An Efficient Non-Viral Vector for Gene Therapy

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

By

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December 2012
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Acknowledgements

I shall forever be grateful to my advisor, mentor and role model, Dr. Aida Metzenberg, for letting me work with her in her laboratory and giving me a project that would lead to my Master’s degree. Her eagerness, passion, positive attitude and her everlasting smile made every difficult situation and bad day into one that would always end with great guidance, a new outlook and a box full of chocolates.

My sincere gratitude and thanks to Dr. Stan Metzenberg, for always having an open door for any questions that I came across and giving innovative ideas for my thesis work. I thank him for his constant support and giving me fresh and innovative approaches for any halts in my project. Many thanks to Dr. Steven Oppenheimer, who would always applaud me in any stage of my work. I thank him constantly for providing me the tools needed to finish the project.

My thesis work could not have been successfully completed without the never ending love and support from my parents, Mayank and Neeta Shah, and my beloved fiancé, Hemang Thakkar, who has spent weekends and weeknights with me in the laboratory to give me company and enthusiasm so I could finish this project successfully on time. Without his support, I could never have followed my dreams.

Of course, no successful work can be possible without the help of dear friends and laboratory members. I would like to thank my dear friend, Andrea Cosco, for always staying by my side, providing me with constant questions, answers and new ideas to keep me moving forward. I would also like to thank Ricardo Gutierrez for always giving me his valuable time for this project. Last but not least, I would like to specially mention Atefeh Rajaei, who provided me with endless encouragement and suggestions.
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Abstract

An Efficient Non-Viral Vector for Gene Therapy in MPSII

By

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Masters of Science

Biology

Gene therapy is a method with great potential for the treatment of heritable disorders. One difficulty with this approach is that the vectors used in early trials were derived from viruses. Viral genomes have several disadvantages as vectors for gene therapy, including the interruption of cancer-causing genes by random insertion into the genome and activation of the immune system. The purpose of this project was to compare vectors that could serve as a foundation for gene therapy that avoids these difficulties. I used as a model the treatment of Hunter syndrome, a lysosomal storage disease to compare efficiencies of non-viral vectors in delivering genes to cultured cells deficient in functional genes. Mucopolysaccharidosis type II (MPSII), also known as Hunter syndrome, is an x-linked recessive disorder that occurs when the IDS gene located on the X chromosome becomes mutated. The IDS gene is responsible for making the I2S enzyme. The lack of I2S or reduced function of this enzyme causes a buildup of glycosaminoglycans (GAGs) within cells everywhere in the body. Due to this accumulation, many cells of the body become swollen, which makes this disorder a progressive debilitating condition. Currently, enzyme replacement therapy (ERT) is the only FDA approved treatment option for affected individuals. Unfortunately, ERT does not treat or provide a cure for this disorder. For a permanent therapy in the future, gene
therapy may be the solution. In this study, I examine the effect of a non-viral vector containing the IDS gene on MPSII fibroblasts. The IDS gene was cloned into a non-viral vector, pIRES-hrGFP-2a. MPSII fibroblasts were transfected with the construct, which encodes the I2S enzyme. A 4-methylumbelliferyl sulfate (4-MUS) assay was performed to test for secreted sulfatases in the conditioned media (CM) after transcription of the IDS gene was allowed to proceed. Fluorescence indicated the release of a fluorophore when the sulfate group on the 4-MUS sulfate was cleaved by sulfatases. The highest sulfatase activity was present in the CM post transfection indicating that the I2S enzyme was successfully produced and secreted. The pIRES-hrGFP-IDS-2a construct yielded I2S enzyme up to 24 hours. MPSII fibroblasts survived with the construct for up to 48 hours. In this study, we show how a non-viral vector can be used to restore gene function and prolong the life of mutated cells. This non-viral construct with modifications can be used to cross the blood-brain-barrier to treat this disease in the central nervous system (CNS), which is not currently available for treatment because of the blood-brain-barrier (BBB).
Chapter 1: Introduction

Lysosomal Storage Malfunctions

To date, there are over thirty known lysosomal storage disorders.¹ These disorders develop because of the debris and waste that accumulates in the lysosomes. Lysosomes are organelles that are found within cells that contain specific hydrolase enzymes that aid in degradation and digestion of any cellular debris and waste materials, which can include lipids, polysaccharides and some proteins.¹ There is a deficiency of an enzyme which is responsible for degrading glycosaminoglycans (GAGs).² There are more than 40 acid hydrolases in the lysosome, and each catalyzes the degradation of a single type of intermolecular bond. In Hunter syndrome, the accumulation of GAGs leads to enlarged lysosomes, which in turn lead to the malfunction of many organs and tissues. Specific genes are responsible for the production of each hydrolase.² When these genes are mutated, the activity of the encoded enzyme is diminished.

Iduronate 2-Sulfatase (IDS)

One such gene that is responsible for diminishing the production of lysosomal enzymes is the Iduronate 2-sulfatase (IDS) gene, which is found on the X chromosome on the q arm at position 28 (Figure 1).⁵ The IDS gene is responsible for making a lysosomal enzyme called iduronate 2-sulfatase (I2S).³ Scientists have resolved that location of this gene is at approximately in Xq28 to a region of 148,560,200 to 148,586,900 base pairs.⁶ This makes the IDS gene 33 kilo-base
pairs in length.\textsuperscript{6} Although this gene may seem very large, the coding region to make the iduronate 2-sulfatase enzyme is only 1,653 base pairs.\textsuperscript{3} Mutations within this gene can cause an X-linked recessive disorder called Mucopolysaccharidosis Type II (MPSII), or Hunter syndrome. Different types of IDS mutations have been characterized with the genomic make up of this gene. The most common mutation seen with the IDS gene is the different changes of nucleotides when the DNA is going through a period of mitosis.\textsuperscript{4}

**Figure 1:** Diagram of human chromosome X, with the IDS gene position marked with a yellow arrow. Karyotypic bands are indicated with vertical stripes. Location of the IDS gene is at Xq28 (Genetics Home Reference)
Clinical Features of MPSII

Mucopolysaccharidosis type II is seen more frequently in males than females because it is a X-linked recessive disorder and males are hemizygous. Individuals with MPSII do not show any clinical features at birth of this disorder until ages from two to four.\textsuperscript{11} Some of the clinical features of the disorder within children at this age are enlarged nose, tongue, cheeks and lips.\textsuperscript{12} Other features include a large spleen and liver.\textsuperscript{11} The vocal cords, located in the neck, also become enlarged resulting in a deep voice.\textsuperscript{13} Sleep apnea can occur because the airway becomes constricted. Individuals with this disorder are often seen with macrocephaly (an enlarged head) and hydrocephalus (an accumulation of fluid in the brain).\textsuperscript{11} This disorder also affects one of the most vital organs.\textsuperscript{13} The heart may become abnormally large and heart valve problems may develop which can unfortunately lead to cardiac arrest. Individuals with this disorder can have a range of intellectual and development disabilities, ranging from mild to severe.\textsuperscript{12} Both of mild and severe forms of this disease lead to the clinical features described above, but the severe form leads to a decline in intellectual ability and faster disease development.\textsuperscript{12} The individuals with the severe form of the disease lose the ability to have the basic performance skills and the life expectancy for these individuals is only from the ages of ten to twenty years.\textsuperscript{12} Those with the mild form of this disorder do not have their Intelligence affected but have a shorter lifespan and live into early adult stages of life.\textsuperscript{12} Figure 2 shows the life span of MPSII individuals versus age.
Dermatan Sulfate and Heparan Sulfate

