

Electroporation of murine thoracic aortas to provide a method of localized gene delivery

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Abstract

Thoracic aortic aneurysm (TAA) disease affects about 15,000 people each year.⁴ However, the cellular mechanism of TAA disease is not known,⁵ resulting in there being a high demand for research on the condition. The Akerman and Ikonomidis laboratory has demonstrated matrix metalloproteinases (MMPs) to be large players in the disease mechanism.¹² As furin has been found to modulate MMP levels, this protein subsequently plays a role in TAA progression.¹⁴ However, an effective means for localized transfection of vectors for these proteins into tissues has not been established.^{8,9} I hypothesize that we can overexpress furin in the murine thoracic aorta through electroporation. An overexpression vector for furin was generated and confirmed by transforming the vector into *E. coli*, purifying the double-stranded DNA (dsDNA) and running it on an ethidium bromide gel, followed by final confirmation via sequencing. Direct electroporation of the murine thoracic aorta was performed. Electrical conditions comprised of 8 square-wave pulses for 10 ms each, at 200 V. These data provide significant steps towards TAA research, as there is currently very little understanding of the molecular mechanism behind TAA development;⁵ by contributing to this research, we are one step closer to defining a noninvasive treatment for the disease.

Introduction

The aorta is the biggest artery in the body, beginning in the left ventricle and descending into the lower abdomen. It is the body's main artery, functioning to propel blood systemically.¹⁰ Due to its large anatomical significance, one can imagine the disastrous effects of this vessel going awry. The overall motivator for my research is the lack of a noninvasive treatment option for thoracic aortic aneurysm (TAA) disease.² About 15,000 people are affected by TAA disease in the United States annually,⁴ but there is no way to treat the disease without performing

surgery.² The cost of the current treatment option--surgical intervention--is disproportionately high, highlighting the importance for more treatment options to be devised. Further, patients with TAAs are generally asymptomatic until the aorta ruptures or dissects; therefore there are likely many more individuals suffering from TAAs than we know of.¹

A thoracic aortic aneurysm (TAA) is defined as a dilation of the supradiaphragmatic aorta by 50%.² During TAA disease the extracellular matrix breaks down, causing the thoracic aorta to become stiff and lose compliance, as the elastin and collagen within the extracellular matrix are degraded.² Dilation of the thoracic aorta accompanies this breakdown of the extracellular matrix. In non-diseased vessels, collagen and elastin do not degrade in a short period of time. However, these proteins can be specifically broken down by matrix metalloproteinases (MMP).² Matrix metalloproteinases are proteolytic enzymes, which are divided into different classes based upon what cellular component they degrade.³ As the degradation of the extracellular matrix seems to be especially important in the progression of TAA, a link may be made between this degradation and the metalloproteinases which assist in this extracellular breakdown.

Of the matrix metalloproteinases, Specifically focused on in my research is membrane type-1 matrix metalloproteinase (MT1-MMP). Previous research has shown it to play a direct role in extracellular degradation and subsequent TAA development.^{11,12} Membrane type-1 matrix metalloproteinase can activate other MMPs as well as cellular signaling pathways.⁷ This is important for aneurysm development, as this activation of metalloproteinases may result in degradation of the extracellular matrix, as seen in aneurysm disease.² Additionally, MT1-MMP has the ability to release cytokines and growth factors, which can exacerbate the dilation of the vessel that occurs in aneurysms, making this metalloproteinase especially significant to the

development of TAAs.¹² For instance, MT1-MMP has been demonstrated to release sequestered TGF- β from the extracellular matrix.¹² Interestingly, multiple other laboratories have defined a direct role for TGF- β in aneurysm development.¹³ As TGF- β is dysregulated in aneurysms and MT1-MMP is expressed in larger quantities in thoracic aortic aneurysms, this suggests that MT1-MMP plays a role in the disease, altering cell-signaling and growth pathways.¹²

