ADENOVIRAL VECTORS AS A TREATMENT FOR SIALIDOSIS

THE COMPARATIVE USE OF HELPER-DEPENDENT AND FIRST-GENERATION ADENOVIRUSES FOR RESCUING SIALIDASE DEFICIENCY USING *IN VIVO* AND *IN VITRO* MODEL SYSTEMS

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MARK MITCHELL, B.Sc.H.

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AUTHOR: Mark Mitchell, B.Sc.H. (Queen's University)

SUPERVISOR: Professor S. A. Igdoura

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Abstract

Sialidosis is caused by the accumulation of the ganglioside GM3 and other sialoglycoproteins within the cells of the liver, kidney and brain. Currently there is no treatment for sialidosis, while other lysosomal storage disorders are being treated through enzyme replacement therapy or bone marrow transplantation. The helper-dependent, or "gutless" adenovirus system (HD) has recently been improved upon with reportedly less immunogenicity than its first-generation (FG) predecessor and lifelong transgene expression produced in its hosts. To this end, the complete mouse lysosomal sialidase gene was cloned into a HD-vector (AdmsialHD) and a FG-vector (AdmsialFG) in an attempt to rescue the sialidase-deficiency and associated phenotype in B6.SM fibroblasts and in the SM/J mouse. Lysosomal sialidase levels were increased to normal levels *in vitro* following both AdmsialHD and AdmsialFG infections while SM/J mouse infections at doses of 5×10^9 particles/mouse did not yield any increase in lysosomal sialidase activity or correct the associated phenotype. Interestingly, AdmsialHD only up-regulated sialidase to high levels in sialidase-null cells whereas AdmsialFG up-regulated sialidase significantly in all cell lines tested. Together, these data suggest that the therapeutic dose for both AdmsialFG and AdmsialHD should be elevated at least 10-fold in order to achieve phenotypic rescue and that FG-vectors possess some viral property, perhaps the E4 gene products, enabling them to attain greater transgene expression relative to HD-vectors.

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List of Abbreviations

Avidin-PE	R-Phycoerythrin Avidin D
BAC	Bacterial Artificial Chromosome
β-gal	β-Galactosidase
bp	Base Pair
cDNA	Complimentary Deoxyribonucleic Acid
CNS	Central Nervous System
CPE	Cytopathic Effect
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DPEC	Diethylpyrocarbonate
ER	Endoplasmic Reticulum
ERT	Enzyme Replacement Therapy
FACS	Fluorescence Activated Cell Sorting
FG-Ad	First Generation Adenovirus
G418	Geneticin
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GSL	Glycosphingolipid
HBS	HEPES-Buffered Saline
HBSS	Hank's Buffered Salt Solution
HD-Ad	Helper Dependent Adenovirus
HDL	High Density Lipoprotein

IFU	Infectious Units
ITR	Inverted Terminal Repeat
kb	Kilo Base Pair
kDa	Kilodalton
LDL	Low Density Lipoprotein
MALII	Biotinylated Maackia Amurensis Lectin II
MAP	2-Amino-2-Methyl-1-Propanol
MEF	Mouse Embryonic Fibroblast
MEM	Minimal Eagle's Medium
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
Mu-Nana	4-Methylumbelliferyl-n-Acetyl-α-D-Neuramide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PI	Post Infection
PFU	Plaque Forming Units
PPCA	Protective Protein/Cathepsin A
RNA	Ribonucleic Acid
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate

SNA	Fluorescein Elderberry Bark Lectin
TBS	Tris-Buffered Saline
VLDLR	Very Low Density Lipoprotein Receptor

Chapter 1

Introduction

1.1 Glycosphingolipids and Gangliosides: Biosynthesis and Catabolism:

Glycosphingolipids (GSL) is one of two classes of molecules (GSL and sphingomyelin) that are derived from sphingolipids, which consist of the long chain amino alcohol sphingosine (Ichikawa & Hirabayashi, 1998). GSLs are amphipathic molecules connected to virtually all eukaryotic cell membranes through the extracellular leaflet (Yamashita et al. 1999). The basic GSL consists of the hydrophobic molecule ceramide, which can be linked to hundreds of different oligosaccharides at various levels of orientation and are projected into the extracellular matrix (Ichikawa & Hirabayashi, 1998). Due to the vast array of oligosaccharides that can be used biologically to form GSLs, there exists between three to four hundred distinct GSLs of the glucosylceramide (GlcCer) type (Ichikawa & Hirabayashi, 1998). GSLs are involved in many different physiological processes, including growth and development, cell differentiation, immune response, cell-cell communication and as targets for microorganisms and toxins (Varki, 1993). Additionally, inborn errors of GSL catabolism, termed the glycosphingolipidoses, occur in nature through defects in enzymes that are responsible for GSL degradation (Jeyakumar et al. 2002). Any enzyme defect within the GSL catabolism pathway leads to an accumulation in the lysosome of the substrate for which the defective enzyme is specific (Jeyakumar et al. 2002).

Ganglioside biosynthesis (reviewed by Chatterjee, 1998) occurs on the inner leaflet of the endoplasmic reticulum membrane and begins after the

condensation of the amino acid serine with the fatty acyl coenzyme, palmitoyl-CoA, which is catalyzed by the enzyme serine fatty acyltransferase. 3-ketosphinganine is thus formed followed by a reduction reaction by NADPH, creating sphinganine. Sphinganine is then acylated by the fatty acyl CoA and oxidized to form a 4-trans double bond, producing ceramide, which is the precursor to GSLs and sphingomyelin. After the transportation of ceramide to the Golgi apparatus, glucose is transferred to ceramide from UDP-glucose by the enzyme UDP-glucose:Cer,β1-4glucosyltransferase (GlcT-1), producing glucosylceramide (GlcCer). A galactose residue is then transferred to the oligosaccharide chain of GlcCer from UDP-galactose by the enzyme UDP-galactose:GlcCer,β1-4-gal-transferase (GalT-2) and/or LacCer producing lactosylceramide (LacCer). LacCer is used as the common precursor to all GSL families as complex gangliosides are formed by the addition of monosaccharides and sialic acid residues to the growing oligosaccharide chains of LacCer. The importance of GSLs in mammalian development and differentiation can be seen through a knockout mouse that is deficient in ceramide glucosyltransferase, which is the initial transferase in GSL biosynthesis. This particular strain of mouse dies *in utero* from widespread apoptosis, demonstrating the vital role played by GSLs (Yamashita et al. 1999).

The biochemical pathway for ganglioside catabolism involves many different enzymes, with a deficiency in any one resulting in a specific lysosomal

storage disorder (Figure 1.1). Gangliosides are based on a hydrophobic ceramide with a hydrophilic oligosaccharide chain that has at least one sialic acid residue. The hydrophobic ceramide acts as an anchor, holding the ganglioside in the cellular membrane, while the hydrophilic oligosaccharide chain projects into the extracellular space. The majority of gangliosides are found in the brain, with twelve gangliosides identified specifically in the human brain, with other tissues in the body containing various different gangliosides (Gravel et al., 1995).

Gangliosides are sent to the lysosomes through routine turnover of plasma membrane components via endocytosis (Figure 1.1). GM1 is the product of the initial catabolic event, perhaps due to membrane-bound sialidase, with all but one sialic acid residue removed. Once inside the lysosome, GM1 is hydrolyzed by the removal of galactose through the activity of β-galactosidase to form GM2. GM3 is then formed from the removal of N-acetylgalactosamine from GM2 via β-hexosaminidase A. Lactosylceramide is formed once the remaining sialic acid residue is cleaved by lysosomal sialidase, which is then followed by the removal of the final galactose by the combined action of β-galactosidase and glucosylceramidase to yield ceramide (Sandhoff & Kolter, 1997).