I2S is necessary for the digestion of Dermatan Sulfate and Heparan Sulfate.5 Dermatan Sulfate and Heparan Sulfate are GAGs, which are made up of unique structures of duplicating disaccharide units. Several enzymes are involved in the degradation pathway of these two GAGs (Appendix A and B).7,8,9,10 The Iduronate 2-sulfatase (IDS) gene is responsible for generating an enzyme called iduronate 2-sulfatase (I2S) that is necessary for the digestion of GAGs. GAGs can also be referred to as mucopolysaccharides. GAGs are un-branched long polysaccharides that have a unique structure of duplicating disaccharide units. There are different types of GAGs that exist in the glycosaminoglycan family. Some familiar examples of GAGs include chondroitin sulfate, heparan sulfate, keratin sulfate, dermantan sulfate and hyaluronan acid. These subfamilies of GAGs differ in the composition of their glycosidic linkages. The enzyme I2S is responsible for diminishing sulfate from an acid called sulfated alpha-L-iduronic
acid. Sulfated alpha-L-iduronic acid is seen in both dermatan sulfate and heparan sulfate (Figure 3 and 4). Iduronate 2-sulfatase (I2S) is found in the lysosomes. Iduronate 2-sulfatase has a robust sequence homology with enzymes such as the *Homo sapiens* glucosamine-6-sulfatase and the enzymes *Homo sapiens* arylsulfatases A, B, and C.\(^7\) Dermatan sulfate is found in various parts of the body. It is found in the skin and various valves, vessels, tendons and the lungs. It has been found that dermatan sulfate plays several roles in carcinogenesis, coagulation, heart disease and fibrosis.\(^8\) Heparan sulfate is the most common form of GAG that is seen in nature. It is found in tissues of all animals. Heparan sulfate is usually seen on either cell surfaces or close to extracellular matrix proteins. Heparan sulfate can bind to several co-factors that are involved for many biological processes which include developmental processes, coagulation and angiogenesis. Heparan sulfate has a very similar homology to heparin since both these molecules carry a consistent repeating disaccharide.
Pathway of Glycosaminoglycan Degradation

The Iduronate 2-sulfatase (IDS) gene is responsible for creating the Iduronate 2-sulfatase (I2S) enzyme. As mentioned previously, this enzyme is responsible for the degradation of specific glycosaminoglycans. Those specific glycosaminoglycans are heparan Sulfate and dermatan Sulfate. The degradation
of these glycosaminoglycans occurs in the lysosomes that are located within cells. Lysosomes are organelles that are located within cells that carry enzymes to break down specific cellular waste and debris and recycle those products. Dermatan sulfate has a unique pathway to degradation before it starts to accumulate in the lysosomes.\textsuperscript{8} Dermatan sulfate with the addition of specific hydrolases is converted into dermatan sulfate disaccharide.\textsuperscript{9} This structure is then sulfated and becomes a structure filled with L-iduronate and N-acetyl-Beta-D-galactosamine.\textsuperscript{9} Iduronate 2-sulfatase enzyme becomes involved in this step to make this molecule desulfated.\textsuperscript{9} Dermatan sulfate that is bound to L-iduronate and N-acetyl-Beta-D-galactosamine is digested into two molecules, L-iduronate and dermatan sulfate with N-acetyl-Beta-D-galactosamine.\textsuperscript{9} The later molecule goes on to become an independent N-acetyl-Beta-D-galactosamine.\textsuperscript{9} Heparan sulfate is degraded by heparanases that cut the heparan sulfate from the proteoglycan protein structure and digests them into small oligosaccharides.\textsuperscript{13} Heparanases are important since they are needed for sites for inflammation and injury.\textsuperscript{14} These enzymes have been successfully removed from chinese hamster ovary cells, platelets and placenta.\textsuperscript{14} Appendix A and B show the degradation of dermatan sulfate and heparan sulfate.

**Bone Marrow Transplant**

MPSII can be treated with Bone marrow transplantation. Bone marrow transplant is the process where the damaged or destroyed bone marrow is replaced with stem cells from a healthy bone.\textsuperscript{15} Bone marrow transplantation for a lysosomal storage disorder was first carried out in 1981 when a one-year-old boy, with
Mucopolysaccharidosis Type I (Hurler Disease), received the bone marrow from his mother. Three to four months after the transplant, iduronidase activity was present in the blood serum and in the urine. This finding implied that GAGs were degraded and excreted through the urine. Since this finding, bone marrow transplantation has proved to be an effective therapy for those who are affected by any lysosomal storage disorders. Although bone marrow transplantation works very effectively, this therapy does have many disadvantages. Graft-versus-host disease can arise where the body of the host can reject anything that is foreign. The difficult of finding a perfect match and the cost of the treatment can leave patients in a very difficult position. To cure those individuals who endure the hardships of this disease, gene therapy can be possible through the replacement of the mutated IDS gene with the non-mutated IDS gene with gene therapy. By understanding the severe clinical phenotypes of this disease, it becomes important to find a therapy for individuals who suffer from this disorder. Fortunately, enzyme replacement therapy (ERT) and gene therapy have proven to be therapies that can be cost effective as well as treat and cure the individuals who have this disorder.

**Enzyme Replacement Therapy and Gene Therapy**

Enzyme replacement therapy (ERT) and gene therapy are available and are currently investigated by scientists for those who suffer from lysosomal disorders such as Gaucher’s or Pompe disease. ERT is a treatment that is used to restore an enzyme that is either missing or deficient. Elaprase, idursulfase, is an enzyme
replacement therapy that is used for patients who have MPSII. This therapy involves injecting the individual with the purified form of the I2S enzyme from a human cell line. This therapy has worked to treat short stature, which showed that the height of patients continually increased when the therapy was constantly delivered for several years. ERT has been shown to be effective in treating respiratory function, the ability to walk, and a higher joint mobility. Unfortunately, ERT does not treat or cure the disease but only takes care of the symptoms that the individuals experience. Gene therapy can be used to treat and even cure patients with genetic and acquired disorders. Most recently advances have been made with genetic diseases such as Hemophilia and Severed Acquired Immune Deficiency (ADA-SCID) and acquired diseases such as Parkinson’s disease, Huntington’s disease and even different types of head and neck cancers. The concept of gene therapy emerged in the 1970s, once there was extensive knowledge of genes and their function. The first gene therapy trials were done with recessive genetic disorders, which included cystic fibrosis, lysosomal storage disorders and several others. Although there have been many great advances in the scientific community using gene therapy, several accidents have occurred at the cost of innocent lives. One primary example is the death of Jesse Gelsinger. Gelsinger suffered from Ornithine Transcarbamylase Deficiency, which is another x-linked recessive disorder. Gelsinger was given gene therapy by an infusion of the adeno-associated viral vector that corrected the non-mutated version of the gene. Gelsinger passed away due to a massive organ failure since his body rejected the viral vector. In 2000, gene therapy in France for x-linked Severed
Combined Immune Deficiency SCID had to be halted, because of the deaths of two children who were treated and developed leukemia.\textsuperscript{21} The viral vector that was used activated a cancer gene.\textsuperscript{21} Although there have been unfortunate incidents in the history of gene therapy, the vectors used in this type of therapy have brought about effective treatment for individuals who have diseases with no cure. In 2005, researchers at the University of Michigan in Ann Harbor, used gene therapy in young adult guinea pigs who were deafened by ototoxic drugs that destroyed the inner ear hair cells.\textsuperscript{22} An adenoviral vector with the Atoh1 gene, to promote hair cells, was injected into the guinea pigs.\textsuperscript{22} With time, inner ear hair cells began to grow and hearing was 50\% to 80\% replaced.\textsuperscript{22} Gene therapy, with the use of specific vectors, has been shown to replace a host’s mutated gene with a fully functioning one in many different cell lines.