While MT1-MMP certainly plays a role in TAA development, the mechanism by which it does so is still unknown.⁵ In order to continue our research on the cellular mechanism of the disease, we must have a way to effectively manipulate the amount of MT1-MMP in the cells and aorta. Furin is a cellular endoprotease which activates many proprotein substrates, including MT1-MMP.¹⁴ My lab has recently focused on studying furin as an upstream regulator of MT1-MMP. By studying levels of furin in cells and tissues, we can indirectly determine the amount of active MT1-MMP and thus study its effect on aneurysm development. An efficient way to locally manipulate the amount of protein in cells and tissues has yet to be discovered for the aorta. Although lipid-based transfection and transduction can transfer genetic material into cells, this is an inefficient method, having very low rates of success.^{8,9} Additionally, these methods do not provide localized gene transfer in tissues, but are rather systemic, affecting other parts of the body besides the diseased tissue.⁸ My lab wants to specifically target the thoracic aorta to modulate gene expression in. This sort of targeted expression has yet to be discovered.

Fibroblasts play a large role in maintaining vascular homeostasis.¹⁵ They also have the ability to differentiate into myofibroblasts. In pathological conditions, fibroblasts have been demonstrated to take on such different phenotypes.¹⁶ When they change it is possible that vascular homeostasis may subsequently change. This leads our lab to focus on fibroblast cells in our studies of thoracic aortic aneurysm development. In order to study the initiation and

progression of TAA, this laboratory has developed a murine model, in which calcium chloride is used to induce thoracic aortic aneurysms. This murine model has been demonstrated to recapitulate hallmarks of the clinical pathologies of the disease.¹² Previously, our lab induced TAAs in mice which were heterozygously deficient in MT1-MMP and found that with this deficiency, the mice did not develop aneurysms as contrasted to control mice, thereby proving the critical role in which MT1-MMP plays in aneurysm development. Our current model, however, makes use of wildtype mice to study the mechanisms of TAA, taking into account the importance that MT1-MMP holds for disease progression. By conducting research to determine an optimal method for gene insertion into aortic tissue, we take one step closer towards developing a therapeutic strategy for the attenuation of the disease.

To recap, especially of concern to our research is the development and progression of TAA disease as well as what biomolecular and cellular processes are associated with the disease. The obvious connection between furin, MT1-MMP, and TAA disease alongside the lack of noninvasive treatment options² for the disease highlights the need for further study on the matter. My research focuses on developing an effective method for inserting furin vectors into aortic fibroblasts and murine aortas. By determining an efficient and reliable method of gene insertion, we can further study how higher protein expression of furin changes TAA development. I hypothesize that we can overexpress furin in the murine thoracic aorta through electroporation.

Methods

Generation of overexpression vectors

Overexpression vectors for MT1-MMP and furin were obtained. These vectors were transformed into NEB 5-alpha Competent E. coli using the following procedures. Each vector tube was centrifuged and placed on ice. The E. coli cells were thawed on ice and aliquoted into 2

tubes of 50 μ L each. One μ L of the overexpression vector was pipetted into each 50 μ L solution of the E. coli cells and each solution was mixed by tapping gently. The sample was incubated on ice for 30 min. Following this incubation, the sample was heat shocked in a 42 °C water bath for 30 s. Samples were then removed from the bath and placed on ice for 2 min. Following this, 250 μ L of pre-warmed S.O.C. medium was added to the sample and the sample was shaken at 225 rpm for 1 hr at 37 °C. Kanamycin agar plates were made up at a concentration of 25 μ g/mL. Three plates were streaked with the sample mixture with the following volumes: 20 μ L, 110 μ L, 200 μ L. Plates were inverted and incubated at 37 °C overnight. The following day, 6 colonies were selected from each plate. They were then grown up overnight in 5 mL of an LB broth and kanamycin solution (concentration of 50 μ g/mL). Each tube was then shaken overnight at 225 rpm at 37 °C. The resulting bacteria was purified to elute dsDNA using the QIAprep® Plasmid Giga Kit (cat. no. 12191).

Confirmation of vectors

Confirmation of the vectors was obtained through a restriction digest. Restriction enzymes MluI and AsiSI were used for the digestion of MT1-MMP and furin vectors. The samples were digested in a thermocycler using the following protocol: 37 °C for 1 hr, 80 °C for 20 min, and finally 4 °C until ready to load into an agarose gel. Digested samples were loaded into a 1% agarose gel containing ethidium bromide, which allows for visualization of DNA under UV light. Gel electrophoresis was performed at 90 V for 60 min using 1x TAE buffer. The furin vector was sent off for chain-terminating sequencing. Once the sequence came back, it was inputted into BLAST for final confirmation of the vector.