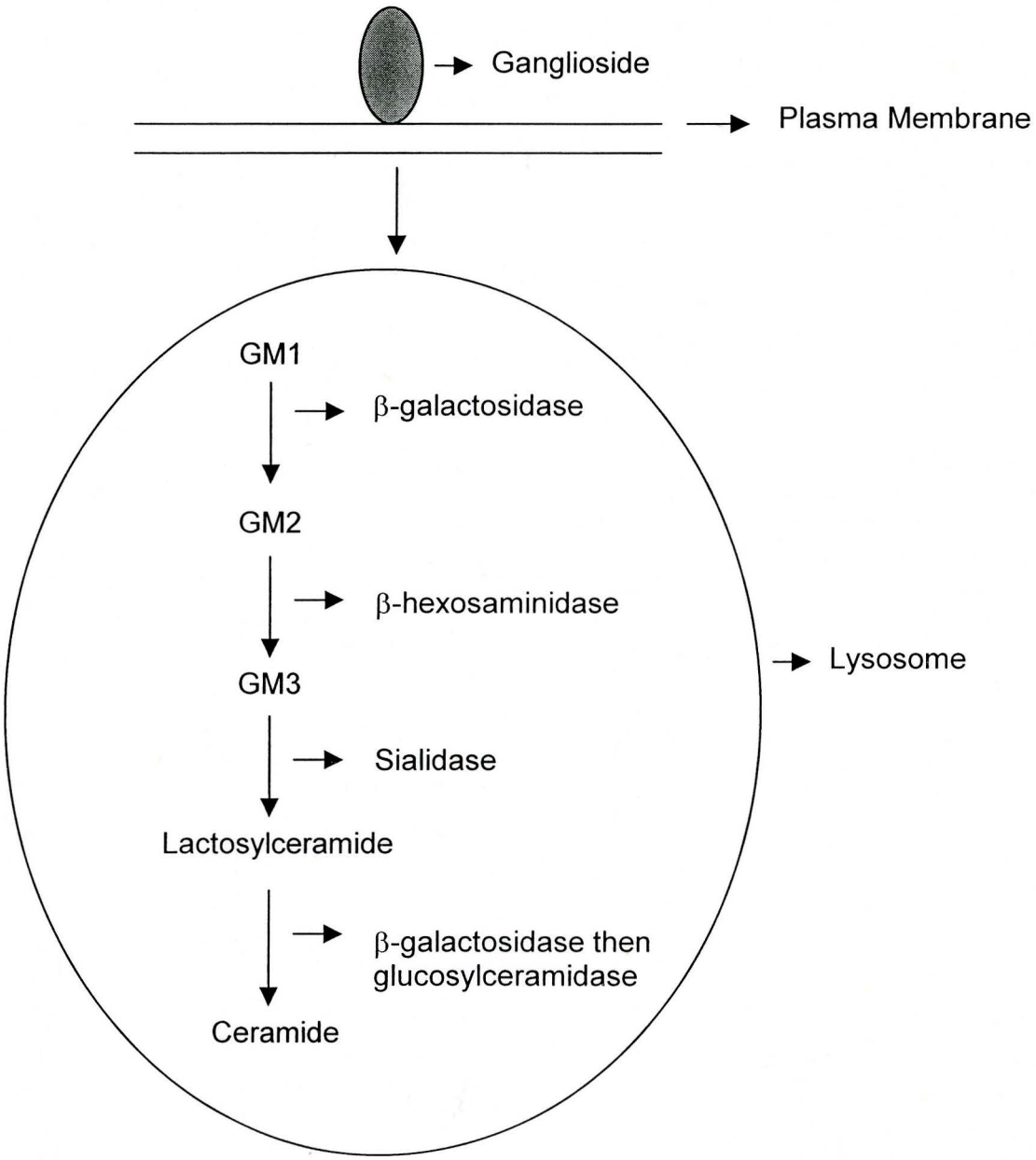

Figure 1.1 Ganglioside catabolism. Gangliosides are routinely sent to the lysosomes via endocytosis and catabolized in the lysosome by degradative enzymes. GM1 is catabolized to GM2 by the removal of galactose by β-galactosidase. GM3 is then formed by the removal of N - acetylgalactosamine from GM2 by β-hexosaminidase. GM3 has its sialic acid removed by sialidase activity, producing lactosylceramide The dual action of glucosylceramide and β-galactosidase removes the final galactose residue, yielding ceramide.

1.2 Sialidases and Disease:

The sialidases are a group of hydrolytic enzymes that cleave terminal α2-3 and α2-6 sialic acid residues from glycolipids, glycoproteins and oligosaccharides. Sialic acids are composed of a nine-carbon chain monosaccharide with more than forty structural forms (Vimr & Lichtensteiger, 2002). The most common sialic acid is *N*-acetylneuraminic acid (Neu5Ac) and is found in most organisms, including mammals, avian species, bacteria, viruses, and some plants (Vimr & Lichtensteiger, 2002). The importance of sialic acid in humans has been acknowledged with respect to its involvement in cellular differentiation, tumorigenicity and antigen masking (Collard et al., 1986; Pilatte et al., 1993 and Varki, 1997). Specifically, sialic acid containing receptors are utilized by the influenza A and B viruses as binding sites for the haemagglutinin glycoproteins, which initiates the viral infection (Wagner et al., 2002). Additionally, influenza neuraminidase cleaves sialic acid residues from cellular receptors to promote further viral spread by the progeny virus (Wagner et al., 2002). Another influenza virus, the *Haemophilis influenzae* also uses sialic acids in a unique way, catabolizing the sialic acid for a source of carbon and nitrogen and also transporting sialic acid residues to the surface for decoration to avoid immune detection (Vimr et al., 2000).

The mammalian sialidase family consists of three different enzymes, membrane-bound (Miyagi et al., 1999 and Wada et al., 1999), cytosolic (Ferrari

et al., 1994; Miyagi et al., 1993 and Monti et al., 1999) and lysosomal sialidase (Bonten et al., 1996; Carrillo et al., 1997; Igdoura et al., 1998; Milner et al., 1997 and Pshezetsky et al., 1996, 1997). Lysosomal sialidase becomes active once in the lysosomal compartment due to the low pH, 4.5, which is necessary for optimal enzyme activity and activation (Igdoura et al., 1998 and Bonten et al., 2000). Furthermore, lysosomal sialidase can either be attached to the lysosomal membrane or remain free in the lysosomal matrix (Achyuthan & Achyuthan, 2001). Cytosolic sialidases on the other hand have an optimum pH activity of 6.5 and have a high level of expression in skeletal muscle (Sato & Miyagi, 1996). Bacterial and mammalian cytosolic sialidases also do not have a signal peptide or lysosomal targeting motif unlike its lysosomal counterpart (Roggentin et al., 1989 and Ferrari et al., 1994). Plasma membrane sialidases, or "ganglioside sialidases", are different from the other two sialidases in that they contain a transmembrane domain and act specifically on membrane gangliosides (Achyuthan & Achyuthan, 2001). Each of these sialidases has been cloned with only the lysosomal sialidase linked to disease, sialidosis and galactosialidosis.

The lysosomal sialidase gene is four kilobase-pairs long with 5 introns and 6 exons, which has been mapped to chromosome 6p21 within the major histocompatibility complex (Pshezetsky et al., 1997). Similarly, the mouse lysosomal sialidase is around four kilobase-pairs long and is found on chromosome 17 (Womack et al., 1981). The lysosomal sialidase gene encodes

a 44kD glycoprotein that is only functional as a part of a multienzyme complex with β-galactosidase and protective protein cathepsin A (PPCA) (d'Azzo et al., 1982 and Verheijen et al., 1982). Cathepsin A first binds to sialidase within the endoplasmic reticulum from whence they are both transported to the lysosomes (van der Spoel et al., 1998). Cathepsin A is thought to maintain sialidase in its catalytically active conformation (van der Horst et al., 1989) as well as to protect both sialidase and β-galactosidase from proteolysis (Vinogradova et al., 1998).

The structure of sialidase has yet to be determined due to the difficulty in its purification, but homology studies between human sialidase and bacterial and viral sialidases indicate that the structure of human sialidase could be very similar to both bacterial and viral sialidases, which would mean that the human sialidase could consist of six four-stranded antiparallel β-sheets arranged in a propeller formation (Crennel et al., 1993). Interestingly, it was found by Igdoura et al. (1998) that the mouse lysosomal sialidase will form the multienzyme complex with human PPCA and β-galactosidase when expressed in human sialidosis cell lines, demonstrating that the human and mouse sialidases share structural similarity. Also, while a deficiency of lysosomal sialidase will result in sialidosis, only a deficiency in lysosomal sialidase activity due to a deficiency in PPCA will result in galactosialidosis (de Geest, et al., 2002).

1.3 Sialidosis:

Sialidosis is an autosomal recessive lysosomal storage disorder that is caused by a deficiency in lysosomal sialidase (Meikle, Clague, & Carey, 1999). The disease has a lower rate of incidence compared to other lysosomal storage disorders, with 1 in approximately 4 million patients diagnosed (Meikle, Clague, & Carey, 1999). The molecular basis for sialidosis is slowly being uncovered as various mutations within the gene have been found to be responsible for the lack of or low levels of lysosomal sialidase, most notably missense mutations (Bonten et al. 2000 and Lukong et al., 2001). Several of these mutations, F260Y, L270F, and A298V, have been hypothesized to alter the surface of sialidase, resulting in a reduction of PPCA binding to sialidase and thus an inability to form the multienzyme complex (Lukong et al., 2001).

Sialidosis is caused by an accumulation of GM3 ganglioside (Figure 1.2), sialyloligosaccharides, sialylglycoproteins and glycolipids in the liver and various other tissues as well as vacuolation in the liver, bone marrow, brain, kidney, as well as several endocrine organs including the thyroid and adrenal gland (Sergi et al., 2001). Sialidase levels usually range between 0-10% of normal levels in patients with sialidosis, but it is important to note that the severity of the symptoms is correlated with the level of residual sialidase activity (Bonten et al., 1996 & Bonten et al. 2000). Bonten et al. (2000) noted this, as the symptoms in a patient with a tyrosine to cysteine mutation at position 370 were quite severe.