\textit{Ex Vivo and In Vivo Therapies}

There are two administration routes at present for gene therapy: \textit{ex vivo} and \textit{in vivo}.\textsuperscript{23} \textit{Ex vivo} route is the isolation of an individual’s cells followed by gene transfer.\textsuperscript{23} Those cells are then placed back into the individual. The \textit{In vivo} route is used when the gene is directly inserted into the individual.\textsuperscript{23} The problem with the \textit{in vivo} route is that there can be unwanted consequences that might affect additional tissues and organs.\textsuperscript{23} The gene may enter unwanted cells that can lead to the failure of that specific organ. Gene transfer, whether done \textit{in vivo} or \textit{ex vivo} is carried out with the use of vectors.\textsuperscript{23}
Vectors

Retroviral, adenoviral, adeno-associated viral and non-viral vectors are just some examples of different vectors that have been constructed for gene therapy.\textsuperscript{23} Viral vectors have been used for \textit{in vivo} transfer of genes.\textsuperscript{23} Retroviral vectors are single-stranded RNA viruses that can incorporate their single stranded genome into the host’s genome.\textsuperscript{23} One potential complication of gene therapy with viral vectors raises questions of whether there could be a possible immune response against the new gene or if the gene transfer could interfere with the function of other nearby genes.\textsuperscript{23} Adenoviral vectors are double-stranded DNA viruses that are not incorporated into the host’s genome but also do not replicate during mitosis.\textsuperscript{23} Adenoviral vectors have high transient transgene expression levels.\textsuperscript{23} Adeno-associated viral vectors are single-stranded DNA viruses that are present in cells in an extrachromosomal state but do not incorporate themselves into the host’s genome.\textsuperscript{23} This may make adeno-associated viral vectors very ideal for gene therapy since there are no undesired side effects. Non-viral vectors are double-stranded plasmid bacterial DNA.\textsuperscript{23} One of the interesting aspects of non-viral plasmids is that they maybe treated in order to cross the blood-brain-barrier to treat diseases that affect the central nervous system and the brain.\textsuperscript{24} The blood-brain-barrier (BBB) is a physical partition between the brain extracellular fluid and the normal circulating blood that flows throughout the body.\textsuperscript{25} It acts to protect the brain and the central nervous system against any viral or bacterial infections. Even certain antibiotics and antibodies are too large to cross through this barrier.\textsuperscript{26} This becomes difficult when rare bacterial infections occur in the
brain, which become very hard to treat. Viral vectors have been shown to not cross the BBB.\textsuperscript{27} This finding leads to immense difficulties to create specific pharmaceutical drugs that can target the brain for brain disorders. Fortunately, non-viral vectors have been shown to cross this barrier.\textsuperscript{27} Unfortunately, individuals who have MPSII are also affected by an abnormal central nervous system, brain and are seen with head fluid abnormalities.\textsuperscript{28} Unfortunately, even ERT has not been successful to treat individuals with this type of infection.

\textbf{pIRES-hrGFP-2a Non-Viral Vector}

There has been much research on the treatment of lysosomal storage disorders with the use of gene therapy. By understanding the mechanisms of the etiology of each storage disorder, it is possible to develop gene therapy appropriately. Since one of the characteristics of MPSII is progressive degeneration of the central nervous system, it is important to develop therapy with factors that will not provoke the immune system, will replicate epigenetically, will be expressed at high levels and will be able to cross the BBB. Non-viral vectors, such as pIRES-hrGFPII, have shown a new light into gene therapy (Figure 5). This plasmid is originally isolated from bacteria, and contains no extraneous viral DNA, except for the promoter. This vector carries the robust cytomegalovirus (CMV) promoter.\textsuperscript{29} It has been shown that the CMV promoter can achieve high expression levels in the brain.\textsuperscript{28} The CMV promoter has been shown to be more effective than other promoters.\textsuperscript{29} The reason why the CMV promoter has a higher expression rate than other promoters is because the promoter and enhancer have
many regulatory and transcriptional elements that can be provided by a wide variety of transcriptional factors. The pIRES-hrGFPII is an unique vector in that they carries the Herpes simplex virus Thymidine Kinase (HSV-TK) gene which can be used for drug binding. HSV-TK has been shown to successfully prevent graft-versus-Leukemia after a bone marrow transplant. HSV-TK has been shown to be used successfully in treating human head and neck cancer, proving that the gene could be vital in crossing the BBB. If a sequence was inserted into this vector that would allow the encoded enzyme to cross the BBB, the pIRES-hrGFPII non-viral vector, with the HSV-TK, would prove to be a very powerful tool for gene therapy to cross the BBB. This vector would treat the part of the disease that results from enzyme deficiency in the CNS. Once the non-mutated version of the IDS gene is placed into the non-viral vector, the vector is then tested out in MPSII affected fibroblast cell strain. The cell strain that will be used to test the non-viral vectors to compare the efficiency of the I2S enzyme production would be in transiently transfected MPSII male’s fibroblasts cells. Once high expression is seen, then either ex vivo or in vivo therapies can be carried out in a specific animal model.
Animal Models

Animal models have been the focus of scientific research for decades. The history of animal testing dates back to the third and fourth centuries where Erasistratus and Aristotle were the first individuals to execute experiments on living animals. Since humans are derived from animal species, both are genetically very similar. It is proposed that any beneficial conclusions that are carried out from the experimentation on animals can have similar results to those carried out in human beings. Initial studies on mouse models can provide to be very effective. Mice have advantages in that they are easy to breed, could be produced in large
quantities and their entire genome has been sequenced. In larger animals, the advantages of size can be effective in clinical or surgical techniques. Physiological and the long-term effects of gene therapy can be seen through the usage of larger animals. Fortunately for scientific research, some animals are already born with lysosomal storage disorders, which make them an excellent candidate to study for developing gene therapy. The first lysosomal storage disorder that was identified, besides in a human, was in 1971 in a Siamese cat. Animals that have naturally occurring lysosomal storage disorders are mice, pigs, goats, sheep, quail, dogs, cats, cattle, guinea pigs and emus. These animals have been continually studied for the effects of gene therapy on the lysosomal storage disorders.

**Purpose of Study**

Gene therapy has been proven successful by the use of vectors that promote the function of a non-mutated gene. Bone marrow transplantation, enzyme replacement therapy and gene therapy have all been used to develop strategies for treating various diseases. Only gene therapy has seen proven to successfully show the path of a proper cure. It is hoped that the use of the non-viral vector, pIRES-hrGFP-2a, will be highly effective in delivering a gene for missing enzymes to cells deficient in Iduronate 2-Sulfatase.
**Hypothesis**

The function of the IDS gene can once again resume to function in affected MPSII fibroblast cells with the non-viral expression vector construct, pIRES-hrGFP-IDS-2a. It is my working hypothesis that the I2S enzyme will be produced, which will be secreted to the conditioned media (CM) and will be positively confirmed by the 4-MUS assay.
Chapter 2: Materials and Methods

Materials

The human *IDS* DNA, to provide as an insert for the pIRES-hrGFP-2a vector construct, was provided in the pOTB7 vector and was kindly provided by Dr. YoungBae Sohn (Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea). The pIRES-hrGFP-2a vector was purchased from Agilent Technologies (Santa Clarita, CA). To take DNA concentration readings, the Nanodrop 2000 Spectrophotometer from Thermo Scientific was used (Wilmington, DE). Absorbance was measured at 360nm and 380 nm. The 360nm/380nm ratio was multiplied by 50 to give the nanograms per microliter concentration of the DNA. The Phusion® High-Fidelity DNA Polymerase (New England BioLabs), with the accompanying buffer, was generously provided by Dr. Stan Metzenberg (California State University, Northridge). The restriction enzyme, XhoI with the accompanying buffer, was also graciously provided by Dr. Stan Metzenberg (California State University, Northridge). The restriction enzyme, SacII with the accompanying buffer, was provided by Dr. Mary-Pat Stein (California State University, Northridge). The Taq DNA Polymerase (1/50) was gifted to the lab by Dr. Michael Summers. The MAX Efficiency® DH5α™ Competent Cells were purchased from Invitrogen (Grand Island, NY). The Wizard® Plus SV Minipreps kit was purchased from Promega (Madison, WI). Mucopolysaccharidosis Type II fibroblasts (GM00140) were purchased from Coriell Institute Cell Repositories (Camden, NY). The
inverted microscope was kindly provided by Dr. Steven Oppenheimer (California State University, Northridge). Fetal Bovine Serum (FBS) (Atlanta Biologicals) and Trypsin-EDTA (Lonza) were purchased. DMEM/Ham’s F-12 powder was purchased from Cellgro (Manassas, VA). Opti-MEM® I Reduced Serum Media and Lipofectamine® were purchased from Invitrogen (Grand Island, NY). The fluorescent microscope was kindly provided by Dr. Rheem Medh. 4-Methylumbelliferone sodium salt and 4-Methylumbelliferone sulfate potassium salt were purchased from Sigma-Aldrich (St. Louis, MO). The 4-methylumbelliferone assays were performed by using the TKO 100 Mini-Fluorometer by Hoefer Scientific Instruments (Holliston, MA).

