, growth, and muscle strength have been improved by inactivating the pro-apoptosis protein Bax by cross-breeding into the dy^{W} background. However, this method was unable to improve regeneration of myofibers, but rather increased the number of myofibers formed in early life that survived. In addition, it has been demonstrated that overexpression of a transgene coding for the anti-apoptosis protein family Bcl-2 reduced the prevalence of apoptosis, producing similar effects as Bax
inhibition. While this transgenic experiment improved multiple signs of pathogenesis, improved regeneration of muscle tissue was not achieved.

Overexpression of another non-homologous protein, $\alpha_7\beta_1$ integrin, the cellular receptor to the $\alpha_2$ chain of laminin, has also been used in transgenic research on the mouse model of CMD (Doe et al., 2011). Previous methods focused on the primary loss of laminin 211/221 in muscle tissue, but had not explored the secondary loss of $\alpha_7\beta_1$ integrin and possible replacement. The Doe et al. study demonstrated that mice who transgenically overexpressed $\alpha_7$ integrin had increased muscle strength and activity, reduced muscle pathology, and increased lifespans. The transgenic overexpression of $\alpha_7$ can reinstate the sarcolemmal stability lost in LAMA2-CMD, as the integrin can re-localize to myofibers deficient in laminin-$\alpha 2$. Increased $\alpha_7$ can also stabilize the ECM by causing increased expression of extracellular proteins galectin-1, galectin-3, and tenascin C. This method did not, however, restore overall body weight in the mouse model.

Clinically, the transgenic method is impossible in humans as it involves genetic manipulation of embryos; a much simpler manipulation would simply be screening for unaffected embryos. Viral gene therapy is similar in that genetic correction is possible, but this technique is more complicated and includes drawbacks as immune suppression, incomplete delivery to all muscle fibers, and delivery to non-target tissues. Therefore, the need for a more feasible treatment is needed, as introduction of viral vectors into human patients for gene therapy would be enormously expensive and potentially controversial. While these studies examining transgenic expression of key proteins involved in CMD have shown success as proof of principle in the mouse models, there is desperate need for a small molecule or protein-based treatment that can be systemically delivered.
The use of clustered regularly interspaced short palindromic repeats (CRISPR) methods to alter the LAMA2 gene has shown success in correcting the CMD mutation (Kemaladewi, Hyatt, Ivakine, & Cohn, 2016). When processing mRNA before translation into protein, cells use exon inclusion and intron splicing to modify the segments of RNA. The gene mutation in the dy2J/dy2J mouse model of LAMA2-CMD involves skipping over exons in mRNA sequences required for α2 formation, and therefore truncates the α2 chain. Using CRISPR and CRISPR-associated protein 9 (Cas9) gene editing technologies, the gene mutation was corrected. This was achieved by introducing specific guide RNAs targeting the intron sites and a Cas9 derived from *Staphylococcus aureus* to adult mice in the adeno-associated virus serotype 9 (AAV9). The correction resulted in restoration of laminin-α2 in muscle tissue and, in contrast to transgenic methods, in peripheral nerve tissue. The CRISPR/Cas9 method was able to correct neurological deficits produced by the disease, which had not been achieved in transgenic studies. However, a more feasible and convenient treatment than gene therapy for human patients remains unestablished.

More recently, a research theme revolving around inhibition of cellular processes and correction of mutations has arisen. The aforementioned Carmignac (2011) study characterized over-activity of the catabolic process of autophagy in laminin-α2 deficient cells. Inhibition of the cell’s mechanisms of autophagy using the autophagy inhibitor 3-methyladenine (3-MA) demonstrated reduction in muscle pathology in the null mutant dy3K/dy3K mouse model of the disease. Apoptosis was reduced with administration of 3-MA, and regeneration was stimulated. Similar to transgenic studies, injections of 3-MA did not improve symptoms of peripheral neuropathy, and there remained a need for exploration of other small molecule or protein-based therapies.
Utilization of the embryonic isoform of laminin, laminin-111, has demonstrated potential as a possible therapeutic treatment of CMD. Laminin-111 is expressed in muscle during embryonic development and is expressed in the adult kidney. Therefore, it would not be recognized as foreign by the immune system, which would be the case with laminin-211 treatments. Injections of laminin-111 was first shown to be an effective treatment in the mdx mouse model of Duchenne muscular dystrophy and in α7 integrin-null mice (Rooney, Gurpur, & Burkin, 2009; Rooney, Gurpur, Yablonka-Reuveni, & Burkin, 2009). Upregulation of α7β1 integrin has been shown to improve muscle pathology of CMD mice (Doe et al., 2011), and there was previously a question of whether laminin can regulate expression of α7β1 integrin. Rooney, Gurpur, & Burkin (2009) showed that myoblasts exposed to laminin-111 exhibited increased expression of α7 integrin, indicating that laminin does indeed play a role in regulation of this integrin. It was shown that injections of laminin-111 could be systemically delivered to the mdx mouse models of Duchenne muscular dystrophy, which protected them from muscle injury during exercise and reduced the degeneration of myofibers.

Mouse models devoid of any α7 integrin, subjected to muscular damage with injections of cardiotoxin to their tibialis anterior muscles, have been shown to have improved regeneration of myofibers after injections of laminin-111 prior to damage (Rooney et al., 2009). The role of α7β1 integrin in the myopathy of CMD has been established and manipulated in other studies as a treatment approach to CMD (Doe et al., 2011), but have not examined the potential of laminin-111. Rooney et al. (2009) showed that delayed muscle repair, reduced myofiber regeneration, post-damage hypotrophic myofibers, impaired myoblast proliferation, and reduced sarcolemmal integrity in α7
integrin-null mice improved with laminin-111 injections. These 2009 studies were among the first to treat the phenotype of CMD, rather than correct or manipulate the genotype, and correct the baseline defects in cellular adhesion. However, they only examined α7 integrin congenital myopathy and Duchenne muscular dystrophy, leaving the question of whether such methods would be effective in LAMA2-CMD.

**Laminin-111 Protein Substitution**

The use of laminin-111 as a protein substitution therapy for mice deficient in α2 has been shown to be beneficial, as laminin-111 is similar in structure and function to the lost isoforms 211/221 (Rooney, Knapp, Hodges, Wuebbles, & Burkin, 2012). Systemically-delivered injections of the embryonic isoform has been shown to increase in lifespan of dyW mice by 3.5-fold, as well as improve mobility and muscle strength, reduce fibrosis and inflammation, and decrease apoptosis of muscle cells (Rooney et al., 2012). The improved cell adhesion as a result of localization of laminin-111 to the basement membrane can stabilize the ECM and reduce the amount of cell-programmed death due to loss of contact between laminin and and muscle cells. In addition to the results indicating the defects in cellular adhesion could be corrected, it has also been shown that this improvement of disease in dyW mice is partially a result of improved muscle regeneration (Van Ry et al., 2014). Following cardiotoxin-induced muscle damage to the dyW mice, cross-sectional area of myofibers was increased, as well as the overall number of myofibers. Like the Rooney et al. (2009) study, treatment with laminin-111 decreased apoptosis, increased α7β1 integrin expression, and prolonged lifespan. However, the Van Ry et al. 2014 research examined in particular the timing and quantity of muscle
regeneration as a result of satellite cell activation and proliferation, and showed the peak times for these activities were early on, after injection with cardiotoxin.