Murine anesthetic induction and maintenance

Prior to being anesthetized, each mouse was weighed. Directly after, each mouse was initially anesthetized by being placed in a customized induction chamber. The induction chamber was constructed of 2 containers stacked on top of each other, with 4x4 gauze being placed in the bottom container. Liquid phase isoflurane--a general anesthetic--was placed onto the gauze, which then vaporized and rose into the top container. Each mouse was placed in the top container so that direct contact with liquid phase isoflurane was avoided, but rather, anesthetic effect was achieved through the inspiration of the vaporized isoflurane. The chamber was constructed in a way which ensured administration of 3-4% isoflurane vapors in room temperature air. Determination of anesthetic depth was conducted through examination of whisker movement, eyelid reflexes, and toe pinch reflexes. The cessation of whisker movement and absence of each reflex indicated that the mouse was asleep. Following the placement of the mouse into the induction chamber, each mouse was moved out of the induction chamber and onto the sterile field. Mice were placed in a supine position and, from this point forward, isoflurane was delivered to the murine through a nose cone. Anesthesia was regulated to ensure the continued absence of whisker movement and reflexes as previously described. Prior to the surgical procedure, 100 μ L of 0.03 mg/mL buprenorphine was also injected into mice for pain management.¹⁷

Murine thoracotomy

The descending aorta was accessed by thoracotomy. In preparation for the thoracotomy, fur in the thoracic area was depilated. Immediately after, the skin on the surgical site was prepped. The skin was wiped with betadine and then 70% EtOH. This was repeated 3 times. Veterinary eye ointment was also applied to each eye to prevent dryness. An incision was made

in between the 4th and 5th ribs. All muscles overlying the intercostal space was dissected free and retracted with 5.0 silk threads. Only intercostals were transected.¹⁷

Electroporation of murine aorta

Following the murine thoracotomy, the adventitial surface of the aorta was exposed. The artery was then soaked by a solution composed of 10 μ L sterile PBS and 90 μ L of the vector of interest. Two 3 mm gold plates were then positioned on either side of the thoracic aorta. The BTX-ECM 830 in vivo square wave electroporation system, Harvard Apparatus was then used to administer 8 electrical pulses. The pulses delivered were each 10 ms in duration and were 200 V in magnitude. Postoperative pain management was carried out through the administration of buprenorphine every 8-12 hours for the first 48 hours after surgery.¹⁷ All animal procedures were approved by the UNC Institutional Animal Care and Use Committee.

Harvest surgery

Two days following the electroporation procedure, the thoracic aorta was extracted using the thoracotomy methods as described above.

Homogenization

Following obtainment of the thoracic aorta, the artery was combined with a PBS + inhibitor cocktail and homogenized using a glass homogenizer.

BCA and Western Blotting

After the thoracic aorta was homogenized, a BCA was completed to determine protein concentration and a western blot was performed using standard procedures. Ten μ g of total protein was loaded onto the gel. The western blot was then analyzed to determine relative protein concentration of furin. Protein concentrations were normalized against GAPDH concentrations from the tissue.

Electroporation of aortic fibroblast cells

Healthy and normal aortic fibroblast cells were grown up in culture using standard cell culture procedures. Fibroblast growth media was used, which contained the growth media, 10 mL of SupplementMix fibroblast growth supplement (cat. no. 395325), 50 mL fetal bovine serum, and 25 μ L gentamicin per 500 mL. Upon becoming confluent, cells were lifted and centrifuged at 500 x g for 4 min. Supernatant was discarded and the pellet was resuspended with an electroporation buffer to a concentration of 4000 cells/ μ L. One of two electroporation buffers was used. The first consisted of 10 mM Tris HCl (pH 7.5), 270 mM sucrose, and 1mM MgCl₂. The second consisted of 10mM Tris (pH 8.0), 1mM EDTA, and 140 mM NaCl.