This tyrosine-370 is known to be the catalytic site of sialidase through homology assessment with bacterial and viral sialidases, thus the Tyr370Cys mutation will produce an inactive sialidase that localizes to the lysosome (Bonten et al. 2000). Sialidosis can be either type I or type II, with the main difference being the age of onset and dysmorphism. Type I sialidosis (normosomatic) generally occurs between the ages of eight to twenty-five with clinical manifestations including a cherry-red spot, myoclonus, mild mental retardation, seizures and neuropathy (Thomas & Beaudet, 1995 and Achyuthan & Achyuthan, 2001). In contrast, type II sialidosis (dysmorphic) is characterized by an infantile onset, with somatic deformations, medial to major mental retardation, bone abnormalities and death before the first decade of life (Thomas & Beaudet, 1995 and Achyuthan & Achyuthan, 2001).

Patients with either type I or type II sialidosis secrete vast amounts sialyloligosaccharides and sialylglycopeptides in their urine as a consequence of their storage (Takahashi et al., 1991). These excreted sialic acid-containing molecules consist mainly of *N*-acetylglucosamine Neu5Ac linked to galactose, at the reducing end and non-reducing end respectively. 70% of these linkages are of the α2–6 type and 30% are of the α2–3 type (Takahashi et al., 1991). The diagnosis for sialidosis is done through cultured fibroblast sialidase activity from suspect patients using Mu-Nana as a substrate (Achyuthan & Achyuthan, 2001).

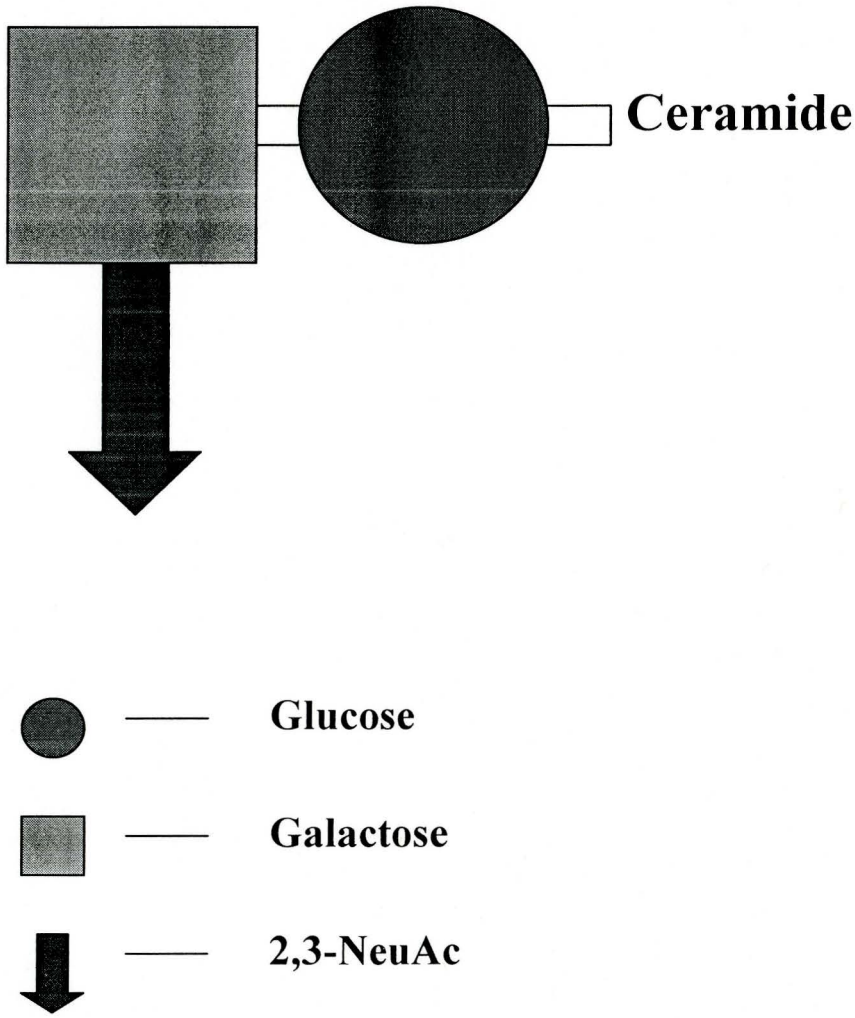

Figure 1.2 Structure of ganglioside GM3. Ceramide backbone attached to a glucose, galactose and 2,3-sialic acid residue moiety

Similarly, prenatal diagnosis may be possible through sialidase activity assay (Mu-Nana as substrate) of cultured fibroblasts or leukocytes from the amniotic fluid of an at-risk parent (Mueller & Wenger, 1981).

1.4 The Adenovirus:

Adenovirus is a linear double-stranded DNA virus with over 100 serotypes identified. The general viral genome for all of the human serotypes contains approximately 30 to 40 kb and has two inverted terminal repeats, one at each end. Adenovirus is nonenveloped with the genome being packaged by capsid proteins, forming a 140 nm in diameter virus. The capsid is icosahedral in shape consisting of three major components, the penton base, hexon and fibre proteins. X-ray crystallography for the adenovirus has also given researchers a proposed three-dimensional structure (Hitt & Graham, 1997).

Adenovirus infects cells through binding to the coxsackievirus and adenovirus receptor (CAR), which is prominent on most human cell types, followed by internalization receptor-mediated endocytosis (Howitt, Anderson & Freimuth, 2003). The C-terminus of the fiber protein is responsible for CAR receptor binding with the fiber shaft being important in fiber-receptor interaction and stability (Wu et al., 2003). Internalization is facilitated by the penton base binding to α_v integrins on the cell surface, which initiates the endocytosis of the viral particle (Hitt & Graham, 1997). Both of these processes have been shown

to be necessary for adenoviral entry into mammalian cells as genetically modified adenoviral vectors with either ablated fiber-CAR interaction or penton base-α_v integrin interaction demonstrated an absence of viral transduction (Bilbao et al., 2003).

The first six to eight hours are important in the expression of adenovirus early proteins, E1A, E1B, E2, E3 and E4 (Hitt & Graham, 1997). Early adenovirus proteins are involved in replication of the virus whereas late transcription is responsible for structural protein synthesis. Looking specifically at the early proteins, E1A functions as a transcriptional regulator while E1B is important in blocking host protein synthesis as well as E1A-induced apoptosis (White et al. 1991). E2 proteins function in viral replication, encoding the DNA polymerase, the DNA-binding protein and the terminal protein precursor (Hitt & Graham, 1997). E3 proteins have been shown to modulate the immune response, especially the 19 kDa protein (Lee et al., 1995). This has been shown to block MHC-I/antigen complexes from reaching the cell surface and ultimately their detection by cytotoxic T lymphocytes (Lee et al., 1995). E4 proteins have relatively unknown functions, with only the ORF6 protein playing a significant part in viral infectivity (Flint & Gonzalez, 2003). The E4 ORF6 proteins have been shown to have similar function to the E1B 55 kDa protein as the ORF6 protein can also inhibit the host cell apoptosis in response to viral infection (Flint & Gonzalez, 2003). Additionally, a complex consisting of the E1B 55 kDa protein

and the E4 ORF6 protein has been implicated in the late protein mRNA's export from the nucleus to the cytoplasm, while simultaneously inhibiting the export of newly synthesized host cell mRNA (Flint & Gonzalez, 2003).

Since only the ITRs and the viral packaging signal are necessary for viral replication in *cis*, all or some of the early and late proteins can be provided in *trans* through either a transformed cell line like the human embryonic kidney cell line 293, which expresses the E1 proteins from Ad5 (Graham et al. 1977), or through a replication defective "helper" virus (Parks et al. 1996 & Hitt et al., 1997). The use of the helper virus is necessary for helper-dependent adenovirus production, which will be discussed later on. First-generation adenoviruses however, can either be E1 or E1/E3 deleted, in which the E1 proteins are expressed in trans from the 293 cell line (Hitt, M 1997). Up to 3.2 kb of a transgene can be inserted into an E1-deleted adenovirus type 5, whereas an E1/E3-deleted vector can encode a transgene up to 8.2 kb without affecting viral packaging, which is due to the adenovirus' ability to package up to 105% of the wild type genome size (Bett, Prevec & Graham, 1993). Recent first-generation adenovirus production methods employ the bacteriophage P1 Cre-lox recombination system (Ng et al., 1999). This method involves co-transfecting a shuttle plasmid containing the transgene and the right ITR with a lox-P site upstream with a plasmid-based E1/E3-deleted adenovirus that contains the cre-recombinase sequence as well as a lox-P site (Ng et al., 1999). Viral rescue

occurs following cre-lox recombination, which replaces the sequence containing the bacterial origin of replication, ampicillin resistance gene and cre-recombinase gene in the adenovirus-based plasmid with the transgene from the shuttle plasmid (Ng et al., 1999). Despite further advances with recombinant adenoviruses, specifically with the helper-dependent adenovirus, first-generation adenoviruses still remain useful due to their relative ease of production compared to helper-dependent adenoviruses, but the elicited immune response following infection does not allow for a sustained duration of transgene expression, which places them in a niche for vaccination purposes, short-term *in vivo* work and *in vitro* work (Danthinne & Imperiale, 2003).