*Protein Production & Purification*

Previous studies have shown how CHO cells are used for production of proteins (J. Kim, Y. Kim, & Lee, 2012) and will serve as a foundation for the production of rhLAM-111 *in vitro*. Transient transfection, or the deliberate temporary introduction of nucleic acids into a eukaryotic cell, is used to produce a high amount of expression of the target gene for a short amount of time, rather than the low expression over generations of cell lines seen in stable transfection. Transient transfection of genes has been accomplished using acidic polyethylenimine (PEI) to reduce costs and save time when producing recombinant proteins (Longo, Kavran, Kim, & Leahy, 2013), and will applied to production of rhLAM-111.

Purification of recombinant proteins is achieved using a variety of columns and appropriate buffers. Self-assembly of laminin *in vitro* has been shown to be dependent on time, temperature, and concentration (Yurchenco, Tsilibary, Charonis, & Furthmayr, 1985), and will occur if laminin is incubated in the presence of a divalent cation such as calcium, and in the absence of a buffer containing ethylenediaminetetraacetic acid (EDTA) or ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). Laminin polymerization is also affected by other basement membrane proteins such as heparin sulfate proteoglycans, as there are binding sites on laminin for these molecules (Fujiwara, Wiedemann, Timpl, Lustig, & Engel, 1984). Due to this interaction, laminin can be purified using heparin affinity chromatography, as seen in other studies purifying proteins with an affinity for heparin sulfate (Nasimuzzaman, Lynn, van der Loo, & Malik, 2016). Therefore,
our purification of laminin will rely on extraction of laminin from CHO cells using EDTA/EGTA washing, and this laminin will be partially purified and concentrated using a heparin affinity column. Purification techniques such as immobilized metal ion affinity chromatography (IMAC) and and gel-filtration have been shown to be successful in purifying proteins (Alajlani, Shiekh, Hasnain, & Brantner, 2016; Cheung, Wong, & Ng, 2012), and will be explored as further purification steps.

Gel filtration utilizes size exclusion for purification of proteins. The resins used in gel filtration columns are composed of small beads containing tiny pores. Large molecules have a lower likelihood of entering the small pores and are therefore pass more quickly through the column than do small molecules. This allows for separation of molecules based on size, as the larger molecules elute from the column before the smaller molecules. Based on the size of the target protein, various resins are available, including the Sepharose resin. Sepharose fast flow resin has been used in purifying proteins other than laminin (Lagerlund, Larsson, Gustavsson, Farenmark, & Heijbel, 1998) as a size-exclusion resin. In addition, the Sepharose resin has also been utilized in the purification of the protein laminin, utilizing a high rate of flow and moderate pressure requirements (Shibata, Peters, Roberts, Goldstein, & Liotta, 1982). Another gel filtration resin used in purification of recombinant proteins is the Superdex resin, for which prep conditions have been established for use of purification (Hellberg, Ivarsson, & Johansson, 1996). The Sepharose and Superdex resins both have established separation ranges that include or are near the molecular weight of laminin. These resins will be useful in purifying a large, 900 kDa protein such as laminin, as it will elute quickly from the columns due to its size.
Other methods that have been shown to successfully purify proteins involve tagging the target protein. IMAC utilizes a specific amino acid tag, such as histidine, on the target protein, which binds covalently to a metal ion chelator in the column resin (Cheung, Wong, Ng, 2012). Metal ion chelators used in IMAC include divalent cations such as Cu$^{2+}$, Zn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$. The affinity of the tag for the metal ion allows for the tagged protein to be retained in the column after the sample is introduced. Proteins that are not bound to the resin are removed by washing with buffer, allowing for their elution off the column. Previous studies have used washes with free imidazole to elute proteins from the IMAC column, as imidazole competes with the histidine tag on the protein for the binding sites on the resin (Bornhorst & Falke, 2000). One study using cells transfected with vectors containing sequences for the α1 chain successfully purified laminin fragments by IMAC using a histidine (His) tag (Santos-Valle et al., 2012). The methods of this study also utilized washes containing imidazole and 0.5 mM sodium chloride (NaCl) to elute proteins from the column, which will be used in our purification of laminin-111.

Ion exchange chromatography is commonly used in purification of proteins, using the differences in charges between protein tags and column resins (Mizuta, Hwang, & Yoshinaka, 2002). Phosphocellulose (PC) columns can utilize a His tag through the binding affinity of the tag for phosphorylated cellulose, or phosphocellulose. An unpublished study by David D. Hackney, Maryanne F. Stock, and Jonathan D. Didier at Carnegie Mellon University, titled “Purification of histidine-tagged proteins on phosphocellulose and application to cleavage of fusion proteins with thrombin” successfully purified proteins containing a His tag. The phosphocellulose chromatography method does not require exposure of the protein to metal ions, and instead utilizes pH changes with EDTA/EGTA
and reducing agents like dithiothreitol (DTT) to elute proteins. The His-tag on the target protein binds tightly to the PC resin at a pH below 6.6 and has a low affinity for the beads at a pH over 7.2; therefore, laminin tagged with histidine could be selectively eluted at a pH > 7.2 using this method.

While many studies have addressed multiple forms of muscular dystrophy, including causes of disease, effects of pathology, and potential treatments, none addressed the use of human embryonic laminin as a treatment for muscular dystrophy caused by the mutation of the LAMA2 gene in the dy<sup>W</sup> mouse model. This gap in the research has led directly to the development of my research question, as it is unknown if human laminin-111 can produce the same effects in the dy<sup>W</sup> mouse model. The methodology used in this study will serve as a reference when answering the question of the effectiveness of recombinant hLAM-111 as a therapeutic treatment.

**METHODOLOGY**

**Cell Lines and Culture Conditions**

Freestyle Chinese hamster ovary suspension (CHO-S) cells were obtained from Life Technologies and cultured according to the manufacturer. Cells were incubated at 37°C in a humidified atmosphere of 92% air and 8% CO2 in ThermoFisher Freestyle CHO expression media supplemented with 4 mM glutamine and 0.5X penicillin-streptomycin. Cells were cultured in disposable polycarbonate shaker flasks, according to the CHO-S manual. Cells were passaged at least 5 times before use in transient transfections, and cell viability was determined by the trypan blue method using a TC20 automated cell counter (BioRad).

**Transient transfections**
CHO-S cells were grown until a total of $1.2 \times 10^9$ viable cells were obtained. Polyethylenimine (PEI HCl Max, MW 40,000, Polysciences Inc.) was prepared from a dry stock in 0.2 M hydrochloric acid (HCl) at 8 mg/mL. Laminin expression plasmids were previously generated in the Burkin Lab and each plasmid contained a complimentary DNA (cDNA) sequence for expression of one of the laminin polypeptides under the control of the constitutively active EF1 promoter. For some experiments (TALON and phosphocellulose purifications) a 6X histidine(His)-tagged laminin C1 construct was generated by adding a thrombin cleavage site and downstream poly-His tag onto the C-terminus of laminin C1. This construct was used identically to the wild type laminin C1 plasmid in transfections. Laminin plasmids were used in equimolar ratios, after accounting for differences in plasmid size. For the transfection mixes, PEI was diluted into 10 mL of sterile DNA compaction buffer (20 mM sodium pyruvate, 150 mM NaCl, pH 4.0) to a final concentration of 120 µg/ml PEI and mixed. A total of 400 µg of DNA was diluted into a separate 10 ml aliquot of compaction buffer and mixed. These 400 µg of DNA was 43.6% laminin a1, 29.4% laminin B1, and 26.97% w/v laminin C1. Diluted PEI was added to diluted DNA dropwise while rocking DNA tube to allow for optimal mixing. Transfection mixes were incubated for 30 min at room temperature to allow for complex formation and DNA condensation. While transfection mixes were incubating, cells were centrifuged and re-suspended in a minimal volume of fresh expression media. The cells were then counted using a TC20 automated cell counter, viable cells determined by trypan blue exclusion, and the cells were diluted to 380 mL at a concentration of $3 \times 10^6$ cells/mL. After the 30 min incubation was finished, the transfection mix was added to the flask of diluted cells dropwise while swirling, and the flask was returned to the CO$_2$ incubator.
**Extraction**