The vector was pipetted into the solution to bring the total vector concentration to either 0.25 μ g/150k cells, 0.5 μ g/150k cells, or 1.0 μ g/150k cells. A cuvette was chilled on ice and the cell solution was pipetted into the cuvette. Cells were electroporated using one pulse of either 250 V, 350 V, or 450 V. Pulses lasted for 10 ms and a square wave shape was used. The gap between electrodes was 4mm for all conditions. The concentration and voltage used in electroporation was dependent on the electrical conditions being tested for a certain trial.

Results

Vector confirmation

Vectors were made to overexpress furin. The furin overexpression vector was confirmed through a restriction digest. Figure 1 shows a vector map, describing the composition of the furin overexpression vector. A restriction digest was performed to verify the composition of the vector. Figure 2 shows the restriction digest for the furin overexpression vector. Because the length of each band on the restriction digest coincides with the composition of the furin overexpression vector, as represented by figure 1, this provides confirmation of the vector.

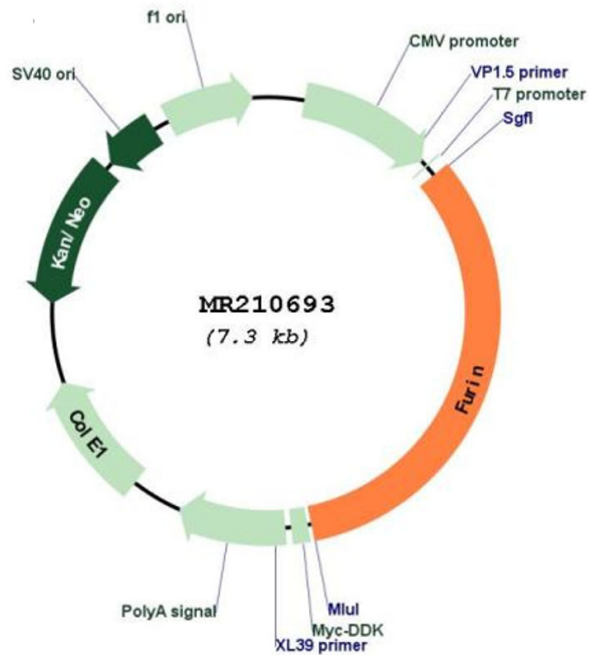


Figure 1. Furin overexpression vector map. This vector includes a Myc-DDK Flag tag and sites for restriction enzymes AsiSI and MluI.¹⁴

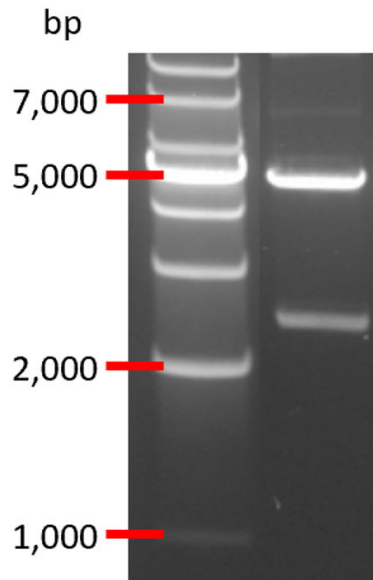


Figure 2. Furin overexpression restriction digest. The ORF for furin in a murine model is 2379 bp and can be represented by the lower band. The remaining band is 4900 bp.

Following the confirmation of the vector by restriction digest, the vector was sent out for sequencing. Chain-terminating sequencing was used to provide the complete nucleotide sequence for the furin vector. After inputting the sequence into BLAST, the sequence of the vector was matched with known sequences for the furin ORF and Myc-DDK tag. An Myc-DDK tag is merely a sequence added onto the vector which codes for a specific protein. Expression of the Myc-DDK protein can then be used as an indicator of the vector's presence. We used the Myc-DDK tag to confirm the presence of furin in downstream applications of our research. The Myc-DDK tag sequence was confirmed as being a part of our vector sequence, as shown as a band on the restriction digest and in our BLAST results. Figure 3 shows the blast results for the furin vector sequence.