1.5 Current Treatment for lysosomal storage disorders:

Currently, treatments being tested for lysosomal storage disorders include enzyme replacement therapy (Naganawa et al., 2002 and Weinreb et al., 2002), bone marrow transplantation (Shiffman & Brady, 2002), and gene therapy utilizing the adenovirus or adeno-associated virus (Akli et al., 1996, Stein et al., 1999, Sferra et al., 2000, Pauly et al., 2001, and Du et al., 2002). Enzyme replacement therapy has provided positive results for Gaucher disease with respect to anemia, thrombocytopenia, organomegaly, bone crises, and overall bone pain as seen from a five year clinical trial (Weinreb et al., 2002). Similarly, enzyme replacement therapy has been tested *in vivo* with Fabry disease mice with the results showing that alpha-galactosidase producing fibroblasts can

successfully reduce the accumulation of ceramide trihexoside after transplantation into the diseased mice (Naganawa et al., 2002). Bone marrow transplants have shown clinical success with several lysosomal storage disorders, including Gaucher disease, some mucopolysaccharidoses and in mild Krabbe disease (Shiffman & Brady, 2002), but the therapy has limited application due to the associated high morbidity and mortality, difficulty finding compatible donors, and the possibility for graft-vs.-host disease after transplantation.

Gene therapy has provided an alternative measure to treat lysosomal storage disorders, since the majority of the clinical symptoms are due to a monogenic defect. Adenoviruses have been used by various researchers for the treatment of lysosomal storage disorders due to their high cloning capacity, high level of transgene expression, and their ability to infect both dividing and non-dividing cells (Akli et al., 1996, Stein et al., 1999, Sferra et al., 2000, Pauly et al., 2001, and Du et al., 2002). Specifically, recombinant adenoviruses have been used to treat mucopolysaccharidosis (MPS) type VII (Stein et al., 1999), Wolman disease (WD) and cholesteryl ester storage disease (CESD) (Du et al., 2002), Pompe disease (Pauly et al., 2001), and Tay-Sachs disease (TSD) (Akli et al., 1996). Recombinant adeno-associated viruses have also been used on MPS type VII as a possible treatment (Sferra et al., 2000).

The recombinant adenovirus and adeno-associated viruses used in MPS type VII both demonstrated that lysosomal storage in the CNS could be dramatically reduced via adenovirus-mediated β-glucuronidase expression with storage reduction and enzyme expression maintained at wild type levels for 16 weeks with the adenovirus vector (Stein et al., 1999) and 3 months with the adeno-associated vector (Sferra et al., 2000). Lysosomal acid lipase (LAL) deficient mice display the characteristic phenotype for both WD and CESD, and thus were suitable for infection with recombinant adenoviruses carrying the gene for LAL (Du et al., 2002). After 20 days post-infection, triglyceride and cholesterol levels had declined by levels greater than 50% of diseased levels, indicating that adenovirus-mediated gene transfer of LAL can correct the diseased state for both WD and CESD (Du et al., 2002). Similarly, a recombinant adenovirus expressing the human lysosomal enzyme acid alpha-glucosidase (GAA) was produced to treat GAA deficient mice and to be used as part of an enzyme replacement therapy *in vitro* (Pauly et al., 2001). GAA deficient fibroblasts were infected with the GAA expressing adenovirus and were plated over uninfected GAA fibroblasts covered by a porous filter. It was discovered that GAA is transferred through the filter from the infected cells into the uninfected cells with the enzyme reaching the lysosomes where it is spliced into its lysosomal forms (Pauly et al., 2001). Enzyme levels in the uninfected cells were detected at levels comparable to normal human fibroblast GAA levels, indicating that enzyme endocytosis by the deficient cells was efficient (Pauly et

al., 2001). Additionally, both human and mouse GAA recombinant adenoviruses were used to infect GAA deficient mice with results demonstrating hepatic transduction with the heart and skeletal muscle being reached by GAA (Pauly et al., 2001).

1.6 Helper-Dependent Adenovirus Gene Therapy of Sialidosis:

The use of adenoviruses in gene therapy is attractive in several respects, including their ability to infect both dividing and non-dividing cells, their high cloning capacity, their achievable titre levels of up to 10^{13} particles/ml, their high level of transgene expression and their ease of production (Hardy et al., 1997, Hitt et al., 1997). One main drawback to the use of adenovirus gene therapy is that the majority of the population has been exposed to the adenovirus. Thus upon therapeutic exposure using the adenovirus serotypes Ad2 or Ad5, which are the most widely used in gene therapy, the host will experience a systemic inflammatory response in which the virus' initial infection will be blocked from host antibodies that are directed towards the virion (McCoy et al., 1995, Hitt et al., 1997). Additionally, the remaining viral proteins that are expressed along with the transgene have both been implicated in creating a host immune response, thus either dampening or blocking any therapeutic benefit (Tripathy et al., 1996, Hitt et al., 1997).

A novel non-immunogenic adenoviral vector was produced by Parks et al., (1996) to improve on the existing adenoviral gene therapy technology. The advantages of this system over the replication-defective adenovirus system are that the Hd vector can accommodate up to 35 kb of foreign DNA and that there are no adenoviral proteins expressed as the vector is devoid of all genes with the exception of the packaging signal and the two inverted terminal repeats (ITRs) (Parks et al., 1996). The general idea behind Hd vector production is that the helper virus, which is a first-generation virus lacking the Ad-E1 region, expresses all other adenoviral proteins, but has its packaging signal flanked by loxP sites. After initial transfection into 293cre cells (which express Ad-E1 and cre-recombinase) with Hd vector DNA (containing transgene), helper virus is used to infect the cells and all expressed adenoviral proteins are used *in trans* by the Hd vector for virus replication and amplification. The helper virus does not amplify as the Cre-recombinase acts upon the loxP sites flanking the helper virus packaging signal, excising it and thus rendering the helper virus DNA unpackageable (Parks et al., 1996). Figure 1.3 displays the process for Hd vector production, with low levels of helper vector contamination (<0.2%) and high titres (10^{10}-10^{12} particles/ml) both possible through this procedure (Parks et al., 1996, Parks et al., 1998, Kim et al., 2001).

With all of the adenovirus coding sequences deleted, the Hd vector toxicity is diminished significantly when compared to recombinant adenoviruses (Morral

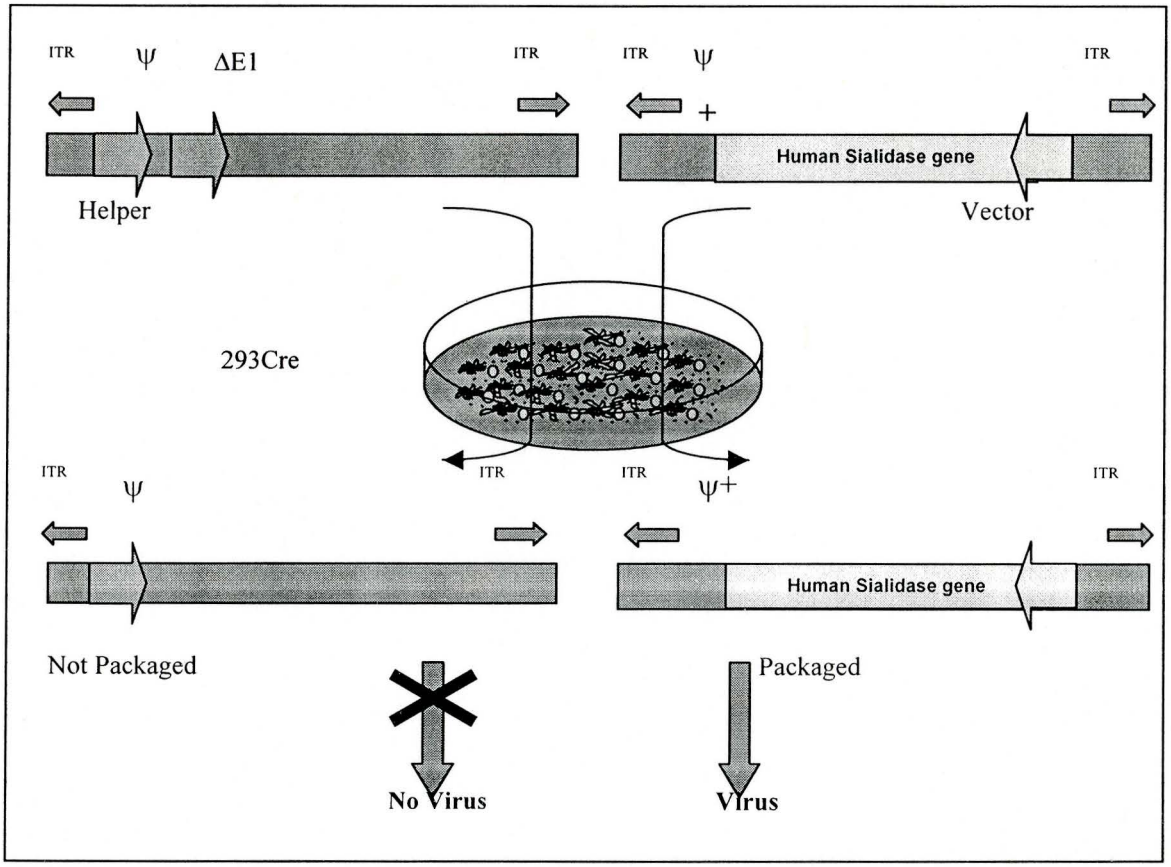

Figure 1.3 Generation of Ad-based vector complemented by a helper virus containing LoxP-flanked packaging signal. Infection of Cre-expressing 293 cells with the helper virus results in excision of the viral packaging signal ψ, rendering the helper virus DNA unpackageable

et al., 1998). Furthermore, the duration of transgene expression with the use of Hd vectors is significantly greater than that of recombinant adenovirus transgene expression, with some researchers reporting lifelong or long-term expression (Morsy et al., 1998, Morral et al., 1999, Kim et al., 2001). In fact, Maione et al., (2001) found that mice that were preimmunized with the same adenovirus serotype as the Hd-vector that was used for their intramuscular infection maintained the same long-lasting transgene expression when compared to mice that were not preimmunized.