CHO cells transfected with laminin constructs were pelleted at 1000 rpm for 20 min, and the conditioned media supernatant was decanted and then chilled on ice. The CHO cell pellet was re-suspended in 50 mL laminin extraction buffer (500 mM NaCl, 50 mM Tris, 10 mM EDTA, 10 mM EGTA, 2 mM PMSF, and 2 mM NEM, pH 7.4) containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and N-ethylmaleimide (NEM). The cells were incubated for 30 min with rocking, and then pelleted again. The supernatant, henceforth referred to as “EGTA wash”, was chilled on ice, and the cells were washed an additional 5 times in this manner. For storage, EGTA washes and cell supernatants were flash frozen in liquid nitrogen and stored at -80 °C until use.

**Antibodies and Reagents**

Antibodies were purchased from Santa Cruz Biotechnology (SC). Primary anti-laminin antibodies used included mouse α1 F8 monoclonal SC-74417, rabbit α1 polyclonal SC-5582, rabbit β1 polyclonal SC-5583, and rabbit γ1 polyclonal SC-5584. All primary antibodies were diluted 1:200 in 5% bovine serum albumin (BSA) in phosphate buffer saline containing Tween 20 (PBST). Secondary antibodies included rabbit anti-mouse SC-A21065, and goat anti-rabbit SC-A32734. Secondary antibodies were diluted 1:15,000 in 5% BSA in PBST.

**Purification**

*Heparin Affinity Chromatography*

Extracted laminin samples of EGTA washes or conditioned media were thawed on ice, and centrifuged at maximum speed to pellet residual cellular debris. The samples were then filtered with a 0.45 µm Millex-HV syringe filter, and diluted with loading buffer (10
mM sodium phosphate, pH of 7.4, 10 mM EGTA, and 10 mM EDTA). The diluted sample was then directly injected onto a 5 mL heparin fast flow column (GE Life Sciences) by using a mixer bypass valve. The column was first pre-equilibrated in loading buffer using a GE Life Sciences AKTA Pure fast protein liquid chromatography system (FPLC). Samples were applied at 1.5 mL/min, and the flow-through was collected. The column was then washed with loading buffer until the absorbance at 280 nm returned to baseline, and then laminin was eluted with a gradient of 0-75% elution buffer (loading buffer supplemented with 2M NaCl) over 20 min at 1 mL/min. Fractions were assayed for laminin by SDS-PAGE or dot immunoblotting as described in text. Fractions with peak laminin elution activity were pooled and purified further by gel filtration chromatography.

**Gel Filtration Chromatography**

Superdex 200 high resolution (HR) 10/300 size exclusion resin (GE Healthcare Life Sciences) was provided by the Burkin Lab in a pre-packed column. After equilibration with buffer (10 mM EDTA, 10 mM EGTA, 50 mM NaPO4, 150 mM NaCl, pH 7.4), 500 µl of pooled heparin fractions were injected onto the column at a rate of 0.25 mL/min. Fractions with peak laminin were collected in 1 mL aliquots.

Sepharose 4 Fast Flow resin (GE healthcare Life Sciences) was packed into an HR16:300 column at 8 ml/min and equilibrated in gel filtration buffer (150 mM NaCl, 50 mM NaPO4, 5 mM EDTA, 5 mM EGTA, at pH 7.4) prior to running heparin fractions through the column at a rate of 1 mL/min. Fractions with peak laminin were collected in 1.5 mL aliquots, and laminin containing fractions were identified as described in the text.

**TALON affinity chromatography**
All samples were on ice in the 4°C cold room during the entirety of the protocol to avoid temperature dependent laminin polymerization, since protein would be in buffers free of metal chelators. A 50 ml aliquot of cell supernatant were dialyzed into 4L of 1X PBS for 2 hours three times. PBS was added to 4 mL of TALON resin slurry until the volume reached 50 mL. The beads were centrifuged at 1000 x g for 5 min, and the supernatant was decanted. This wash process was repeated 4 times. Cell supernatant was centrifuged at 700 x g for 30 min and the pellet was saved. The cell supernatant was then added to the equilibrated beads. The bead/supernatant mixture was rotated end-over-end at 4°C for 3 hours. The mixture was then added to a gravity column and flow-through was collected. Wash buffers (PBS with increasing concentrations of imidazole, pH 7.4) were prepared, and the column was washed with 10 mls of each buffer. 10 ml fractions were collected and assayed for laminin.

Phosphocellulose column

Five grams of phosphocellulose (PC; Sigma Aldrich, fibrous form) was suspended in 0.5 M sodium hydroxide (NaOH) supplemented with 0.05% Triton-X 100 and stirred for 5 min. The resin was collected using a sintered glass funnel, and re-suspended in the alkaline wash. The process was repeated until the pH of the suspension was greater than 12. The beads were then washed with water 3 times, and then re-suspended in 0.5 M HCl. The beads were washed with acid buffer until the pH was less than 4. The beads were then washed with water until the pH was ~7, and then equilibrated in MT100 buffer (15 mM 2-(N-morpholino)ethanesulfonic acid, MES; 15 mM Tris; 100 mM NaCl; 10 mM EGTA; 10 mM EDTA; pH 6.6). EGTA washes or cell supernatants were clarified by centrifugation and filtering, and then used to re-suspend the washed and equilibrated PC resin. The pH of
this suspension was adjusted to 6.6, and the mixture incubated overnight with end over end rotation.

The next day, the resin was washed by batch binding. The resin was centrifuged at 1000 x g for 5 min, supernatant was decanted, and the beads were washed 3 times in MT100 buffer. The beads were then re-suspended in a minimal volume of MT100 buffer. For pH mediated elution, this buffer was adjusted to pH 8.0, incubated for 15 min with rotation, and then collected. For ionic strength mediated elution, the pH was not changed from 6.6 and instead the suspension was poured into a 10 ml disposable gravity flow column. To initially determine what salt concentration would elute laminin, the column was washed with 10 ml volumes of MT buffer containing 100 mM increases in NaCl, and 10 mL fractions were collected. In later experiments, the column was washed with MT200 buffer (MT buffer with 200 mM NaCl), and laminin was eluted with MT600 buffer (MT buffer with 600 mM NaCl), and 1 ml fractions were collected.