Isolation of the IDS Gene

The IDS gene was isolated from the pOTB7 vector (Open BioSystems). To isolate the DNA, primers were designed to contain 5’ and 3’ ends of the gene and 5’ and 3’ ends of the pIRES-hrGFP-2a vector (Table 1). PCR was then performed to amplify the IDS gene using the primers described in Table 1.
Table 1: Primer sequences used to amplify the IDS gene from pOTB7 vector

<table>
<thead>
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<td>ACCCGGGA</td>
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<tr>
<td></td>
<td>CCGGCCGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>TGGAACGT</td>
<td>54 bp</td>
<td>65.9° C</td>
<td>40.7%</td>
</tr>
<tr>
<td>Primer</td>
<td>CATATGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TACTCGAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTACTTAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACTTTCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTAGATGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGTCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR reaction reagents were used to carry out the PCR reaction in this exact order (Table 2) and then placed into a thermocycler with specific PCR conditions, temperature and time for each step (Table 3).
Table 2: PCR reaction mix for the amplification of IDS gene

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-pure H2O</td>
<td>36 μL</td>
</tr>
<tr>
<td>5x Phusion HF Buffer</td>
<td>10 μL</td>
</tr>
<tr>
<td>25 mM dNTP</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>Forward Primer (5 pmoles)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse Primer (5 pmoles)</td>
<td>1 μL</td>
</tr>
<tr>
<td>pOTB7 Vector (1 ng/μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Mineral Oil</td>
<td>30 μL</td>
</tr>
<tr>
<td>Phusion® High-Fidelity DNA Polymerase*</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>Total</td>
<td>80 μL</td>
</tr>
</tbody>
</table>

*Phusion® High-Fidelity DNA Polymerase was added after the PCR reaction was denatured at the beginning of the reaction in the thermocycler. The reaction was paused at the beginning of the annealing reaction at 54°C. 0.5 μL of the polymerase was then added to the tube containing the other reagents, quickly mixed and centrifuged and the reaction was once again started.

Table 3: PCR conditions

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>97</td>
<td>5 Minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>97</td>
<td>15 Seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing*</td>
<td>54</td>
<td>10 Seconds</td>
<td>10</td>
</tr>
<tr>
<td>------------</td>
<td>----</td>
<td>-------------</td>
<td>----</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>90 Seconds</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>97</td>
<td>15 Seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>90 Seconds</td>
<td>25</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>13 Minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

*Right before the annealing step is to start, the thermocycler was paused and 0.5uL of Phusion® High-Fidelity DNA Polymerase was added to the tube containing the PCR reaction reagents, quickly mixed and centrifuged and the thermocycler was started again.

6.5% Polyacrylamide Gel Electrophoresis

Once the reaction was completed, 10 uL of the reaction were mixed together with 2 uL of 6X loading dye (Fermentas). The samples were electrophoresed on a 6.5% Polyacrylamide Gel Electrophoresis (PAGE) in lanes 2 through 6. GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) was used as a reference in lane 1 to estimate the size of electrophoresis bands. Once the electrophoresis was completed, it was removed from the apparatus and stained with 0068 ethidium bromide (Sigma-Aldrich) to observe bands under the UV Transilluminator.

DNA Gel Extraction and Purification

A gel slice containing the amplified IDS gene was crushed and the DNA was eluted using 350uL of an elution solution. The elution solution contained 10mM Tris, 200 mM NaCl, and 1 mM EDTA in an eppendorf tube. The tube was
incubated overnight at room temperature. The following day, the eppendorf containing the crushed gel and elution buffer was spun down at 20,800g for 10 minutes. The supernatant, containing the DNA, was then transferred into a new eppendorf tube and was used for ethanol precipitation. 1 mL of cold 100% ethanol and 1 uL of 20 mg glycogen were added to the tube containing the DNA. The solution was then vortexed until white pellets or a cloudy solution started to appear. The solution was then centrifuged for 20 minutes at 20,800g. At the end of centrifugation, a white pellet had formed. The supernatant was discarded without disturbing the pellet. The pellet was then washed with 1 mL of cold 70% ethanol and then centrifuged at 20,800g for 5 minutes. The supernatant was then removed and discarded. The pellet was left to air dry for 30 minutes and then re-suspended with 6 uL of nano-pure water. The pellet was then stored in -20°C.

Digestion and Purification of pIRES-hrGFP-2a Vector

The vector, pIRES-hrGFP-2a was digested with the restriction enzymes SacII (NEB) and XhoI (NEB). The vector was digested by a single digestion each time. First the vector was digested with SacII. In an eppendorf tube, the reagents were added in the following order: 63 uL of nano-pure water was added, then 10 uL of 10x Buffer 4 (NEB), 25 uL of pIRES-hrGFP-2A (59.7 ng/uL), 1 uL of BSA and 1 uL of SacII (Table 4). This mixture was quickly mixed, centrifuged briefly and then set on a thermocycler to digest at 37°C for 12 hours.
Table 4: Restriction enzyme digest mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-pure H2O</td>
<td>63</td>
</tr>
<tr>
<td>10x Buffer 4 (NEB)</td>
<td>10</td>
</tr>
<tr>
<td>pIRES-hrGFP-2a (59.7 ng/uL)</td>
<td>25</td>
</tr>
<tr>
<td>Acetylated BSA (10 mg/mL)</td>
<td>1</td>
</tr>
<tr>
<td>20,000 units/mL SacII (NEB)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

After the reaction was completed, 1 uL of 20,000 units/mL XhoI (NEB) was added to the previous reaction and was digested at 37°C for 12 hours. After the reaction was completed, the reaction was treated with 1 uL of Shrimp Alkaline Phosphatase (Promega) to remove the 5’ phosphate groups, thus preventing the vector from re-circularizing and re-ligating of linearized coning vector DNA during ligation. The reaction was incubated in the thermocycler at 37°C for 60 minutes and then heat-denatured at 80°C for 20 minutes. To confirm that the DNA was properly digested, 10 uL of the digest reaction was mixed with 2 uL of loading dye and electrophoresed on a 0.8% agarose gel. Once the EtBr staining of the gel confirmed that the digestion was complete, the remainder of the reaction was ethanol precipitated. To perform ethanol precipitation, 5 uL of 5M NaCl were added to the remainder of the reaction and incubated for ten minutes at room temperature. After the incubation, 1 mL of cold 100% ethanol and 1 uL of 20mg/mL glycogen were added to the reaction. The solution was then vortexed.
until white pellets appeared or the solution turned cloudy. The reaction was then incubated at 4°C for 20 to 30 minutes. The reaction was then centrifuged at 20,800g for 20 minutes. The supernatant was carefully removed and discarded. In order to wash the precipitated DNA in the pellet, 100 uL of 70% ethanol was added and the pellet was gently vortexed for one minute and centrifuged 20,800g for 10 minutes. The supernatant was once again carefully removed and discarded. The pellet was then set to air-dry and then re-suspend into 6 uL of nano-pure water. A nano-drop spectrophotometric reading was taken to measure the concentration of DNA.

**Subcloning of the IDS Gene**

Insertion of the IDS gene into a new vector, isolated from pOTB7 vector, and the digested vector, pIRES-hrGFP-2a, was performed by the One-step Sequence and Ligation-Independent Cloning method, with the components shown in Table 5.