There have been a diverse group of transgenes cloned into an Hd-vector, including the neurotrophin gene (Zou et al., 2001), the very low-density lipoprotein receptor (VLDLR) (Oka et al., 2001), and the mIFNα gene (Aurisicchio et al., 2001), all reporting long-term transgene expression with limited adenovirus-associated toxicity. The neurotrophin gene study is relevant to sialidosis as the Hd-vector was targeted to the brain. Zou et al., (2001) reported that regardless of the injection site in the rat, either intraventricular or intrahippocampal, neutrophin was expressed in the brain for 6 months with low levels of cytokine expression and immune infiltration compared to a first generation neurotrophin expressing adenovirus. This demonstrates the ability for Hd vectors to reach the brain and maintain transgene expression over a long period of time. Similar findings were reported involving an Hd-vector expressing the VLDLR (Oka et al., 2001). VLDLR expression in LDLR-deficient mice was

recorded to last a minimum 6 months with no hepatic toxicity compared to the acute liver toxicity produced by the first generation adenovirus carrying the VLDLR gene (Oka et al., 2001). In addition, the mice plasma cholesterol levels remained low over the 6-month period and no atherosclerosis was seen in the LDLR-deficient mice that received the Hd-vector (Oka et al., 2001). Using a different approach with the Hd-adenovirus, Aurisicchio et al., (2001) developed a Hd-adenovirus containing the mIFNα gene controlled by tetracycline, which would allow for mIFNα activation upon tetracycline induction, and also allowing the levels of mIFNα to be controlled. Tetracycline induction was shown to promote the expression of mIFNα 3 months post-infection in mice and was capable of prolonging the survival of these mice when infected with a lethal dose of coronavirus (Aurisicchio et al., 2001). Furthermore, viral toxicity was minimal as indicated by the low levels of liver damage in Hd vector infected mice (Aurisicchio et al., 2001). More recent experiments utilizing the helper-dependent system have revealed similar results as those previously mentioned. Specifically, transgenes as diverse as human factor VIII (Reddy, et al. 2003), human apolipoprotein A-I (Belalcazar, et al. 2003) and neuroD-betacellulin (Kojima, et al. 2003) all displayed successful transgene expression over an extended period to treat or reverse the respective disorders, hemophilia (9 months), atherosclerosis (7 months) and diabetes (4 months).

1.7 The SM/J mouse:

The mouse model chosen for experimental work with adenoviral constructs is the SM/J mouse. The SM/J mouse is an inbred mouse strain that is ideal for the study of transgenic delivery of sialidase due to their low endogenous levels of the lysosomal enzyme (Rottier, Bonten & d'Azzo, 1997). Interestingly, it was because of this deficiency that the chromosomal mapping of sialidase, *NEU-1*, was carried out and found to be located at the histocompatibility locus on chromosome 17, which is the mouse equivalent to the histocompatibility locus on chromosome 6p21 in humans (Womack, Yan & Potier, 1981). This chromosomal mapping allowed for the cloning of the human sialidase gene entitled G9. The cause of the lysosomal sialidase deficiency in SM/J mice was recently discovered to be due to a single amino acid substitution, L209I, which was proposed to induce a reduced affinity of the enzyme towards its substrate (Rottier, Bonten & d'Azzo, 1998). Due to the low endogenous levels of lysosomal sialidase, any increase in enzymatic activity should be found with relative ease. In addition, phenotypic rescue can be assessed and possibly related to the increase in transgenic sialidase activity.

There are several phenotypic traits associated with the SM/J mice, including the lysosomal sialidase deficiency (Rottier, Bonten & d'Azzo, 1998), decreased lifespan (Storer, 1966), slow growth from 3 to 10 weeks of age (Cheverud et al., 1999), low levels of HDL-C (Pitman et al., 2002), susceptibility

to aortic lesions on a high fat diet (Nishina et al., 1993), low levels of sialidase in activated T cells (Landolfi et al., 1985), low levels of IL-4 production (Chen et al., 1997) and hyperresponsive B cells (Engel et al., 1981). The lysosomal sialidase deficiency has been directly linked to several of these phenotypes, including the IL-4 deficiency, T cell activation, and tumor metastasis (Landolfi et al., 1985, Chen et al., 1997, & Tokuyama et al., 1997).

The immune response in the SM/J mouse has been compromised mainly due to the low levels of lysosomal sialidase (Rottier, Bonten & d'Azzo, 1998). Specifically, the T_{h2} response, which is responsible for IL-4 production, is defective in the SM/J mouse leading to surface antigens from T_{h2}-committed cells to be abnormally desialylated and leading to a lack of antibody production, IgG1 and IgE, following immunization (Chen et al., 1997). Additionally, T cell sialidase is involved in the production of macrophage activating factor, through the conversion of vitamin D_3 binding protein (Yamamoto & Kumashiro, 1993). Furthermore, Chen, Ding & Daynes, (2000) found that increased levels of the ganglioside GM3 on the surface of T hybridoma cells inhibited the IL-4 response possibly through alterations in the calcium response produced after T cell activation. They also discovered in B10.SM mice, which have T cells that are deficient in lysosomal sialidase and IL-4 and have higher levels of GM3 on their surface, that following T cell priming the calcium response associated with T cell

activation was significantly lower than in control cells (Chen, Ding & Daynes, 2000).

It remains unclear on whether lysosomal sialidase has a direct role on atherosclerosis, but several findings have suggested that sialidase and sialic acid residues are important in the onset of atherosclerosis. Fujioka et al., (2000) found that low-density lipoproteins (LDL) from coronary artery diseased patients that were desialylated were taken up by monocyte-derived macrophages to be degraded twice as much compared to control LDL's. Furthermore, they found that cholesterol esterification within the monocyte-derived macrophages was stimulated two-fold with the desialylated LDL compared to the sialylated controls. With this in mind, it is possible that the low level of lysosomal sialidase in the SM/J mouse has an implication in the increased susceptibility that the SM/J mouse has towards atherosclerosis. Several researchers have discovered this increased susceptibility to aortic lesions through high fat diet comparisons with control mice (Nishina et al., 1993 & Pitman et al., 2002). Nishina et al., (1993) found that after feeding on a high fat, high cholesterol diet, the SM/J strain developed aortic lesions at a higher rate than controls, despite a maintenance of its HDL levels. However, the natural levels of HDL in the SM/J strain were found by Pitman et al., (2002) to be significantly lower than control mice before and after a high fat diet. Normally, HDL levels increase in mice following a high fat

diet regimen as HDL levels are generally negatively correlated to susceptibility to atherosclerosis.

1.8 Research Objectives:

The objective of this thesis is based on the principle that the helper-dependent adenovirus is a good choice for the introduction of transgenic lysosomal sialidase in sialidase deficient cells and more importantly in the SM/J mouse. Due to the large cloning capacity of the helper-dependent vector, the entire mouse and human lysosomal sialidase genes can be cloned with relative ease. Human sialidase-deficient cell lines (WG544 and Wall) are also suitable for *in vitro* assessment of transgenic lysosomal sialidase activity from both first-generation and helper-dependent adenovirus vectors. Additionally, using the SM/J mouse as the *in vivo* model is also acceptable due to their low levels of lysosomal sialidase expression and associated phenotypes, including low weight levels in the first few months of life relative to C57/Bl6 mice, low levels of IL-4 expression, susceptibility to atherosclerosis and tumor metastasis. The SM/J mouse will provide evidence of transgenic sialidase activity through performing sialidase activity assays on the kidney, liver and spleen, through RT-PCR of RNA within the aforementioned organs, through measuring body weights for the second to third month of age, through measuring blood cholesterol, liver enzyme levels and through FACS analysis of monocyte surface glycosylation levels. In addition to this, the helper-dependent vector will be compared to the first-

generation vector in terms of immune response through adenovirus antigen levels and in terms of duration of transgene expression following a one-month experiment. With no current treatment for sialidosis patients, this study could provide a first step in the treatment of sialidosis utilizing the helper-dependent adenovirus as a means of gene therapy.