**Gel Electrophoresis and Protein Analysis**

The fractions collected from purification were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% Bis Tris polyacrylamide gels under reducing conditions, and on 3-8% tris-acetate polyacrylamide gels for non-reducing conditions, according to the protocol provided by gel manufacturer (Life Technologies). For some dilute protein samples, sodium deoxycholate/trichloroacetic acid (DOC/TCA) precipitation was conducted using a previously published protocol (Bensadoun & Weinstein, 1976). After electrophoresis was complete, gels were stained with Coomassie Brilliant Blue R overnight and washed with de-stain (525 mL deionized H2O; 75 mL methanol, MeOH; 50 mL acetic acid) the following day, as described by Mahmood & Yang
(2012). For Western blot analysis, proteins separated on the acrylamide gels were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) as described by Mahmood & Yang (2012). Fractions containing rhLAM-111 were also spotted in 2.5 μL aliquots onto a nitrocellulose membrane. Dot blot membranes were dried at room temperature for 20 minutes before blocking, and PVDF membranes were placed in block immediately after transfer. For both types of blots, membranes were incubated for one hour in a blocking buffer composed of 5% instant dry milk in PBS. Membranes were then incubated in anti-laminin primary antibodies overnight at 4°C. Primary antibody incubation was followed by four 5 minute washes in tris-buffered saline with 0.05% Tween 20 (TBST). Membranes were then incubated in the appropriate secondary antibodies for one hour. Secondary antibody incubation was followed by four 5 min washes in TBST, and membranes were imaged with the 800 channel on a LiCor Odyssey imaging system using Image Studio software.

RESULTS

Detection Sensitivity Optimization by Antibody Titrations
The primary and secondary antibodies used for detection of the laminin subunits have established dilution ranges which are established by the manufacturer, but the ideal dilution for optimal sensitivity must be established for each application and laboratory. The primary antibodies have a recommended dilution range of 1:100-1:1000 and the secondary antibodies have ranges of 1:10,000-1:20,000. To optimize the antibody concentrations for maximal sensitivity in laminin dot blot and western blot assays, titrations were completed for both primary and secondary antibodies. Samples from an EGTA wash were serially diluted 1:2 in PBS to create a 10 sample serial dilution series. 2.5 µLs of each serial dilution was spotted onto two nitrocellulose membranes in quadruplet. After blocking the membranes, the membranes were cut into four strips, with one replicate of each serial dilution on each strip. Strips were incubated in rectangular 4-well plates with increasing concentration of either alpha or beta primary anti-laminin antibody. To titrate the primary antibodies, they
were diluted in 5% BSA in 1X PBST at dilutions of 1:200, 1:450, 1:750, and 1:1000, as the recommended range for both the alpha and beta antibodies are 1:100-1:1000. The blot strips were then washed and incubated with the same concentration of secondary antibodies. A similar titration was completed for anti-mouse and anti-rabbit 800 secondary antibodies, except the blots were cut after probing with a single concentration of primary antibody, and then probed with different concentrations of secondary antibodies, with dilutions of 1:10,000, 1:15,000, 1:20,000, and 1:25,000. To determine which antibody concentration was the most sensitive, the dilution which allowed detection of the most dilute sample concentration was identified. For the laminin sample dilutions of (1) 1:2, (2) 1:4, (3) 1:8, and (4) 1:16, a primary antibody dilution of 1:200 was the most sensitive concentration of primary antibody for detection of laminin (Fig. 4). No noticeable difference in signal sensitivity was observed between different concentrations of secondary antibody (Fig. 5), suggesting that changing the concentration of secondary antibody did not affect the signal sensitivity, and did not need optimization. Based upon these results, all subsequent immunological studies used primary antibodies diluted 1:200 and secondary antibodies diluted 1:15,000.

Expression of Laminin-111 by Transient Transfection of CHO Cells and Extraction

*Warm wash buffer improves laminin extraction*

The EDTA and EGTA contained in laminin extraction buffers bind to and chelate calcium and magnesium. Laminin polymerization is calcium dependent (Yurchenco, Tsilibary, Charonis, & Furthmayr, 1985). Therefore, we hypothesized that adding these chelators to the extraction buffers would help to depolymerize laminin into monomers and smaller oligomers, which would increase the amount of laminin extracted by allowing it to be
released from the cell in vitro. Additionally, the chelators were included in all downstream buffers to prevent laminin polymerization during adsorptive chromatographic methods, where laminin is concentrated on chromatographic beads. Laminin polymerization is also temperature dependent; temperatures above 4°C are required for laminin polymerization, and the temperature dependent polymerization step is believed to be upstream of the calcium-dependent step in laminin polymerization (Yurchenco, Tsilibary, Charonis, & Furthmayr, 1985).

Increasing the temperature of the EGTA and EDTA extraction buffers increases the free energy of the in vitro environment, which allows for increased release of the divalent cations to allow their chelation by EGTA/EDTA. However, increased temperature also shifts the equilibrium towards polymerized laminin. We hypothesized that changing the temperature of laminin extraction could increase the amount of laminin being extracted from the surface of cells. To determine if more laminin could be extracted by using warm EGTA rather than cold EGTA, cells were harvested by centrifugation as described in methods. The cell pellet was then re-suspended in either ice-cold or 25°C EGTA, and incubated for one hour with rocking to keep the cells in suspension. As measured by dot immunoblotting, the laminin signal for the warm EGTA samples using laminin α1, β1, and γ1 antibodies was on average 1.6-fold higher than for the cold EGTA samples (Fig. 6),
indicating washing with warm EGTA was more successful in extracting laminin. However, it is possible that warm washing also led to increased extraction of protein contaminants. Because the purification strategy was to use a heparin affinity column followed by gel filtration for size separation, to use time efficiently, we crudely separated the proteins by heparin affinity chromatography (see detailed separation results below) and then separated these fractions by SDS-PAGE in order to evaluate the purity of the EGTA washes and cell supernatant. The gels were stained for total protein using Coomassie Brilliant Blue R dye as described in methods. The results of the gels showed more contaminants in both the warm EGTA wash and the cold EGTA wash (Fig. 7) than in the supernatant samples (Fig. 8). However, when comparing the warm and cold EGTA washes, the total protein in warm washes is higher, and the contaminating proteins are largely in the same proportions.
overall, after accounting for differences in protein concentration. The overall number of bands in the supernatant appeared to be less than in the EGTA wash samples, and included bands of high molecular weight with sizes similar to laminin. This suggests that using 25°C as the EGTA extraction temperature increases the yield of laminin extracted without selectively isolating laminin and thus does not affect the purity. Additionally, although the cell supernatant is a much purer starting extract, the laminin contained in the supernatant was far more dilute. From these results, all future cell extraction experiments were conducted using warm EGTA conditions. Additionally, both warm EGTA and cell supernatants were used as sources of laminin to obtain the maximum yield.

**Time course of Laminin-111 expression after transient transfection**

When previous researchers in the Burkin lab had produced laminin by transient transfection, cells were harvested 4 days after transfection. However, the time course of laminin production had never been evaluated. To determine if laminin could be extracted with a higher yield by allowing cells to incubate longer post-transfection, cells were transfected and aliquots were harvested and frozen daily for 8 days, until cell viability started to decline. To determine when the levels of laminin production would peak, a dot immunoblot analysis was performed using samples from each day. Laminin expression was highest around 7-8 days post-transfection (Fig. 9), indicating
that cells should be incubated for a longer period of time. After 8 days, cell viability quickly decreased (data not shown). These results suggest that by increasing the time cells produce laminin, overall yield could be increased by at least 2-fold. Therefore, following this experiment all subsequent transfection samples were incubated for 7-8 days before harvesting.