**Table 5: Experimental ligation mixture**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-pure H2O</td>
<td>2.3</td>
</tr>
<tr>
<td>Digested pIRES-hrGFP-2a (237.1 ng/uL)</td>
<td>0.5</td>
</tr>
<tr>
<td>Insert: IDS Gene (66.9 ng/uL)</td>
<td>5</td>
</tr>
<tr>
<td>10X Acetylated BSA</td>
<td>1</td>
</tr>
<tr>
<td>10X Buffer 2 (NEB)</td>
<td>1</td>
</tr>
<tr>
<td>T4 DNA Polymerase (3 units/μL) (NEB)</td>
<td>0.2</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

The ligation method also included a negative control where the insert was replaced with nano-pure water. The reaction proceeded at 50°C for 1 hour on a thermocycler.

**Transformation**

Transformation was performed by adding 1 μL from each ligation reaction to 100 μL of MAX Efficiency® DH5α™ Competent Cells (Invitrogen) in 15 mL falcon tubes. The tubes were gently tapped to mix the components together. The falcon tubes were placed on ice for 30 minutes. After the incubation, the cells were heat shocked in a water bath at 42°C for 45 seconds and placed back on ice for 2 minutes. 900 μL of pre-warmed Super Optimal Broth (S.O.C.) was added to the falcon tube and then were set to shake at 37°C at 225 RPM for 1 hour. 100 μL, 300 μL, and the rest of the cultures were plated onto Luria-Bertani (LB) agar plates with 50 μg/mL Ampicillin. The LB/Ampicillin plates were then incubated at 37°C overnight.

**Colony Screening and Confirmation**

Colonies that were grown on LB/Ampicillin plates were picked and screened via PCR for the insert. Conditions for the PCR screening are shown in Table 6.
Individual colonies were picked and placed in eppendorf tubes that contained 50 
μL of Luria Bertani Broth (LBB) with Ampicillin. 0.5 μL of each colony was 
taken for Touchdown PCR as shown in Table 7.

Table 6: Reaction mix for PCR Screening of Colonies

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-pure H2O</td>
<td>7.95</td>
</tr>
<tr>
<td>Standard Taq Buffer (10X)</td>
<td>12.5</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>3</td>
</tr>
<tr>
<td>dNTP (10 mM)</td>
<td>0.62</td>
</tr>
<tr>
<td>Forward Primer (5 pmol)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse Primer (5 pmol)</td>
<td>0.5</td>
</tr>
<tr>
<td>Colony Sample</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

Table 7: Reaction Conditions for Touch-down PCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>5 Minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>30 Seconds</td>
<td>2</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>60 Seconds</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>30 Seconds</td>
<td>2</td>
</tr>
<tr>
<td>--------------</td>
<td>----</td>
<td>------------</td>
<td>---</td>
</tr>
<tr>
<td>Annealing</td>
<td>58</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>60 Seconds</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>56</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>60 Seconds</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>54</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>52</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>60 Seconds</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>51</td>
<td>30 Seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>60 Minutes</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5 Minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

After the Touchdown PCR was completed, 10 uL of the reaction were added to 2 uL of 6X loading dye and the samples were run on a 6.0% PAGE to see if the insert was amplified.
**Isolation and Purification of pIRES-hrGFP-IDS-2A**

Vector DNA, from a confirmed colony, was grown overnight in LB/Ampicillin at 37°C at 225 RPM. The clone was isolated and purified using the Wizard® Plus SV Minipreps Kit (Promega). The cell culture was pelleted by centrifugation for 5 minutes at 5000 RPM and the supernatant was discarded. The pellet was re-suspended with 50 uL of Cell Resuspension Solution. 50 uL of Cell Lysis Solution was added and the sample was inverted 4 times to mix. 3 to 4 uL of Alkaline Protease Solution were added to the sample and inverted 4 times to mix. The sample was then set for incubation at room temperature for 5 minutes. 100 uL of Neutralization Solution were added to the sample and inverted 4 times. The sample was then centrifuged at 20,800g for 10 minutes at room temperature. Meanwhile, the Spin Column was inserted into the Collection Tube. After centrifugation, the cleared lysate or supernatant was put into the Spin Column. The Spin Column with the Collection Tube that carried the lysate was spun down for 2 to 3 minutes at 20,800g. 750 uL of Wash Solution with ethanol was added to the Spin Column and centrifuged for 2 to 3 minutes at 20,800g. The flow-through in the Collection Tube was discarded and the Spin Column was reinserted into the Collection Tube. Then 250 uL of Wash Solution with ethanol was once again added to the Spin Column and centrifuged for 2 to 3 minutes at 20,800g. The Spin Column was then transferred to an eppendorf tube and 100 uL of Nuclease-Free Water were added to the Spin Column. This was centrifuged for 2 to 3 minutes. The Spin Column was discarded and the vector DNA was taken for DNA
concentration readings on the Nano-Drop 2000 Spectrophotometer (Thermo Scientific). The pIRES-hrGFP-IDS-2a vector construct was stored in -20°C.

**Confirmation of pIRES-hrGFP-IDS-2a**

To confirm that the pIRES-hrGFP-2a vector has the IDS gene insert, the vector was subjected to double restriction enzyme analysis. Components of the restriction enzyme reaction are shown in Table 8.

**Table 8: Double-digest mix of pIRES-hrGFP-IDS-2a**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-pure H2O</td>
<td>62</td>
</tr>
<tr>
<td>10x Buffer 4 (NEB)</td>
<td>10</td>
</tr>
<tr>
<td>pIRES-hrGFP-2a (59.7 ng/uL)</td>
<td>25</td>
</tr>
<tr>
<td>Acetylated BSA (10 mg/mL)</td>
<td>1</td>
</tr>
<tr>
<td>20,000 units/mL SacII (NEB)</td>
<td>1</td>
</tr>
<tr>
<td>20,000 units/mL XhoI (NEB)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>100 uL</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated in a thermocycler at 37°C for 16 hours. 10 uL of the reaction were mixed with 2 uL of 6X loading dye (Fermentas) and the samples were then put to run on a 0.8% agarose gel. The GeneRuler® 1 kb DNA Ladder (Fermentas) was used for comparison, in order to estimate the size of the
digested DNA in fragments. Other samples that were loaded on the gel were the pIRES-hrGFP-2a and pIRES-hrGFP-IDS-2a uncut.

**Cell Culture**

Mucopolysaccharidosis Type II (MPSII) fibroblasts (GM00140) were purchased from Coriell Cell Repository and were cultured in a 37°C/5% CO₂ incubator with DMEM/Ham’s F-12, 18% Fetal Bovine Serum (FBS) (Atlanta Biologicals), L-glutamine and with penicillin-streptomycin (Invitrogen). This was the complete media. Fibroblast cells slowly started receiving several passages every 3 to 4 days without antibiotics to proceed towards transient transfection. Before each passage, the cells were first observed under an inverted microscope to check for 90% to 100% confluency. The spent medium was aspirated and 10 mL of Versene (PBS with EDTA) were added to the flasks to wash the cells. The Versene was then aspirated and replaced with 4 to 5 mL of pre-warmed Trypsin-EDTA (Lonza). The bottoms of the flasks were gently tapped so the adherent cells would start detaching from the bottom surface. The flasks were then incubated for 5 to 7 minutes in the 37°C/5% CO₂ incubator. The flasks were checked under the microscope after the incubation to see if the cells had been detached. Five to seven mL of complete media were then added to the trypsinized flasks to inactivate the Trypsin-EDTA. The cells were then transferred to a falcon tube and centrifuged for 5 minutes at 200g. The supernatant was discarded and 1 mL of complete medium was gently added to the pellet. A small amount of cells were aliquoted for cell quantification and viability. The appropriate amounts of cells
were added to new flasks that contained pre-warmed 20 mL of fresh complete media.