Mus musculus furin (paired basic amino acid cleaving enzyme) (Furin), transcript variant 2, mRNA

Sequence ID: [NM_001081454.2](#) Length: 4171 Number of Matches: 1

Range 1: 277 to 2655 [GenBank](#) [Graphics](#)

[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
4394 bits(2379)	0.0	2379/2379(100%)	0/2379(0%)	Plus/Plus
Query 1	ATGGAGCTGAGATCCTGGTTGCTATGGGTGGTCGCAGCAGCAGGAGCCGTGGTCCTGCTG	60		
Sbjct 277	ATGGAGCTGAGATCCTGGTTGCTATGGGTGGTCGCAGCAGCAGGAGCCGTGGTCCTGCTG	336		
Query 61	GCAGCTGATGCTCAAGGCCAGAAGATCTTCACCAACACCTGGGCCGTGCACATTCTCGGA	120		
Sbjct 337	GCAGCTGATGCTCAAGGCCAGAAGATCTTCACCAACACCTGGGCCGTGCACATTCTCGGA	396		
Query 121	GGCCAGCTGTGGCTGATAGGGTGGCGCAGAAGCATGGCTTCCACAACCTGGGCCAGATC	180		
Sbjct 397	GGCCAGCTGTGGCTGATAGGGTGGCGCAGAAGCATGGCTTCCACAACCTGGGCCAGATC	456		
Query 181	TTCGGTGACTATTACCACTTCTGGCACAGAGCAGTGACAAAGCGGTCCCTGTCGCCTCAC	240		
Sbjct 457	TTCGGTGACTATTACCACTTCTGGCACAGAGCAGTGACAAAGCGGTCCCTGTCGCCTCAC	516		
Query 241	CGCCCGCGGCACAGCCGGCTACAGAGGGAGCCTCAAGTAAAGTGGCTGGAGCAGCAGGTA	300		
Sbjct 517	CGCCCGCGGCACAGCCGGCTACAGAGGGAGCCTCAAGTAAAGTGGCTGGAGCAGCAGGTA	576		

Figure 3. BLAST results for furin overexpression vector. Query rows indicate the searched sequence. Subject rows indicate the known sequence for the furin vector. Vertical lines between the rows indicate the same base pair being present in each sequence. The plethora of these vertical lines indicates a perfect match between our furin overexpression vector and the known dsDNA sequence for furin.

Vector production and purification

Following the confirmation of the vector, I produced large amounts of the vector by transduction into E. coli and dsDNA purification. The QIAprep® Plasmid Giga Kit (cat. no. 12191) was used to grow up bacteria and purify large amounts of the vector. A nanodrop spectrophotometer was used to determine the final vector concentration and purity. The purity of the final vector sample measured between 1.8 and 2, measured by an optical density of A_{260/280}.

Protein quantification

Following the electroporation of the furin vector into mice, thoracic aortic tissue was homogenized and collected for protein. A BCA was run to determine the concentration of total protein. Concentrations obtained from the BCA were then used to calculate and load 10 µg of total protein into a western blot. The concentration of Myc-DDK protein present in the murine

thoracic aorta as compared to control GAPDH was then analyzed. Figure 4 shows an image of the western blot. Figure 5 shows a graph which quantifies protein concentration. After analyzing protein concentrations, there was determined to be a 3-fold increase in furin expression from a murine thoracic aorta which was electroporated, as compared to the control.

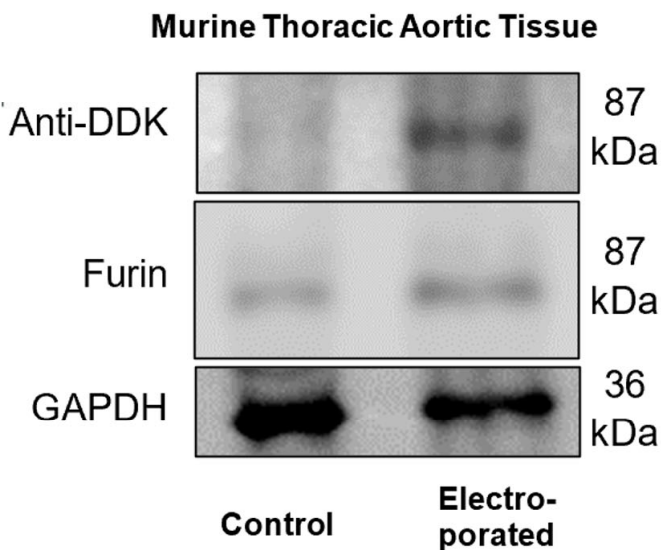


Figure 4. Western blot results for Myc-DDK and furin in a mouse thoracic aorta. Protein was collected 2 days following electroporation.¹⁴