**Higher post-transfection temperature increases protein yield**

When CHO cells are commonly used for transient transfections, the incubator temperature is often lowered to 30-32°C the day after the transfection. This reduces the growth rate of the cells, which allows them to divert energy into protein production, and often leads to increases in protein yield. To determine if decreasing the incubator temperature would increase laminin production, aliquots of the main transfection were taken and moved to either 37°C or 32°C incubators, and were harvested at 8 days. To determine the amount of laminin which had been produced under each condition, a dot immunoblot analysis was performed to compare relative expression levels. Surprisingly, in contrast to many published reports, lower incubator temperatures actually decreased the amount of laminin which was present in the cultures. The density of laminin in the 37°C culture was 1.3-fold higher than in the 32°C culture (Fig. 10). Because of these results, all future transfections were maintained at 37°C for the entirety of the incubation.
Purification of Laminin-111

Heparin Affinity Chromatography

Laminin-111 binds to the myocyte plasma membrane by multiple interactions with integral membrane proteins, as well as through interactions with heparin sulfate proteoglycans (Carey, Rafferty, & Schramm, 1987; Sarrazin, Lamanna, & Esko, 2011). Because of the interaction between heparin sulfate and laminin, the laminin heterotrimer can be purified from solutions using a heparin affinity column. In order to purify laminin from the conditioned media and EGTA washes, a 5 mL heparin fast flow column (GE Healthcare Life Sciences) was first equilibrated in a low salt binding buffer (10 mM NaPO₄, 10 mM EGTA, pH 7.4). Previous studies demonstrated that laminin binds to heparin with a low affinity, and is one of the first proteins to elute during a gradient of increasing salt concentration (Sakashita, Engvall, & Ruoslahti, 1980; Talts, Andac, Göhring, Brancaccio, & Timpl, 1999; Timpl et. al, 1979). Therefore, in order for laminin to bind to the column the salt concentration of the laminin solution needed to be reduced.
To avoid dialyzing the large volume of conditioned media, the sample was instead diluted in loading buffer. To determine the dilution required while maintaining the smallest possible sample volume, the conductivity of the sample was measured by injecting a small volume of the sample through the FPLC conductivity monitor using a line to bypass the heparin column. The sample was then diluted 3-4 fold with the low salt buffer, to achieve a conductivity less than 10 mS/cm. This diluted sample was then loaded onto the heparin affinity column by direct injection, overnight. The column was then washed until the absorbance at 280 nm returned to baseline levels. The laminin was then eluted with increasing salt concentrations, and 1 mL fractions were collected.

**Optimization of elution gradient**

Early experiments purifying EGTA washes and conditioned media on
the heparin column utilized a 0-75% gradient of loading to elution buffer over 20 min. This resulted in chromatograms with elution peaks that were not well separated and blended together (Fig. 11, 12). Fraction collections from these peaks underwent dot immunoblot analysis, which showed higher laminin content in the first part the first elution peak, consistent with Yurchenco et. al 1985 (Fig. 13). These results indicated a need for possible changes in elution buffer gradient for optimization of heparin column purification. To achieve more distinct separation of elution peaks, the gradient profile was altered with two goals. First, to maintain a high concentration of laminin, the elution started with a step elution to 15% elution buffer. Secondly, to better separate the remaining peaks, the slope of elution buffer gradient was slowed (15-50% elution buffer over 20 min). To remove remaining contaminants and regenerate the column, the gradient elution ended with a 50-100% gradient over 25 min. The column was washed with 100% elution buffer for 5 min, and then equilibrated in loading buffer. After optimizing this elution gradient, both the EGTA wash chromatogram (Fig. 14) and conditioned media/supernatant chromatogram (Fig. 15) showed more distinct peaks. The dot immunoblot analysis of the fractions collected from the separated peaks showed higher laminin signal in the fractions from the first peak of the conditioned media/cell supernatant chromatogram (Fig. 16). The fractions collected from the second, smaller peak in the chromatogram contained little to no laminin,
as seen by the drop in signal in the C1-D2 fractions in Figure 16. With the improved separation of peaks, all future heparin column purification was conducted using similar gradients of increasing elution buffer concentration.

**Flow-through binding**

Many of the proteins found in the cell media or EGTA washes do not bind heparin and flow through the column, as evidenced by the large increase in absorbance at 280 nm during loading of samples (Fig. 11, 12). However, it is also possible that all of the binding sites on the column were saturated with laminin and other heparin binding proteins, and that laminin remained in the flow through fractions. To test if the flow through contained laminin, flow-through fractions collected from initial heparin affinity column purification were injected again into the heparin column in the same manner as before. The column was then washed with elution buffer using the same gradient as the first column run. The absorbance at 280 nm trace showed a small peak of protein which eluted at the same retention time as laminin. However, this peak was small, which indicated there was very little, if any, laminin remaining in the flow-through (Fig. 17). Dot immunoblot analysis of fractions from this small peak was not performed due to the extremely low protein content.

**Gel Filtration Chromatography**
Heparin affinity column was especially useful in concentrating laminin from the dilute cell supernatant and providing a partial purification of the laminin. The resultant pool from the heparin column contained many proteins with a broad distribution of molecular weights. Because of this broad distribution of molecular weights, the post-heparin pool was further purified by gel filtration. To purify laminin from other heparin binding proteins, we injected an aliquot of the heparin pool over two types of gel filtration columns, a Superdex 200 HR 10/300 column and a Sepharose 4 fast flow column. The Superdex 200 HR column partially separated 2 main peaks, but baseline separation was not achieved (Fig. 18). Although the peaks were somewhat well separated on the chromatography, we were unable to detect any laminin in the fractions by dot immunoblot analysis. Fractions from the heparin column were included as positive controls on the immunoblot in duplicate, labeled as “1” in Figure 19, and were positive for laminin. Because gel filtration is a non-adsorptive method which
is known to result in more dilute protein, we concluded that the Superdex 200 HR 10/300 gel filtration fractions were too dilute for detection by dot immunoblot.

The Superdex 200 HR gel filtration column produced partial peak separation and fractions too dilute for detection by dot immunoblot, indicating the need for an alternative gel filtration column. To determine if a Sepharose 4 fast flow column would give better peak separation and more concentrated fractions, pooled heparin fractions were run through a gel filtration column containing Sepharose 4 fast flow resin. The resulting chromatogram showed more distinct peaks that had baseline separation, suggesting this column would give a more complete separation (Fig. 20). As this column was also bigger in size, it also allowed for larger volumes of heparin fractions in each run, which would decrease the total time needed for purification. The dot immunoblot analysis of the collected fractions indicated the fractions from this column were also too dilute, as the only spots with detectable signal were spots “1”, “2”, “3”, and “4”, which...
were the positive controls: pre-column pooled heparin fraction, EGTA wash #1, EGTA wash #2, and EGTA #3, respectively (Fig. 21). As expected based upon the published separation ranges, the Sepharose 4 fast flow column was much better at separating the two peaks, but eluted fractions that were ultimately too dilute.