**Transient Transfection**

Lipofectamine® Reagent and Opti-MEM® I Reduced Serum were purchased from Invitrogen. A 24 well-plate was used to grow the MPSII fibroblasts. One day before Transfection, 5 x 10⁴ cells were plated in 500 uL of complete media without antibiotics in 15 wells. The cells were observed the following day to see 50% to 80% confluency according to manufacturer’s directions. The following procedure is for one Transfection reaction. 0.8 uL (200 ng) of pIRES-hrGFP-IDS-2a DNA was diluted in 25 uL of Opti-MEM® I Reduced Serum and mixed gently. In another tube, 0.8 uL of Lipofectamine® Transfection Reagent was diluted in 25 uL of Opti-MEM® I Reduced Serum and mixed gently. The diluted DNA and Lipofectamine® Reagent were gently mixed together and incubated at room temperature for 45 minutes. After the incubation, 150 uL of Opti-MEM® I Reduced Serum were added to the tube containing the DNA and Lipofectamine® Reagent. In the 24 well-plate, the complete media was aspirated and replaced with 200 uL of complete media without any serum. Then 200 uL of the diluted complexes were added to the wells and mixed gently by rocking the plates. The cells were then placed in a 37°C/5% CO₂ incubator for 2 to 72 hours. The media post Transfection, conditioned media, was collected after 2 hours, 24 hours, 48 hours and 72 hours. All Transfection experiments carried control wells as well as
triplicates for accurate results. The conditioned media (CM) was collected at different time points to measure sulfatase activity.

Fluorescence Microscopy

After 24 hours post Transfection, the conditioned media in the wells from the 24-well plate was saved and the MPSII fibroblasts were then washed with 500 uL Versene. After washing, the Versene was aspirated. The cells were trypsinized with 100 uL of Trypsin-EDTA and re-suspended in 300 uL of complete media. The cells with the complete media were then placed in an eppendorf tube and treated with 3uL of 20 mg/mL Hoechst 33258 stain (Invitrogen) to stain the nucleus. The cells were then subjected to incubation for 20 to 30 minutes at 37°C. 20 uL of the stained cells were placed onto a microscope slide and viewed under the fluorescence microscope. Fluorescence was observed by using filters for the Green Fluorescent Protein (GFP) and for the Hoechst stain. The ProgRes CapturePro 2.6 (Jenoptik) (Jena, Germany) software was utilized to take snapshots and save the fluorescent pictures.

4-Methylumbelliferyl Sulfate (4-MUS) Assay

The 4-Methylumbelliferone Sulfate (4-MUS) assay was performed using the TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments). Before the assay was performed, the fluorometer had to be calibrated. A stock solution of 1 uM 4-Methylumbelliferone Sodium (4-MU) was made and protected from sunlight since it is photosensitive. The fluorometer had to be on for 15 minutes before
taking any measurements. The sensitivity detector monitor was equilibrated at 50% by turning the Scale knob about 5 full clockwise turns from the fully counter clockwise position. 1.9 mL of the carbonate stop buffer (0.20M Sodium carbonate, anhydrous) was added to the cuvette and placed into the cuvette well. The Zero was adjusted until the display read 000. 100 uL of the 1 uM 4-MU were added to the cuvette and quickly mixed. The Scale knob then was adjusted quickly to display 500. This set the fluorometer to display 500 for a 50 nM 4-MU final concentration. For the Iduronate 2-Sulfatase assay, 1 uM of 4-MUS was made. 95 uL of CM sample was taken from each control and time (2h, 24h, 48h and 72h) and was incubated with 5 uL of 1 uM of 4-MUS for 5 minutes. After the incubation, the reactions were stopped with the addition of 1.9 mL of the carbonate stop solution (0.20M) and the fluorescence readings were recorded. A standard curve of 4-MUS was generated and the data were plotted. A trendline was created to see how much Methylumbelliferone was in solution after the assay.

**Generating a Standard Curve of 4-MU**

A standard curve of 4-methylumbelliferone (4-MU) fluorescence was created to calculate the amount of 4-MU produced after the sulfatases cleaved the sulfate from the 4-MUS. Once the fluorometer was calibrated and blanked, a series of concentrations were measured by fluorescence intensity as seen in Table 9.
Table 9: 4-MU standard concentrations and readings

<table>
<thead>
<tr>
<th>Carbonate Buffer (mL)</th>
<th>1 uM 4-MU (uL)</th>
<th>Sample Concentration (nM)</th>
<th>Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.9</td>
<td>100</td>
<td>50</td>
<td>500</td>
</tr>
<tr>
<td>1.8</td>
<td>200</td>
<td>101</td>
<td>1010</td>
</tr>
<tr>
<td>1.7</td>
<td>300</td>
<td>152</td>
<td>1523</td>
</tr>
<tr>
<td>1.6</td>
<td>400</td>
<td>198</td>
<td>1983</td>
</tr>
</tbody>
</table>
Chapter 3: Results

PCR of pOTB7 Vector Containing IDS Gene

The IDS gene was located on the pOTB7 vector between the restriction enzymes EcoR1 and XhoI (Figure 6). The gene was successfully isolated and amplified from the pOTB7 vector by performing PCR with IDS gene specific primers that attached to the 5’ and 3’ ends of the gene. The amplified fragment was 938 bp long (Appendix C) and the size was confirmed by a 6.5% PAGE (Figure 7).

Figure 6: pOTB7 vector carrying the IDS gene between the restriction enzymes EcoR1 and Xho1
Figure 7: IDS gene amplified with PCR

Lane 1 shows the GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) used for comparison. Lanes 2 through 6 show bands that represent the amplified IDS gene from the pOTB7 vector (Figure 2). The DNA was then eluted and purified for ligation.

**Restriction Enzyme Digestion of pIRES-hrGFP-2a**

The pIRES-hrGFP-2a vector was successfully digested by two restriction enzymes, SacII (NEB) and XhoI (NEB), by double digestion reactions and treated
with Shrimp Alkaline Phosphatase. Other samples of the pIRES-hrGFP-2a vector were also digested by a single restriction enzyme to confirm that the digestion was taking place. From these digestions, the vector was completely linearized and did not re-circularize. This could be confirmed by the single bands seen on the 0.8% agarose gel (Figure 8). With this confirmation, the digested vector was purified and used for ligation.

Figure 8: Restriction enzyme digestion of pIRES-hrGFP-2a
Lane 2 shows GeneRuler™ 1 kb Plus DNA Ladder (Fermentas). Lane 4 shows the pIRES-hrGFP-2a vector cut with both SacII and XhoI restriction enzymes. Lane 6 shows the pIRES-hrGFP-2a vector cut with SacII. Lane 8 shows the pIRES-hrGFP-2a vector cut with XhoI.

**Ligation of IDS Gene with pIRES-hrGFP-2a and Transformation of Clone**

The double digested pIRES-hrGFP-2a vector and the IDS gene insert were set for ligation using the One-step Sequence and Ligation Independent Cloning method. After ligation, the sample was subjected to transformation where the competent cells produced colonies, containing the pIRES-hrGFP-IDS-2a (Figure 9). Colonies forming on the LB agar plates containing Ampicillin shows that ligation may have worked.

**Figure 9: Transformation plate post ligation of the pIRES-hrGFP-2A vector and IDS gene insert**
Colony Screening with Touchdown PCR

Colonies produced from transformation (Figure 9) were individually screened to see if the pIRES-hrGFP-IDS-2a was in fact carrying the IDS gene insert.

Screening was performed on single colonies with Touchdown PCR and using PAGE with a 6.0% polyacrylamide gel (Figure 10). Out of the 8 colonies screened, lane 10 of the polyacrylamide gel showed a positive band indicating the IDS gene insert. The colony in lane 10 was then grown overnight in LB/Ampicillin media.

Figure 10: 6.0% PAGE showing screens of transformed colonies

Lane 1 shows the GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) used for size determination. Lane 2 is the negative control used in the ligation method. The
negative control is the colony produced from a ligation reaction that consisted of vector alone, without the insert. A band did not appear in lane 2, confirming the negative control. Lanes 3 through 10 each contained results of screening individual colonies. Only lane 10, the colony had the IDS gene insert in the pIRES-hrGFP-2a vector.

**Purification of pIRES-hrGFP-IDS-2a and Confirmation of IDS Gene**

The colony with the insert was grown overnight in LB/Ampicillin media and purified using the Wizard® Plus SV Minipreps Kit (Promega). For further confirmation that the pIRES-hrGFP-2a vector carried the IDS gene, double restriction enzyme digest reaction (SacII and XhoI) was performed on the vector and the sample was run on a 0.8% agarose gel (Figure 11).
Figure 11: Double digestion of pIRES-hrGFP-IDS-2a confirming the IDS gene.