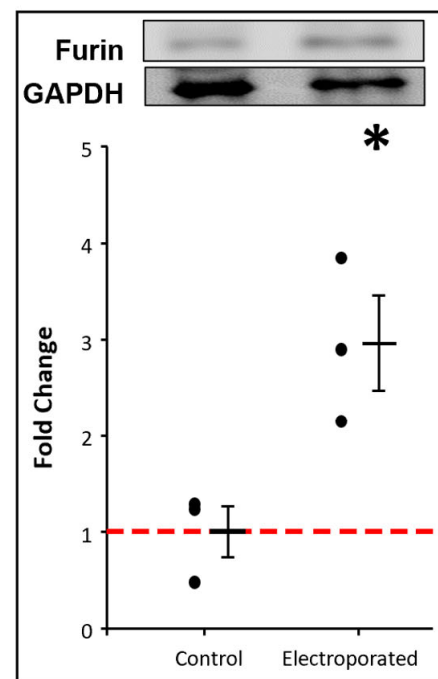


Figure 5. Three-fold increase in furin protein in the murine thoracic aorta, as compared to the control sample, which was not electroporated.¹⁴

Discussion

As previously discussed, MT1-MMP has been shown to be a large player in thoracic aortic aneurysm development. Furin is an upstream regulator of MT1-MMP, so manipulating it and studying the downstream effects would be valuable for further understanding the disease mechanism of TAA disease. However, as there is no efficient way of locally transfecting furin vectors into thoracic aortic tissue, I hope to provide a reliable method for doing so. The aim of my study was to prove electroporation as being an effective method for transfecting vectors into murine thoracic aortic tissue. As high concentrations of Myc-DDK tag proteins were shown to be

present in experimental murine thoracic aortic tissue, as compared to control tissues, this shows that electroporation provides a successful method for gene transfer in aortic tissue. Because the concentrations of Myc-DDK proteins coincide with those of furin, my data shows the successful transfection of a furin overexpression vector by means of electroporation. This proves electroporation to be a promising method for localized gene transfer in the thoracic aorta, supporting my hypothesis that we can overexpress furin in the murine thoracic aorta through electroporation.

Upon growing up and purifying large quantities of the vector, a restriction digest was performed to confirm the vector's presence. The 2 bands which resulted from the restriction digest were 2379 and 4900 bp. As the ORF for furin is 2379 bp, this confirms the presence of furin in the vector. Additionally, the size of the remaining band coincides with the expected size, as determined through the vector map. The total vector should measure to be about 7300 bp, and since both bands add up to equal this amount, this confirms the presence of the vector in our dsDNA. Furthermore, the consistent pairing-up of nucleotides between the searched and known sequences for furin in BLAST further confirms the vector.

Following mass production of the vector, final concentration was determined through a nanodrop spectrophotometer. In addition to obtaining the vector concentration to be used in downstream applications, the nanodrop provided us with a number indicating the dsDNA purity. Because our purity value was in between 1.8 and 2, this indicates an acceptable value, ensuring that the dsDNA was not contaminated with extraneous elements.

As previously discussed, following the electroporation of murine thoracic aortic tissue, the tissue sample was taken and homogenized for protein collection. These protein samples were loaded and run in the western blot, as displayed by figure 4. The presence of an 87 kDa band

indicates that furin protein is being expressed. Additionally, due to the difference in brightness of furin and Myc-DDK bands in the electroporated sample, versus the control, protein concentrations were quantified. Figure 5 shows this quantification. The 3-fold increase in concentration of furin in the electroporated sample, as compared to the control proves electroporation to be an effective method for localized transfection in the thoracic aorta.

After showing successful electroporation in murine thoracic aortic tissue, electroporation was taken a step further and performed on the cellular level. Following the electroporation of furin vectors into murine thoracic aortas, we have begun repeating the process with MT1-MMP vectors. Optimization of electroporation should be similar between furin and MT1-MMP due to their similar sizes. We have constructed a MT1-MMP overexpression vector to include a GFP tag, allowing us to rapidly visualize successful transfection of the vector. The MT1-MMP vectors were confirmed using the same process as completed with furin.