Slot Blot concentration of samples for dot immunoblotting

Because the samples were too dilute to analyze by conventional dot immunoblotting, we first turned to vacuum manifold slot blotting to concentrate the proteins onto nitrocellulose membranes. Previous attempts with conventional dot immunoblotting used 2.5 μL aliquots of each sample on a nitrocellulose membrane. The vacuum manifold blotter was used to concentrate 10 μL and 50 μL aliquots of the same Sepharose 4 fast flow fractions onto a smaller area than could be achieved by conventional spotting. This was performed in triplicate, with the pooled heparin sample blotted in duplicate. The membrane was then incubated and washed as described in Methods. Unfortunately, there was as much or more variability between replicates of the same samples than there was between samples. The results of the blot indicated the slot blotter produced too much variability, as spot intensities were not reproducible between the triplicate aliquots of each individual Sepharose sample (Fig. 22). Therefore, we could not use this method to quantitatively concentrate samples onto the dot immunoblot membrane.
Purification of Histidine-tagged Laminin-111

*TALON affinity chromatography*

Because of the known losses of laminin by heparin affinity chromatography, we used another method of protein purification; immobilized metal ion affinity chromatography (IMAC). IMAC utilizes a genetically encoded amino acid tag on the desired protein that binds to a metal ion chelator in the column resin. One type of IMAC resin used in purifying proteins is TALON, a resin charged with cobalt. Resins containing cobalt have been shown to bind to a His-tag with greater affinity than other IMAC resins containing metals such as nickel or copper. Because the majority of laminin does not bind to heparin columns, we wanted to determine if adding a His-tag would improve the affinity of laminin for a chromatography resin, and if TALON could purify His-tagged laminin-111 with greater success than previous methods using the untagged protein. To do this, the laminin solutions were first dialyzed into PBS, to remove EGTA and EDTA. This was an essential step because these chelators would also chelate and reduce the cobalt in the Talon column. Transfection samples were run through a gravity column containing TALON resin, and the column was washed with increasing concentrations of imidazole, which competes with the histidine tag for binding, effectively eluting the His-tagged laminin. Since dot immunoblotting was not sensitive enough for
previous methods, we instead analyzed the fractions by SDS-PAGE analysis. The gel of the fractions showed bands of high molecular weight, but these bands did not align with the laminin protein standard (Fig. 23). Additionally, it seemed as if the protein slowly leached off the column, rather than eluting in a discrete band at a given imidazole concentration. These results suggest that the laminin was unstable in solution not containing EGTA/EDTA, and likely polymerized and precipitated. This conclusion was supported by a large amount of precipitate in the laminin solution before loading the column. A dot immunoblot analysis of the same fractions showed a large amount of protein in the pre-bead pellet and pre-bead supernatant samples; however, the samples from the imidazole washes of increasing concentration contained very little laminin (Table 1), further suggesting the laminin had polymerized and precipitated in solution not containing EGTA/EDTA.

### Phosphocellulose column purification

Proteins tagged with histidine are known to have a high binding affinity for phosphorylated cellulose, or phosphocellulose (PC). This affinity is highly pH dependent; binding will occur when the pH of the system is below 6.6; binding is low and proteins can be eluted when the pH that is higher than 7.2. Phosphocellulose has the additional advantage that it is not dependent on metal cations for binding, therefore this affinity method can be used to

### Table 1. Dot immunoblot signal values and respective amounts of protein in fractions collected from TALON purification using imidazole washing.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of protein (ng)</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-bead pellet</td>
<td>41.45</td>
<td>84587.91</td>
</tr>
<tr>
<td>Pre-bead supernatant</td>
<td>28.46</td>
<td>59659.24</td>
</tr>
<tr>
<td>Flow-through</td>
<td>17.76</td>
<td>39098.07</td>
</tr>
<tr>
<td>0 mM imidazole wash</td>
<td>5.01</td>
<td>14623.38</td>
</tr>
<tr>
<td>10 mM imidazole wash</td>
<td>-1.06</td>
<td>2967.28</td>
</tr>
</tbody>
</table>
purify proteins in the presence of EGTA/EDTA and reducing agents such as dithiothreitol (DTT). To determine if use of a PC column is an effective alternative to IMAC in purification of histidine-tagged laminin, PC beads were washed and equilibrated in binding buffer (15 mM MES, 15 mM Tris, 100 mM NaCl, 5 mM EGTA, 5 mM EDTA, pH 6.6). The beads were added to cell supernatant, the mixture’s pH was adjusted to 6.6, and the mixture was incubated overnight at 4°C with end-over-end rotation. The beads were then added to two gravity flow columns. The first column was washed with binding buffer and then the laminin was eluted with the same buffer adjusted to pH 8.0 (pH 8 elution buffer). The second column was washed with loading buffer supplemented with increasing concentrations of NaCl, and fractions were collected. The collected fractions underwent SDS-PAGE and were also spotted on a nitrocellulose membrane to undergo a dot immunoblot. The resulting Bis-Tris gel contained multiple bands in the high molecular weight (HMW) range, indicating the presence of laminin. Very little laminin is visible on the gel in the “Pre-binding supernatant” sample before the column (Fig. 24). In contrast, although the eluted sample contains many of the same contaminant bands, the amount and proportion of laminin has been greatly enriched, demonstrating the increased purification of this step. However, as the salt concentration was increased, high molecular weight bands
with sizes consistent with laminin were eluted, with an especially dark band eluted with 300 mM NaCl. Additionally, the amount of contaminant proteins in these fractions were much less than the fractions from the pH 8.0 elution (Fig. 24).

In both forms of elution, there are many high molecular weight bands of similar size to laminin. Laminin is known to be proteolytically processed. Additionally, the CHO cells could be secreting heterodimers or monomers of the polypeptide chains. Therefore, to confirm that these high molecular weight bands are laminin and to determine where the intact trimer elutes, the samples were also analyzed by non-reducing SDS-PAGE. The samples from both the pH elution and NaCl elution experiments which we suspected to contain laminin were subjected to this non-reducing SDS-PAGE analysis. Surprisingly, very little intact trimer was found in the pH 8.0 elution fractions. In contrast, the 500 mM NaCl elution fraction contained a prominent band running at a molecular weight consistent with laminin (Fig. 25). Importantly, very few other bands were found in this region, providing further support that this band was laminin.

**Optimization of NaCl Elution**

In the first salt elution experiment, we surveyed the range of salt concentrations which could elute laminin. Laminin was best eluted at 500 mM salt, but many other proteins were
also contained in the elution fraction (Fig. 25). Additionally, it appeared as though laminin began eluting at 300 mM NaCl. Because this fraction was 10 mL of the total salt elution, it is possible that some proteins in the fraction would be eluted more rapidly than others. Therefore, to determine if we could further refine this fractionation, the experiment was repeated. However, rather than performing increasing salt washes, we washed only with 200 mM NaCl. We then eluted laminin with 600 mM NaCl, and collected much smaller 1.5 mL fractions to attempt to further separate the proteins. After DOC/TCA precipitation and SDS sample buffer re-suspension, samples were run on a non-reduced gel, in order to assess laminin as a non-reduced heterotrimer. Again, laminin eluted and was detected at 900 kDa on a non-reduced gel (Fig. 26, 27). Unfortunately, there was not a great separation of laminin from other proteins, but we were able to reduce the volume that
laminin eluted in, thus maintaining a higher concentration. Due to the higher concentration of the separated laminin, further purification using gel filtration was indicated. However, the study was concluded prior to this gel filtration analysis being performed.
LAMA2-CMD is a type of congenital muscular dystrophy caused by defects in the LAMA2 gene, resulting in severe muscle pathology and early mortality. The defects of the LAMA2 gene cause an absence of the laminin isoforms 211 and 221, which are essential components of the extracellular matrix and are required attachment in the basal lamina of muscle. Recent research in the Burkin lab has shown potential for treatment with mouse laminin-111 to reduce pathology caused by LAMA2-CMD in the mouse model of this disease (Rooney et. al 2009; Van Ry et al. 2014), however, research has not yet shown the effects of recombinant human laminin-111 treatment on the mouse model. For human clinical trials to commence, preclinical data on the the efficacy and safety of hLAM-111 needs to be collected, as the mouse protein would promote an immune response and have greatly decreased stability if injected into human patients. The purpose of this study was therefore to produce recombinant human laminin-111 to test for efficacy in the mouse model of LAMA2-CMD, as successful treatment with rhLAM-111 could facilitate the development of a treatment for human patients with LAMA2-CMD.