Lane 1 and 10 shows the GeneRuler® 1 kb DNA Ladder (Fermentas) for size determination. Lane 3 is the pIRES-hrGFP-IDS-2a vector after being digested with SacII and XhoI. Lane 5 is the uncut circularized pIRES-hrGFP-IDS-2a vector. Lane 7 is the uncut circularized pIRES-hrGFP-2a vector without carrying the IDS gene insert.
Mucopolysaccharidosis Type II Fibroblasts Cell Culturing

Mucopolysaccharidosis Type II (MPSII) fibroblasts (GM00140, Coriell) were successfully cultured in a 37°C/5% CO₂ incubator with DMEM/Ham’s F-12, 18% Fetal Bovine Serum (FBS) (Atlanta Biologicals), L-glutamine and with penicillin-streptomycin (Invitrogen). Passages were performed every 3 to 4 days once the fibroblasts were 90% to 100% confluent (Figure 12). MPSII fibroblasts were eventually introduced into complete media without any antibiotics. MPSII fibroblasts were then cultured into multiple wells on a 24 well-plate.

Figure 12: 400X magnification of MPSII fibroblasts at 90% confluency.

Transient Transfection of pIRES-hrGFP-IDS-2a into MPSII fibroblasts

Transient Transfection was performed in 12 wells on a 24 well-plate that contained MPSII fibroblasts using Lipofectamine (Invitrogen) as described in the
manufacturer’s protocol. At least 50% confluency of MPSII fibroblasts had to be observed before Transfection could be performed (Figure 13). Figure 14 shows 400X magnification of MPSII fibroblasts post Transfection after 24 hours. Figure 15 shows 400X magnification of MPSII fibroblasts post Transfection after 48 hours and figure 16 shows 400X magnification of MPSII fibroblasts post Transfection after 72 hours.

Figure 13: 400X magnification of MPSII fibroblasts at 50% confluency in a well of a 24 well-plate.
Figure 14: 400X magnification of MPSII fibroblasts post transfection after 24 hours.

Figure 15: 400X magnification of MPSII fibroblasts post transfection after 48 hours.
Fluorescent Microscope Images of Transfected MPSII Fibroblasts

After Transfection, the MPSII fibroblasts were washed, trypsinized, re-suspended in complete media without antibiotics, and placed onto microscopic slides to be viewed under a fluorescent microscope. The different filters on the fluorescent microscope were successful in detecting fluorescence from the Green Fluorescent Protein (GFP) and the nucleus stained with the Hoechst 33258 stain (Figure 17). The images were captured by ProgRes CapturePro 2.6 (Jenoptik) software.
Figure 17: 400X magnification of transfected MPSII fibroblast cells

Figure 17a shows a 400X image of a Transfected MPSII fibroblast cell. Figure 17b shows a 400X image of a Transfected MPSII fibroblast fluorescing. Figure 17c shows a 400X image of a Transfected MPSII fibroblast under the GFP filter at a UV emission of 520 nm to see the GFP. Figure 17d shows a 400X image of a Transfected MPSII fibroblast under the Hoechst filter at a UV emission of 461 nm to see the nucleus. Figure 17e shows a merged image of figure 17c and figure 17d to see which parts of the cell are expressing the pIRES-hrGFP-IDS-2a construct.
4-Methylumbelliferyl Sulfate Assay on Secreted Sulfates

Secreted sulfates were measured in the conditioned media (CM) from transfected MPSII fibroblasts after 2 hours, 24 hours, 48 hours and 72 hours. To measure the sulfates, a 4-Methylumbelliferyl Sulfate (4-MUS) was performed. 4-MUS was added to the CM and the emitted fluorescence was measured on the TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments). The amount of 4-MU in nM was determined using a standard curve, as shown in Table 10. The Standard curve was plotted, as shown in Figure 18. Figure 19 shows the amounts of sulfates cleaved on 4-MUS over different time periods in control cells and cells that were transfected.

Table 10: Fluorescence Intensity from the 4-MUS assay

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Fluorescence Intensity</th>
<th>Methylumbelliferone, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0.759902176</td>
</tr>
<tr>
<td>2</td>
<td>98</td>
<td>9.843551607</td>
</tr>
<tr>
<td>24</td>
<td>324</td>
<td>32.4029447</td>
</tr>
<tr>
<td>48</td>
<td>330</td>
<td>33.00186664</td>
</tr>
<tr>
<td>72</td>
<td>320</td>
<td>32.00366341</td>
</tr>
</tbody>
</table>
Figure 18: Standard curve of 4-MUS

\[ y = 10.018x - 0.6127 \]
\[ R^2 = 1 \]

Figure 19: Fluorescence Intensity of 4-MUS from secreted sulfates from media collected from control, 2 hours, 24 hours, 48 hours and 72 hours.
Chapter 4: Discussion

The purpose of this project was to develop a mechanism for comparing non-viral vectors, in order to determine which plasmid vector to use as a basis for building a construct for gene therapy. Viral vectors are commonly used in gene therapy because of efficiency but may cause difficulties with immune response and interruption of native genes. It is hoped that the resulting non-viral vector would be easily administered, and could cross the blood-brain barrier (BBB). Such a construct could be used to treat a variety of disorders that affect both the central nervous system (CNS) and other organ systems. The method which was developed in this study uses MPS II as a model system for disease treatment, and a fluorescent assay for gene activity.

One-step Sequence and Ligation-Independent Cloning Method

An innovative method was used for cloning the IDS gene into the vector to be tested. The one-step sequence and ligation–independent cloning method was taught by Dr. Stan Metzenberg (CSUN). Instead of using the traditional restriction enzyme digestion and ligation process, this new and versatile method provides a simple alternative that saves time as well as material cost. The method is especially useful for cloning because multiple genes can be put into a vector of choice, in a single cloning step. Primers were designed to PCR-amplify the IDS gene from a non-viral construct gifted by Dr. YoungBae Sohn (Seoul, Korea) (Figure 6). The IDS gene was then subcloned into the pIRES-hrGFP-2a non-viral
vector. The most efficient molecular ratio insertion of the amplified IDS gene into the pIRES-hrGFP-2a vector was about 3:1 (insert to vector).

Properties of pIRES-hrGFP-2a

The pIRES-hrGFP-2a vector was chosen to carry the IDS gene insert because of the many useful properties it carries. This particular vector carries the Internal Ribosome Entry Site (IRES) sequence that permits the translation in the middle of mRNA to have a high production of protein synthesis, in all mammalian cells tested. The multiple cloning site (MCS) is located directly after the human Cytomegalovirus (CMV) promoter. The CMV promoter is known to be a strong promoter when used in mammalian cells. It is made of a viral sequence, but is not of enough substance or significance to cross any of the common problems of viral vectors. The IDS gene insert was placed directly after the CMV promoter sequence to generate high levels of gene transcription. Evidence in support of gene transcription is present when the Green Fluorescent Protein (GFP) produces a bright green signal (Figure 13c), observed through fluorescent microscopy. Transcription of the gene must simultaneously occur since the GFP is being transcribed at the same time.