Chapter 2

Materials and Methods

2.1 Cloning of Mouse Sialidase Gene into Helper-Dependent Vector

A genomic fragment containing 10.6 kb from the *Neu1* locus (gene and promoter) was previously cloned into a Bluescript (pBSKS+msial) in order to clone the sialidase gene and promoter in the helper-dependent vector pC4HSU (kindly provided by F. Graham, McMaster University). An adaptor was designed (5'-CGATCGGACCGC-3' and 5'-TCGAGCGGTCCGAT-3') containing a *ClaI* compatible 5' end, a *XhoI* compatible 3' end and a *RsrII* restriction site. The adaptor was cloned into pBSKS+msial through a *ClaI/XhoI* digest of the vector and ligated through the compatible cohesive ends, resulting in the subclone pBSKS+msialR. Following plasmid amplification and CsCl purification, pBSKS+msialR was digested with *NotI* and *RsrII* yielding a 10.6 kb fragment containing the mouse sialidase gene. The helper-dependent plasmid pC4HSU was similarly digested with *NotI* and *RsrII*, producing a 19.3 kb fragment containing the adenoviral components necessary for viral encapsidation, the left and right inverted terminal repeats (ITRs) and the packaging signal (Ψ). The 10.6 kb mouse sialidase fragment and 19.3 kb adenoviral vector fragment were both purified using the Geneclean II with Spin kit (Q-BIOgene). The 10.6 kb mouse sialidase fragment was then ligated into the 19.3 kb adenoviral vector fragment, producing a 29.9 kb helper-dependent vector containing the mouse sialidase gene, pC4HSUmsial. The vector was verified by restriction digestion with both *HindIII* and *EcoRI*.

2.2 Subcloning the Human Sialidase Gene

The human sialidase gene required PCR amplification and subsequent cloning into the pCR2.1 vector. The reaction (forward primer CTGGAGCTGTCTCTGACTGCAT and reverse primer CTGGGACAGGATTCCCATCT) was performed using the human sialidase BAC as a template (NCBI accession #AF134726) and produced an 8 kb product (Figure 2.1). The PCR product was gel purified as before and ligated into the TA-cloning site of pCR2.1 (Invitrogen), yielding a plasmid pCRhsial, which was verified by restriction digestion with *HindIII* and *EcoRI*.

2.3 Subcloning the Mouse Sialidase Gene into the Adenovirus Shuttle Vector pDC312

The adenovirus shuttle vector pDC312 was purchased from Microbix. The plasmid pBSKS+msialR was used for cloning the mouse sialidase gene. pDC312 was digested with *StuI* and *SalI* generating a 3.2 kb vector. pBSKS+msialR was digested with *ScaI* and *XhoI* producing a 7.8 kb mouse sialidase fragment. Both fragments were gel purified and ligated. Restriction analysis with *HindIII* and *BamHI* verified the correct clones, pDC312msial.

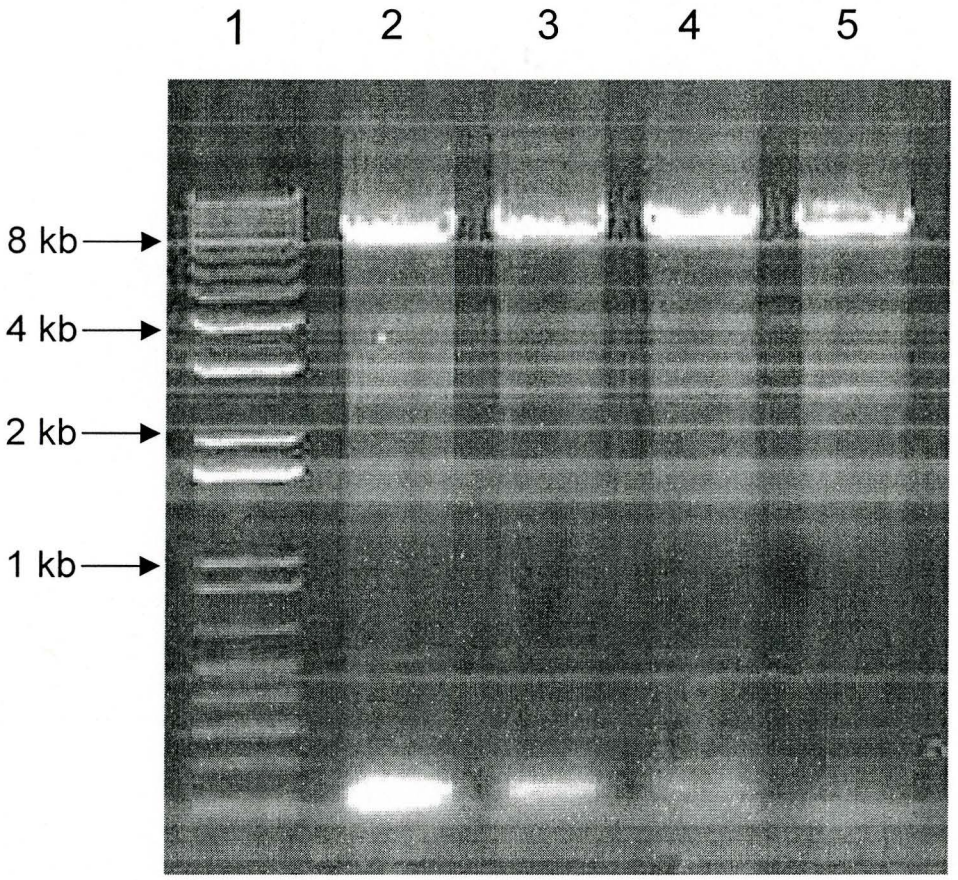

Figure 2.1 Human Lysosomal Sialidase PCR Product: Human lysosomal sialidase PCR product purified through a 0 7% TAE agarose gel Lane 1 1 kb plus DNA ladder Lanes 2-5. PCR product at various annealing temperatures, 57°, 59°, 61°, or 63° C for lanes 2, 3, 4, and 5 respectively

2.4 Cell Culture and Adenoviral Infections

The human embryonic kidney cell lines, 293 and 293Cre4, were generous gifts from Dr. Graham (McMaster University, Hamilton, ON). 293 cells were grown in F-11 medium supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/ml) and fungizone (2.5 µg/ml). 293Cre4 cells were grown in F-11 medium supplemented with 10% fetal calf serum, penicillin/streptomycin, fungizone and 0.4 mg/ml G418. To propagate adenoviruses, 293 cells were cultured in F-11 medium supplemented with 5% horse serum, penicillin/streptomycin and fungizone (maintenance medium). The sialidase-deficient fibroblast cell lines (WG544 and Wall) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/ml) and fungizone (2.5 µg/ml). MEF cells were isolated in our laboratory and were cultured in DMEM as above. B6.SM fibroblasts that were either heterozygote or homozygote for the lysosomal sialidase deficiency were isolated directly from the respective animals in our laboratory and were propagated in DMEM as above. Adenoviral infections were carried out by adding the appropriate amount of adenovirus in PBS++ (0.5 ml for 60-mm dishes, 1 ml for 100 and 150-mm dishes) and aliquoting in 90% confluent dishes immediately after removal of cell medium. Adenovirus was allowed to adsorb to the cell monolayer for either 30 minutes (first-generation vectors) or 60 minutes (helper-dependent vectors).

2.5 Propagation of the Mouse Sialidase-Containing Helper-Dependent Adenovirus

The helper-dependent plasmid pC4HSUmsial was digested (25 μg) with *PmeI* to release the bacterial amplification properties, ampicillin resistance and the origin of replication, producing a 27.2 kb fragment and exposing the 2 ITRs on the ends of the fragment. Following heat inactivation (65° C for 20 minutes), the digested plasmid was transfected into 293Cre4 cells by calcium phosphate transfection. Briefly, the medium was replaced on 5 60-mm dishes at 70-80% confluency with fresh G418+ F-11 1 hour prior to transfection. The transfection mixture containing 5 μg of plasmid, 0.5 ml Hepes-buffered saline (HBS), 5 μg salmon sperm (Eppendorf) and 25 μl $CaCl_2$ was then added directly to the cell medium and mixed. Following overnight incubation at 37°, the cell medium was removed and the cells were washed twice with G418+F-11. The helper virus, AdLC8Cluc (Dr. Graham, McMaster University, Hamilton, ON) was then added to the cells at an MOI of 5 PFU/cell in 0.2 ml PBS++. After a 30 minute adsorption, 5 ml of maintenance medium was carefully added to each infected dish. Complete cytopathic effect (CPE) was seen 48 hours post-infection (pi). The cells were then scraped and transferred into a 15 ml polypropylene tube along with 0.1 volume of 40% sucrose for -70° C storage. This initial lysate was labelled passage zero, P0. The P0 lysate was thawed at 37° C and 0.4 ml was used to co-infect a 90% confluent dish of 293Cre4 cells along with the helper virus at an MOI of 1 PFU/cell as before. After complete CPE was seen, the cell

lysate (P1) was harvested as before and stored at -70° C. Co-infections continued serially up until 8 lysates (P0 - P7) had been harvested. 100-mm dishes were used for P2 - P7 to increase the quantity of lysate. Two 100-mm dishes were used for P5 and P6 while 4 100-mm dishes were used for P7, ensuring that enough P7 lysate was available for high titre production. After each lysate had been collected, pronase/SDS was added to the dishes to digest the remaining adenovirus in order to confirm correct adenoviral propagation. Phenol extraction and ethanol precipitation purified the adenoviral DNA and restriction digestion confirmed proper adenoviral sequence. Sequencing of the purified adenovirus DNA for the mouse sialidase promoter also confirmed the presence of the mouse sialidase gene within the viral genome. The helper-dependent vector AdC4HSULacZ at P4 was provided by Chang-Xin Shi (McMaster University, Hamilton, ON) and was serially passaged as above.