In this study, production of rhLAM-111 was completed using CHO cell lines with transient transfections and subsequent purification on various columns, including gel filtration and heparin affinity chromatography. The purification of laminin was monitored using protein assays, western blotting, gel electrophoresis, and dot immunoblotting. Native laminin has previously been purified from the Engelbreth-Holm-Swarm (EHS) tumor, which secretes extremely large amounts of basement membrane (Wick & Timpl, 1980). The classical purification scheme for mouse laminin isolated from this tumor utilizes a heparin affinity step, followed by gel filtration and cation exchange chromatography.
(Timpl et al., 1979). A major drawback of this purification scheme is that ~70% of laminin does not bind to the heparin column, and a large amount does not bind the cation exchange resin. This is most likely due to the inherent heterogeneity in charge amongst laminin molecules. Laminin is known to be heavily glycosylated, and glycosylation is a very heterogeneous post-translational modification. Therefore, laminin molecules likely exist in many different charged states, many of which are unable to tightly bind heparin sulfate proteoglycans or ion exchange resins.

EHS tumors contain large amounts of basement membranes, with 5-10% of the tumor mass consisting of collagen alone (Timpl et al., 1979). Basement membrane extracts of the tumor often contain 60% laminin by mass (Hughes, Postovit, & Lajoie, 2010). In contrast, even in our transfections which had been optimized for expression timing kinetics, laminin was never observed on gels in samples which had not been concentrated by heparin or phosphocellulose affinity chromatography. Our transfection conditions could have been optimized by titrating DNA/PEI ratios, construct ratios, and many other factors, but it is unlikely that our starting material would have ever contained as much laminin in EHS tumors with as great starting purity. With this initial setback, a large loss of non-binding laminin over the heparin column was a major setback.

Previous studies have shown successful purification of proteins using Sepharose 4 Fast Flow or Superdex 200 gel filtration for size exclusion chromatography, and have used Sepharose columns specifically for laminin purification (Fu, Wang, Hao, Zhu, Sun, 2014; Lagerlund, Larsson, Gustavsson, Farenmark, Heijbel, 1998; Shibata, Peters, Roberts, Goldstein, Liotta, 1982). Some of these studies have successfully utilized these methods for large proteins of over 200 kDa. Molecules purified by gel filtration do not bind to the
column resin and they do not elute from the column based on pH or salt concentration of elution buffer. Gel filtration columns are made of small beads, which contain tiny pores. Larger molecules are less likely to enter the pores than small molecules, and are thus less likely to be retarded as they travel through the column. Therefore, the molecules of the highest molecular weight elute first, and the molecules of the lowest molecular weight are eluted last. The Sepharose 4 fast flow column was able to more successfully purify the 900 kDa laminin protein than the Superdex 200 HR column, largely due to the Sepharose 4 fast flow column having a more ideal separation range (40 kDa–30,000 kDa) than the Superdex column (10 kDa – 600 kDa). In addition to this, the Sepharose 4 fast flow column was larger, which allowed for more efficient separation, and also allow for larger loading volumes due to its longer column size, and the longer column allowed for better peak separation, as the sample moved through more resin. Gel filtration always dilutes samples because diffusion of the sample in and out of the resin molecules is inevitable. After using these gel filtration methods in this study to purify laminin--111, we obtained laminin at concentrations that were too dilute or too contaminated with other proteins to be used for treatment.

Attempts to concentrate the Sepharose 4 fast flow fractions included the use of a vacuum manifold slot blotter; however, this method did not produce consistent results in the triplicate samples. Further optimization of this method may have been constructive, as the Sepharose fractions were likely purer than fractions collected from the Superdex column and could have had potential for treatment if concentrated enough. One major drawback to concentrating laminin is that polymerization of the protein is concentration dependent (Yurchenco, Tsilibary, Charonis, & Furthmayr, 1985). Previously, centrifugal
concentrators were used to purify laminin extracts. These units contain a semi-permeable membrane, contained in a manifold that fits into the top of a centrifuge tube. Solutions are place in the top of the unit, and centrifuged. Molecules including water and EGTA that are smaller than the pore size can pass through the membrane and are collected in the bottom of the centrifugation tube. Molecules larger than the membrane pore size, such as laminin, will accumulate on top of the membrane. For non-oligomeric proteins, these units work well for concentration. When these are used for laminin, the localized concentration of laminin on top of the membrane becomes so high that polymerization into a gel occurred, which was irreversible. When previously attempted, EGTA was not included in buffers. We could try using our EGTA buffers, but one challenge would be constantly adding EGTA to the laminin-containing top solution, as EGTA would also be constantly passing through the membrane.

Another method used in this study, IMAC, has been previously used for protein purification by also using a histidine tag on the target protein (Cheung, Wong, Ng, 2012). Fractions run through the TALON resin column did not elute sharply with imidazole, suggesting the HMW proteins seen in the TALON gels were likely slowly leaching off the column. Although the column was also washed with 0 mM imidazole, the gel lane containing the eluate from this wash also contained the same HMW bands. Dot immunoblot analysis of the TALON fractions showed a high concentration of laminin in the pre-column samples, suggesting the laminin polymerized without the presence of the chelator EGTA. The amount of laminin detected in the flow-through, 0 mM imidazole, and 10 mM imidazole samples also indicated the protein was binding and leaching off the column non-specifically, as there was no laminin detected in higher concentration
imidazole wash eluate, which was not expected for a protein interacting with the column specifically. Additionally, precipitate found in the dialyzed samples before they were filtered and applied to the column contained laminin, further suggesting that the protein was unstable in the absence of EGTA.

Other studies have shown effective use of a phosphocellulose column for purifying histidine-tagged proteins, including an unpublished study by David D. Hackney, Maryanne F. Stock, and Jonathan D. Didier at Carnegie Mellon University. Their study, titled “Purification of histidine-tagged proteins on phosphocellulose and application to cleavage of fusion proteins with thrombin”, showed successful extraction of histidine-tagged proteins using a phosphocellulose column at a pH below 6.6, and efficient elution at a pH over 7.2. This pH-dependent purification process provides an alternative to IMAC that is less expensive and would not expose the laminin to metals. Initial experiments with the PC column produced gels that contained a smaller amount of bands in the lower molecular weight region than TALON gels, suggesting the laminin was more successfully purified. The initial reduced gel showed the laminin was eluting at 300 mM NaCl, which indicated starting the wash step with 200 mM NaCl would be successful in eluting laminin. The abundance of bands in the HMW region of the reduced gel indicated the laminin from the PC column may have been fragmented, so a non-reduced tris-acetate gel was completed to examine laminin as a complete heterotrimer. In this form of gel electrophoresis, the disulfide bonds which hold the trimer together are not broken, and the trimer will migrate at an apparent molecular weight of ~900 kDa. After completion of the gel, the heterotrimer appeared to be enriched with the 500 mM NaCl wash, rather than the 200 mM NaCl wash. This fraction also had very few other protein contaminants. The reduced gel also showed
successful enrichment of laminin in a similar range of salt wash concentration, but the reduced gel indicated this was at 300 mM with a dot immunoblot showing a range of 300-600 mM NaCl. Although the pH 8.0 elution was expected to show a laminin band, the fraction did not illustrate a pure heterotrimer on the non-reduced gel. These experiments could be repeated using a 400 mM NaCl wash with a 500 mM NaCl elution wash, due to the non-reduced gel showing an elution peak at 500 mM NaCl. From these experiments, we learned that analysis by non-reduced gel electrophoresis gave a much clearer pictures of where the intact trimer was eluting. In retrospect, running non-reduced gels for the entire project would have greatly improved the purification process.