Measurement of Transcription Levels of I2S from Cultured Transfected MPSII Fibroblasts

Fibroblasts from an individual with MPSII were transfected with the construct created by an insertion of the Iduronate Sulfatase (IDS) gene into pIRES-hrGFP-
2a. Conditioned media (CM) was collected from the transfected cells to test and to quantify the secreted sulfatases. Sulfatases could only be produced and secreted by fibroblasts if there was transcription of the IDS gene occurring. A 4-Methylumbelliferyl Sulfate (4-MUS) assay was performed to quantify the amount of sulfatases secreted in the CM. When a sulfate group is cleaved from 4-MUS, the resulting 4-MUS a 4-MU is released, and a fluorescent signal is produced. This fluorophore is most active at 449nm, and is visualized under UV light. Fluorescence of high intensity predicts that there are many sulfatases that were secreted in the CM which were produced by the IDS gene from the pIRES-hrGFP-IDS-2a construct. The CM was measured at different time points after transfection to assess the transcription of the IDS gene. CM was collected at 2 hours, 24 hours, 48 hours and 72 hours. At 2 hours, the fluorescent intensity was 98 and at 24 hours, the fluorescent intensity was 324. The fluorescent intensity increased quite drastically between 2 and 24 hours with an increase of nearly 3-fold. At 48 hours, the fluorescent intensity was 330 and at 72 hours, the fluorescent intensity was 320. Between 24 hours and 72 hours after transfection, there was no appreciable increase in the intensity of fluorescence. The transcription rate of the IDS gene plateaus after 24 hours (Figure 16). An increase in the degree of fluorescence of the I2S enzyme was observed up to 24 hours after the pIRES-hrGFP-IDS-2a construct was transfected into MPSII fibroblasts. This increase in fluorescence reflected transcription of the subcloned IDS gene. No change in the intensity of fluorescence was seen between 24 hours and 72 hours. Cells appeared to begin dying 72 hours after transfection, resulting
in a great deal of cellular debris in the cultures. This may be why no increase in fluorescence was observed at or after this point. The study succeeded in assessing a test for the efficiency of a plasmid vector in transferring a gene into a cell. It was also important to determine the degree of expression and longevity of the plasmid construct containing the IDS gene that was transiently transfected into IDS minus cultured cells. It appears that the vector used in this construct will not be appropriate for gene therapy, because it does not persist in vitro after 24 hours.

**Significance of Study**

It was found that the pIRES-hrGFP-IDS-2a construct was not efficient in transferring an IDS gene to a deficient cell with sufficient longevity to constitute a basis for gene therapy in MPSII. This implies that investigators must continue the search for a more appropriate foundational vector for developing gene therapy. Therefore, this research has resulted in diminishing the time and effort needed to develop this type of gene therapy. This will save investigators time and money. The ultimate goal of this work is to produce a construct for gene therapy that will direct adequate production of the enzyme encoded by the construct. It is hoped that the construct will be able, by virtue of additional insertion, to cross the BBB and effectively treat the CNS as well as somatic organs. A construct produced using the pIRES-hrGFP-2a as a foundation is unlikely to be successful, because the vector does not support long-term gene expression, as shown in this study. Gene therapy can treat and cure patients with many genetic disorders and would provide a permanent solution for lysosomal storage
disorders. pIRES-hrGFP-2a was a non-viral vector that might have eliminated the side effects of viral vectors in gene therapy. However, this construct was found not to be an effective vector for gene therapy, because although it supports expression of the otherwise deficient enzyme, this expression does not last long enough.

**Limitations**

Although this study was successful in transferring a construct encoding a deficient I2S enzyme via a non-viral vector, it still presented limitations. Contamination of the cells in the tissue culture hood and 37°C/5% CO2 incubator was a constant problem, resulting in cell death. This problem was solved when the incubator was calibrated, and cleaned with appropriate solutions using proper techniques. Sterilization equipment methods were used including a UV lamp to sterilize the inside and outside of the cell and tissue culture hood.

**Future Studies**

In this study, the MPSII fibroblasts transiently transfected with a non-viral construct containing the IDS gene produced active enzyme. Enzyme activity was assayed using an artificial sulfatase substrate, 4-MUS. The results showed a plateau in the amount of I2S produced in the time frame that was set for the experiment. Using the anti-Hemagglutinin (HA) tag antibody, western blot analysis could be performed in the future in order to confirm that the IDS gene encodes an active form of the I2S enzyme. The IRES sequence in this vector
could increase transcription and translation of certain proteins during mitosis or apoptosis. In the future to study the effect of the IRES sequence on the IDS gene, assays can be performed to see the levels of transcription with and without the IRES sequence. Further modifications to this vector construct could allow it to cross the BBB for gene therapy.
References


Appendix A: Dermatan Sulfate Degradation

Dermatan Sulfate

H₂O

Dermatan Sulfate Disaccharide

[Dermapan-sulfate]-2-O-sulfo-alpha-L-iduronate

H₂O

Iduronate 2-sulfatase (IDSi)

2H⁺

[Dermapan]-alpha-L-iduronate

H₂O

1-iduronidase (IDUA)

Alpha-L-iduronate

[Dermapan]-N-acetyl-D-galactosamine

N-acetyl-beta-D-galactosamine

H₂O

Alpha-glycan

N-acetyl-beta-D-galactosamine
Appendix B: Heparan Sulfate Degradation

**Alpha-iduronic Acid**

- Iduronate sulfatase
- Alpha-iduronidase

**Alpha-linked glucosamine**

- Heparan Sulfate
- Acetyl-CoA Transferase
- N-acetylglcosaminidase

**Beta-linked glucuronic Acid**

- Beta glucuronidase
- Glucuronate sulfatase
- N-acetylglucosamine 6-sulfatase

**Alpha-linked N-acetylglucosamine**
Appendix C

Homo sapiens Iduronate 2-Sulfatase mRNA Sequence:

939 aa

ATGCCGGCACCCGGACCGGGCCAGGCTTCTCTGGCTGGGTCTGGTT
CTGAGCTCCGTTCTGCCGCCTCGGATCCGAAACGCAGGCCAACCTGC
ACCACAGATGCTCTGAACATGCCTCATCCTATCCTTGAGATGACCTGC
CCCTCCCTGGGTATTGAGGATAAGCTGGTGAGGTCCCAATATT
GAACAACTGGCATACTGGCTTCCTTCCCGAAATGCTCTTGGCAGC
CAAGCAGTGTGCGCCGCCGCCGTATTTTTTTCTCAACTGGCCAGGAGA
CCTGACACCACCAGGGCCCTGTACGACTTCAACTCCTACTGGAGGTGAC
GCTGGGAAACTTCTCCACCAGGCAAGTCAACCTCAGGAGAATGGCT
GATGACCATGTGGATGACCTGCGC

CCCTCCCTGGGCTGTTATGGGGATAAGCTGGTGAGGTCCCCAAATATT
GACCAACTGGCATACTGGCTTCCTTCCCGAAATGCTCTTGGCAGC
CAAGCAGTGTGCGCCGCCGCCGTATTTTTTTCTCAACTGGCCAGGAGA
CCTGACACCACCAGGGCCCTGTACGACTTCAACTCCTACTGGAGGTGAC
GCTGGGAAACTTCTCCACCAGGCAAGTCAACCTCAGGAGAATGGCT
GATGACCATGTGGATGACCTGCGC

ATGCCGGCACCCGGACCGGGCCAGGCTTCTCTGGCTGGGTCTGGTT
CTGAGCTCCGTTCTGCCGCCTCGGATCCGAAACGCAGGCCAACCTGC
ACCACAGATGCTCTGAACATGCCTCATCCTATCCTTGAGATGACCTGC
CCCTCCCTGGGTATTGAGGATAAGCTGGTGAGGTCCCAATATT
GAACAACTGGCATACTGGCTTCCTTCCCGAAATGCTCTTGGCAGC
CAAGCAGTGTGCGCCGCCGCCGTATTTTTTTCTCAACTGGCCAGGAGA
CCTGACACCACCAGGGCCCTGTACGACTTCAACTCCTACTGGAGGTGAC
GCTGGGAAACTTCTCCACCAGGCAAGTCAACCTCAGGAGAATGGCT
GATGACCATGTGGATGACCTGCGC

61
Appendix D

Homo sapiens Iduronate 2-Sulfatase Amino Acid Sequence:

313 aa

MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIIVDDLRSPLGCYGDKLVRSPNDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVHPGIISSNHTDDSPYSWSFPPYHPSSEKYENTKCRGPDPHELHNLCPVDVLDPVTLPDKQSETQAIQLLEKMTSASPFFLAVGYHKPHPRYPKEFQKLYPLENITLAPDP
EVPDGLPPVAYNPWMDIRQREDVQALNISVPYPVQDFQEDQSSSTGFRL
KTSSTRKYK*

* is the stop codon.