2.6 Propagation of Mouse Sialidase-Containing First-Generation Adenovirus

293 cells were grown in 60 mm dishes to 60-80% confluency and were transfected as above with 5 μg of both pDC312msial and pBHGlox(ΔE1,E3)Cre (plasmid containing adenovirus genome minus the E1 and E3 regions) and left overnight at 37° C. The following day, the medium was replaced with 10% horse serum/20% yeast extract containing F11 mixed 1:1 with 1% agarose in water and allowed to solidify. The infected dishes were incubated at 37° C with viral

plaques forming anywhere between 5 and 10 days post-transfection. Viral plaques were collected via suction through Pasteur pipettes and by aliquoting the plaque into 1 ml of PBS++/10% glycerol followed by storage at -80° C. The viral plaques were amplified by infecting 60 mm dishes of 293 cells with 0.2 ml of viral plaque. Proper adenoviral sequence was confirmed as before through restriction analysis.

2.7 High Titre Production and Purification of Helper-Dependent and First-Generation Adenovirus

First-generation and helper-dependent adenoviruses were purified by CsCl ultracentrifugation. With respect to first-generation adenovirus vectors, 1 ml of adenovirus lysate from a 60-mm 293 dish was diluted 1:5 in PBS++. This diluted adenovirus was then used to infect a minimum of 20 150-mm dishes of 293 cells. Diluted adenovirus (1 ml) was used to infect each 150-mm dish. CPE was reached 3-4 days later with the cells being scraped into 0.5 ml 10 mM Tris-HCl, pH 8.0/150-mm dish. 5% sodium deoxycholate was added to lyse the cells, followed by the addition of 2 M $MgCl_2$ and DNAase I to digest any unpackaged viral DNA as well as cellular DNA. The lysate was then spun with the supernatant collected and centrifuged twice through CsCl density gradient (Hitt, *et al.* 1995). The first CsCl centrifugation was for 1 hour at 35000 RPM and the lower viral band was collected with an 18-gauge needle and syringe through the side of the tube. The second CsCl centrifugation was left overnight at 35000

RPM and the lower viral band was collected with an 18-gauge needle and syringe through the side of the tube. The collected adenovirus was then injected into a Slide-A-Lyzer dialysis cassette (Pierce) where the virus was dialyzed against 3 changes of 500 ml 10 mM Tris-HCl pH 8.0 over 24 hours at 4° C. The adenovirus was collected from the dialysis cassette and sterile glycerol was added to a final concentration of 10%.

2.8 Determining Concentration of CsCl-Purified Adenovirus

The concentration of both first-generation and helper-dependent adenoviruses were determined through fluorometric analysis using Hoechst dye (Boehringer Mannheim). 20 µl of CsCl-banded adenovirus was treated with 20 µl of pronase/SDS overnight at 37° C to digest the viral capsid. The following day, 20 µl of the pronase/SDS-treated virus was mixed with the Hoechst dye and fluorescence was measured using a Hoefer Fluorometer (Hoefer). Adenoviral particle count was based on the fluorometric result (µg/ml) using the following equation:

$$\frac{\text{Viral DNA Concentration (µg/ml)} \times 9.48 \times 10^{11}}{\text{Length of Viral DNA (kb)}}$$

2.9 Determining Infectious Units of AdC4HSULacZ

Following CsCl-purification, AdC4HSULacZ was used to infect 293 cells at an MOI of 10 particles/cell. After a 24 hour infection period, the medium was

removed and the cells were fixed with 1 ml of β-gal fix solution (2% paraformaldehyde) with a 5 minute incubation at 37° C. The fix solution was then removed and 2 ml of β-gal stain solution, supplemented with X-gal at a final concentration of 0.5 mg/ml, was added onto the monolayer. The cells were then incubated with the stain solution at room temperature overnight. The blue stained cells were counted under a light microscope the next day with the infectious units per ml (IFU/ml) determined using the following equation (Eykholt, Mitchell & Marvin, 2000):

$$\frac{\text{(Blue Cells/Field) x (Fields/Well)}}{\text{Volume of Virus (ml) x (Dilution Factor)}}$$

2.10 Determining Sialidase Activity from *In Vitro* and *In Vivo* Adenovirus Infections

WG544, Wall, MEF, and B6.SM cells were infected with adenovirus (AdmsialFG, AdmsialHD, AdC4HSULacZ or AdS+, which was previously produced in our laboratory) and lysed 96 hrs pi to determine lysosomal sialidase activity induced by the transgenes. The cells were washed twice with cold phosphate-buffered saline (PBS). The cells were then scraped once with a rubber policeman in 1 ml of cold PBS. An additional 1 ml of cold PBS was added and the cells were scraped again to ensure maximum cell harvesting. The cells were centrifuged at 1500 RPM for 10 minutes at 4° C with the pellet being resuspended and lysed in 150 µl of distilled water by continuous pipetting. 4-methylumbelliferyl-n-acetyl-α-D-neuramide (Mu-Nana) was used as the substrate

for the cell homogenates to determine lysosomal sialidase activity. Cell homogenates were incubated with the substrate for 1 h at 37° C (Potier et al., 1979). Reactions were stopped following the addition of 1 ml of 0.1 M MAP, pH 10.5. The resulting fluorescence was measured using the LS Reader Plate Fluorometer (Perkin Elmer). The Bradford protein assay (BioRad) was utilized to determine the protein concentrations of the cell homogenates in order to determine lysosomal sialidase activity per µg of protein. Any additional lysate was prepared for subsequent Western Blot analysis. SM/J tissues (kidney, liver, and spleen) that were collected upon termination of the *in vivo* AdmsialFG, AdmsialHD, and AdC4HSULacZ experiment were homogenized in 1 ml of ddH_20 using a Polytron (Kinematica AG). 50 µl of the tissue lysate was used per enzymatic reaction assay with the rest of the protocol being followed as stated above.

2.11 Preparation of Mouse Monocytes from Blood

Heparin solution (1000 units/ml) was aliquoted as 2 µl aliqouts into a sterile microfuge tube. Blood was collected from infected mice once every other week using the retro-orbital bleed technique. Approximately 120 µl was collected into the heparin-containing tube for each mouse. 120 µl of blood was combined with 400 µl of Tris-NH_4Cl and incubated for 10 minutes at 37° C to lyse the red blood cells. Cells were then pelleted at 1200 RPM for 3 minutes, after which the supernatant was removed and an additional 400 µl of Tris-NH_4Cl was added with

a 10 minute incubation at 37° C. Cells were pelleted again and the supernatant was removed. Cell pellets were washed once with either cold PBS or FACS buffer depending on if the monocytes were being tested for sialidase activity or for fluorescence activated cell sorting (FACS) respectively.

2.12 Fluorescence Activated Cell Sorting of Mouse Monocytes from Blood of Adenovirus-Infected Mice

Blood monocytes were prepared as stated above and were suspended in the labeling solution containing FITC-SNA and Biotinylated-MALII in HBSS and were incubated at 4° C for 30 minutes in the dark. The monocytes were pelleted and washed three times with HBSS. The secondary labeling solution containing avidin conjugated PE in HBSS was added to the cells and the mixture was incubated for 30 minutes in the dark. The monocytes were pelleted and washed three times with HBSS and once with PBS. The monocytes were then resuspended in 500 µl of 2% formaldehyde in HBSS to be analyzed by a FACSorter machine.

2.13 Antibodies

The antibody used in these experiments was the anti-75 antibody, which is specific for the carboxy-terminus of human lysosomal sialidase. The homology between the human lysosomal sialidase carboxy-terminus and the mouse lysosomal sialidase carboxy-terminus is 87%, and thus was thought to possibly

act as the primary specific antibody for the Western Blots of the Admsial-Hd and Admsial-Fg-infected cell lysates.