Due to the salt elution ranges indicated by early PC experiments, another purification was conducted using 200 mM salt wash and 600 mM salt elution, which showed successful enrichment of laminin in the elution fractions. Because phosphocellulose contains many highly charged phosphate groups, it is resistant to pH changes. However, it is not resistant to increasing ionic strength. This is likely why the purity of salt-eluted fractions was higher than the pH 8.0 eluted fractions. Consistent with this, the original paper by Hackney, Stock, and Didier suggests performing the pH 8.0 elution method in batch rather than column purifications, due to this buffering capacity. We initially attempted batch purification, but as expected it resulted in rather dilute fractions. This is because of the larger volume required for batch elution. Because our next step was a gel filtration step which would further dilute the protein, we used the salt elution method due to its increased concentration.

We were able to optimize many parts of the laminin expression and purification during the study. By titrating the detection antibodies, immunological techniques were
optimized using 1:200 dilution of primary antibody and 1:15,000 dilution of secondary antibody. The antibodies were purchased from a manufacturer which sells rather dilute antibodies; however, we were able to compensate for this using a prolonged incubation time, allowing for maximum binding of the antibody to the laminin protein. Laminin extraction methods were also adjusted after experiments with post-transfection incubation time and temperature and extraction buffer temperature. Higher post-transfection temperature improved the overall protein yield during extraction, contrary to other published studies utilizing lower temperatures for maximum protein yield (Lin et al., 2015). Longer incubation time post-transfection also improved protein yield. Optimization of extraction of the laminin from other cell proteins included altering wash temperature of EGTA, ultimately allowing for more successful elution using warm EGTA. These improvements in post-transfection and pre-purification steps for more protein yield allowed us to use more concentrated samples in the purification steps. Without these optimization steps, the more dilute samples would have made concentration by heparin and phosphocellulose affinity more difficult.

The inherent problem with purifying laminin is the gross variability in the molecules within a population due to extensive glycosylation. Even by adding a His-tag, we were not able to overcome this variability, as a lot of laminin was still found in the flow through of the phosphocellulose column. Most modern purifications for laminins work around this step by using an antibody based purification. Antibodies have extremely high affinities for their epitopes, and these interactions are much stronger than most other biomolecular interactions. This contrasts with the interaction between laminin and phosphocellulose, as other proteins eluted at the same salt concentration as laminin. If salt
was used to elute laminin from an antibody column, we would expect that it would require an extremely high concentration of salt, and that no other proteins would elute at this high concentration.

There are two main types of antibody purifications described for laminin in the literature. In the first, an antibody that directly recognizes the primary sequence of laminin is used, and this antibody directly recognizes laminin. An example of this purification is purification of native human laminin from the human placenta using antibody-conjugated affinity chromatography (Mizuta et al., 1996). The advantage of this technique is that native laminin can be purified without a tag. A major disadvantage of this technique is that a laminin specific antibody must be obtained in very high amounts. Additionally, if the region of laminin recognized by the antibody is heavily glycosylated, the glycosylated molecules may not bind the antibody.

In a second type of antibody based purification, an antibody epitope tag is added to laminin in the same manner that we added a poly-histidine tag. One example of this is a FLAG tag (Sigma-Aldrich). The protein can then be purified using a column containing antibodies raised against the epitope tag, in this case anti-FLAG antibodies. The protein can then be eluted using somewhat harsh conditions, or, can be eluted by adding peptides containing the epitope. Anti-FLAG columns are often eluted by adding 3X FLAG peptide to the column (Santos-Valle et al., 2012). The FLAG peptides compete for antibody binding with the FLAG-tagged protein, which promotes very specific elution compared to pH or ionic strength elution methods. The advantage for us using this method is that we could easily engineer a FLAG tag into our construct, and the column and reagents are easily available from commercial sources. This method would have given us great purity in one
step, and potentially could have been the only step needed. The major disadvantage of this method, which prevented us from initially trying it is the large upfront cost of the anti-FLAG column, and FLAG elution peptide.

We ended this project because of the inherent difficulty of the project and because commercially available human laminin was available at a reduced price. In agreement with our conclusions regarding the need for antibody based purification schemes, the company from which we purchased the human laminin actually uses 3 different epitope tags, one on each polypeptide to purify their laminin. We had initially tried purifying laminin through conventional methods and more inexpensive tags. A major lesson learned over the course of this project was that spending more money up front would most likely have given us the results we wanted, and in the long run saved us money by not wasting time.

Future research of this topic could include making further adjustments in extraction from CHO cells for optimal laminin yield, similar to our methods of optimization. These adjustments could include changing salt concentrations in the buffer used for extraction to improve the amount of laminin extracted without lysing cells. In supplement to adjustments in extraction buffer, future studies could also examine whether the increased yield of protein using a buffer with a higher salt concentration extracts only extracellular matrix proteins or if the high salt concentration would further contaminate the laminin with extracted cytosolic proteins. During extraction, it is unknown if we are removing surface proteins only as desired, or if the cells are being lysed and cytosolic proteins are also being extracted. We could determine this by mass spectrophotometry, which could guide our process development in future. In addition to this, laminin could be released and extracted
from the ECM using EGTA only after purposely lysing cells and washing to remove the cytoplasm and its constituent proteins. The laminin released from this process could potentially have a higher starting purity. Washing cells with a non-EGTA buffer such as PBS could also potentially remove unwanted ECM proteins before extraction from the CHO cells with EGTA. Then undergo further purification with potential for higher yield. Although previous studies have successfully purified other laminin isoforms from human cell lines rather than CHO cells (Sroka, Chen, & Cress, 2008), the use of human cell lines for production of laminin-111 for treatment has not been used. There is no human equivalent of the EHS tumor, and it is also likely due to the need for placental tissue to produce the embryonic isoform, which would be required in large amounts for human treatments. The amount of placental tissue required would not be feasible to produce the amount of laminin-111 needed to treat the population of humans with LAMA2-CMD. In addition to this, the placental tissue could potentially introduce unwanted pathogens into the laminin-111, secondary to exposure to animal and other human cell lines (Klimanskaya et al., 2005). Therefore, the production and purification of laminin is likely best achieved using the CHO cell lines with optimized extraction techniques.

Other future directions could also include the use of aforementioned antibody tags on the laminin protein, if expenses were considered. Based on the results, if laminin was a non-glycosylated protein of lower molecular weight, any of our purification strategies would using a histidine tag would likely produced purified and concentrated laminin samples. However, due to the large size and heavy glycosylation of the laminin protein, conventional purification methods are less effective and more difficult to achieve. To optimize gel filtration, different bead and bead pore sizes could be used in future attempts.
to purify the protein. Additional attempts to concentrate the laminin after gel filtration using heparin or phosphocellulose affinity chromatography could be performed with potentially more concentrated laminin samples. The expenses of pursuing these methods with a protein that is very difficult to purify are comparable to the expenses of purification using an antibody tag, and further studies purifying laminin could benefit from initial use of the antibody tag.

Finally, there remains a gap in the literature examining the effects of rhLAM-111 on the murine model of LAMA2-CMD for pre-clinical testing, and future research in the Burkin lab will need to investigate this question. If it appears successful in pre-clinical testing, the efficacy of treatments with laminin-111 could be moved forward to large animal trials. After this and toxicology/safety studies, laminin could potentially succeed, allowing it to be used in clinical trials as treatment for patients with the currently incurable untreatable disease LAMA2-CMD.
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