Following the creation and confirmation of the MT1-MMP overexpression vector, these vectors were transmitted into aortic fibroblast cells using electroporation. We have since begun optimizing the electrical conditions for electroporation. Several trials of electroporation will be performed to determine the overall optimal electrical conditions for administering vectors into aortic fibroblast cells. The optimal electrical conditions are those conditions which result in the highest percentage of vector being expressed in the aortic fibroblast cells. Because the MT1-MMP vector not only overexpressed MT1-MMP, but also included a GFP tag, the presence of MT1-MMP could be visualized under a microscope, as its abundance is represented by bright green fluorescence. This allows the percent expression of the MT1-MMP vector to be determined via fluorescent microscopy. Several variables can be altered which affect the efficiency of electroporation: (1) voltage, (2) type of electroporation buffer used, (3) distance between the

electrodes, (4) duration of electrical pulse, (5) number of electrical pulses administered, and (6) concentration of vector used. Trials will be performed in the order stated above to determine the optimal electrical condition.

We expect to generate parabolic curves for each variable, holding the voltage constant after the optimal voltage is determined in the first trial. The peak of each curve indicates the optimal level for each variable tested. Ultimately, values for each of the 6 variables previously stated will be obtained, allowing us to determine an efficient method for electroporating vectors into aortic fibroblast cells.

Electroporation is favorable as contrasted to other transfection methods due to its ability for localized transfection as compared to systemic gene transfer. Findings from previous research indicate difficulty in transfecting at high efficiency using other means of gene transfer (e.g. lipid-based transfection, transduction). While transduction performed through viral vectors provides a very efficient means of gene transfer, this method is systemic.⁸ The inability to localize gene transfer to a particular tissue results in the method being difficult to apply therapeutically. Introducing a vector directly into a tissue of interest may be favorable over the widespread nature of transduction. Why introduce a treatment systemically for a condition which is localized to an individual tissue?

While lipid-based transfection provides a means for localized gene transfer, its efficiency is low in primary cells.⁹ The high efficiency exhibited by my results proves electroporation to be a promising method of gene transfer. Additionally, as electroporation has never been completed in the thoracic aorta, my findings are novel in that they prove the method to be applicable to this tissue.

Even such, our methods of electroporation do pose their own limitations. Following electroporation, it is not known how long vector expression will last. Our lab's murine model of thoracic aneurysm development requires 4 weeks to pass in order for TAA disease to develop. If electroporated vectors do not remain in the target tissue for over this amount of time, this presents a challenge for studying the disease attenuation. Additionally, as previously mentioned, the optimal electrical conditions for electroporation must still be discovered. There may be other off-target effects of electroporating furin into tissue, however, more research must be conducted to specifically identify these caveats. While we know that electroporation is an efficient method of gene delivery, we must determine optimal values for several variables, each of which independently change electroporation efficiency.

Our research holds great significance with regards to thoracic aortic aneurysm disease. Not only do our findings fit into the greater research about furin and MT1-MMP, but they provide a greater insight as to methods for localized gene transfer. Currently, not much is understood about the molecular mechanisms of thoracic aortic aneurysm disease. Because our results provide evidence for a more efficient method of vector transfection, these methods can be applied towards other research to further understand the mechanisms behind TAA disease, thus ultimately working towards a reliable treatment method for TAA disease.

Approximately 15,000 people in the United States are affected by TAA.⁴ Although this number, at first, seems small, this value is likely underestimated as aneurysms often go undiagnosed due to them being characteristically asymptomatic until they become life-threatening. When a patient is diagnosed with thoracic aortic aneurysm disease, the only treatment options involve surgery and monitoring the aneurysm to determine if its size puts a patient at risk for rupture. Additionally, when one requires a surgical procedure for a TAA, he or

she undergoes the risks which come with surgery. Furthermore, these treatments do not address the underlying workings of the disease, but rather, attempt to prevent the aneurysm from rupturing, which leads to rapid hemorrhaging and a subsequent high risk of fatality. Our research, however, provides a step towards the development of a thorough understanding of the molecular and cellular characteristics associated with TAA, which could potentially provide a new therapeutic strategy towards disease attenuation.

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