2.14 Western Blot of Admsial-Hd and Admsial-Fg-Infected Cells

After determining sialidase activity, an appropriate volume of 5x loading buffer was added to the leftover cell lysate (40 µl) and the samples were boiled for 10 minutes. A 12% SDS-polyacrylamide gel with a 4.5% stacking gel was prepared and 30 µl of each sample was added to each well. The gel was run for approximately 2 hours and the samples were transferred to a nitrocellulose filter at 4° C for 2 hours. The nitrocellulose filter was blocked with 10% nonfat dry milk in TBS (10 mM Tris-Cl, pH 8.0, 150 mM NaCl) for 30 minutes at room temperature. The nitrocellulose filter was then incubated with a 1:200 diluted rabbit-anti-human sialidase antibody in 10% milk/TBS at 4° C overnight. The following day, the nitrocellulose filter was washed once with TBS for 5 minutes, four times with ddH_2O for 10 seconds each, and finally with TBS for another 5 minutes. The nitrocellulose filter was then incubated with the goat-anti-rabbit IgG conjugated to horseradish peroxidase at a 1:10,000 dilution for 2 hours at room temperature. The filter was washed as before with TBS, ddH_2O and TBS. The filter was then developed by adding 2 ml of ECL Western Blot Detection Reagent I and 2 ml of ECL Western Blot Detection Reagent II (Amersham Biosciences) for 1 minute. The filter was drip-dried using forceps and wrapped in saran wrap

before being exposed to Kodak X-OMAT AR scientific imaging film for approximately 5 minutes.

2.15 Adenovirus Injections into SM/J Mice

SM/J mice were purchased from Jackson Laboratories at 3 to 6 weeks old. Mice were allowed to acclimate for 7 days prior to adenovirus injection. AdmsialHD, AdmsialFG and AdC4HSULacZ (5×10^9 particles) were diluted into separate microfuge tubes to a final volume of 100 µl with PBS. Four sets of four mice were carefully injected with 100 µl of AdmsialHD, AdmsialFG, AdC4HSULacZ or PBS into the venous plexus under the eye following avertin anesthesia. All mice were monitored for survival and body weights measured each day for the first 14 days, and approximately every third day up until the termination of the experiment at day 29. At day 29, tissue sections from the kidney, spleen and liver were collected from each mouse to assess sialidase activity (as previously stated), perform histological analysis and conduct RT-PCR analysis. Approximately 500 µl of blood was also obtained from each mouse prior to death through retro-orbital bleeding for FACS analysis.

2.16 Isolation of RNA from B6.SM Cells

Cells were prepared for the lysosomal sialidase activity assay and were split into 2 tubes of equivalent volume of PBS, with the cells being tested for sialidase activity centrifuged and lysed as previously stated. An additional tube

was prepared for RNA isolation by centrifugation at 1200 RPM at 4° C. The PBS was removed from the tube and 1 ml of TRIzol (GibcoBRL) was aliquoted per 50 – 100 mg of cells per tube followed by homogenization using a Polytron (Kinematica AG). The homogenized samples were incubated for 5 minutes at room temperature to dissociate the nucleoprotein complexes. The samples were then subjected to 0.2 ml of chloroform for every 1 ml of TRIzol used, with the samples vigorously shaken for 15 seconds and then incubated at room temperature for 3 minutes. The samples were centrifuged at 12000 g for 15 minutes at 4° C leading to 3 separate phases in each sample. The upper aqueous phase was collected (RNA remains in this phase exclusively) and transferred to a new sterile tube. The RNA was precipitated by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent used with an incubation period of 10 minutes at room temperature. The precipitated RNA was centrifuged at 12000 g for 10 minutes at 4° C. After centrifugation, the supernatant was discarded and the RNA pellet was washed once with 1 ml of 75% ethanol (in DPEC H_20) per 1 ml of TRIzol used. The samples were vortexed and centrifuged at 7500 g for 5 minutes at 4° C. The RNA was then dried, but not completely, and dissolved in 50 µl of DPEC-treated H_20 followed by an incubation at 55° C for 10 minutes to help dissolve the RNA.

2.17 Real-Time PCR of Isolated RNA from AdmsialFG and AdmsialHD-Infected B6.SM Cells

Isolated RNA from all samples was reverse transcribed and frozen at -20° C for real-time PCR. All real-time PCR samples were prepared in triplicate with one non-template control. Each sample was used for two different probes, either a Neu1 specific (Applied Biosystems) or GAPDH specific probe/primer kit (Applied Biosystems). The GAPDH probe kit was used as a control for the expression of a general protein with the Neu1 probe kit used to detect mouse lysosomal sialidase transcripts. The real-time PCR procedure was carried out using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) for 2 hours.

Chapter 3

Results

3.1 Cloning of Helper-Dependent Adenovirus Vector pC4HSU-msial.

The plasmids pBSKS+msialR and pC4HSU were used for the mouse lysosomal sialidase gene and adenoviral growth components respectively. The 10.6 kb mouse lysosomal sialidase gene was ligated via compatible cohesive ends into the 19.3 kb adenoviral vector containing the two ITRs and the packaging signal (Ψ). The resultant plasmid, pC4HSU-msial, was digested with *HindIII* (Figure 3.1 A) to produce the expected fragment sizes of 5680, 5192, 4728, 3231, 2791, 2694, 2501, 1992, 815, and 308 bp (Figure 3.1 B). The mouse lysosomal sialidase gene resides within the 5192, 4728, and 1992 bp fragments collectively. Additional digestion with EcoRI was performed to provide further assurance that the selected clones were correctly verified (Figure 3.2 A). Expected fragment sizes from *EcoRI* digestion were 7752, 7732, 7182, 3389, 2678, and 1189 bp fragments (Figure 3.2 B).

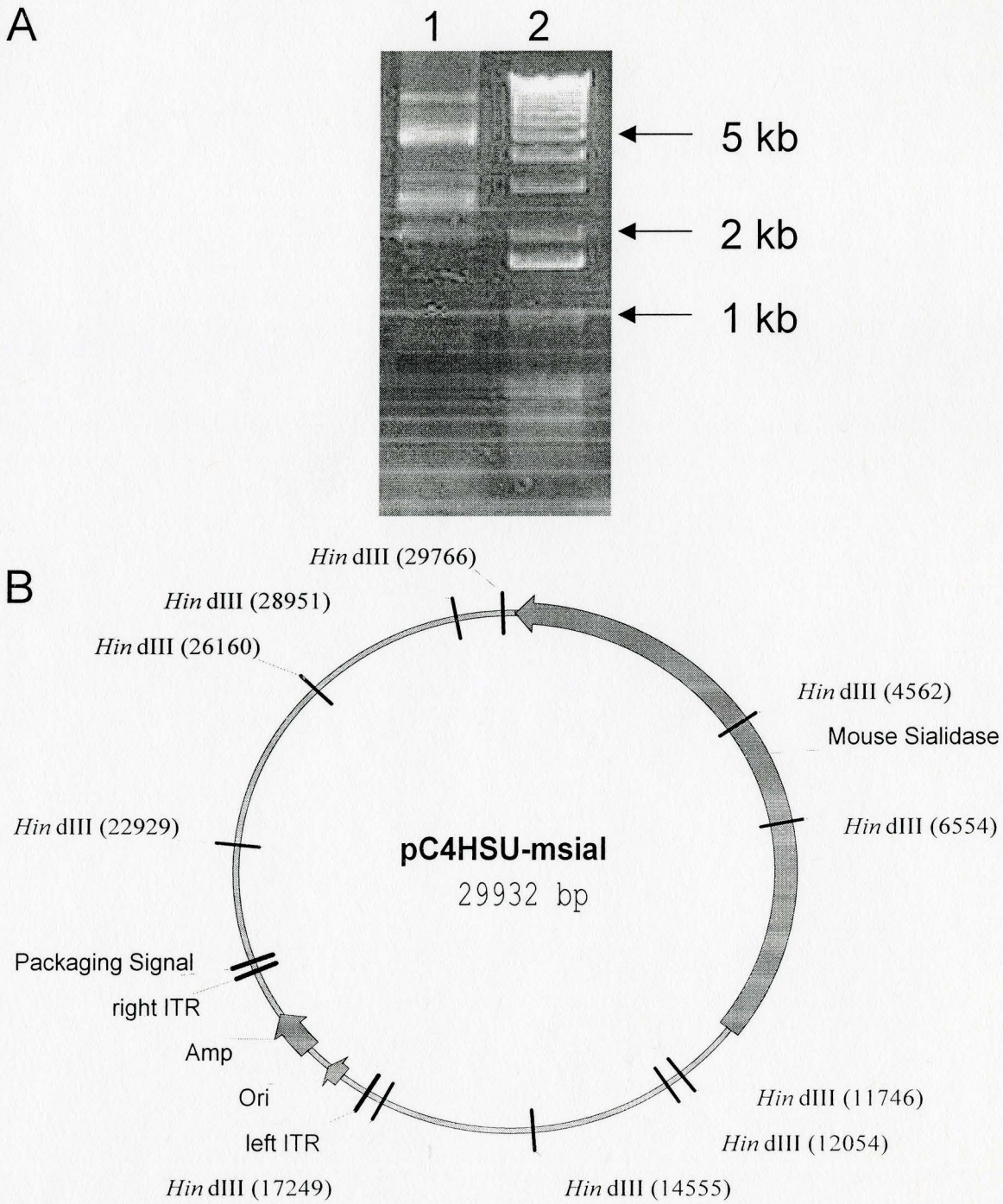

Figure 3.1 (A) Restriction analysis of pC4HSU-msial using *HindIII* revealing the appropriate fragments following digestion (lane 1 pC4HSU-msial, lane 2 1 kb plus ladder (B) Plasmid map displaying fragment sizes corresponding to a *HindIII* digestion

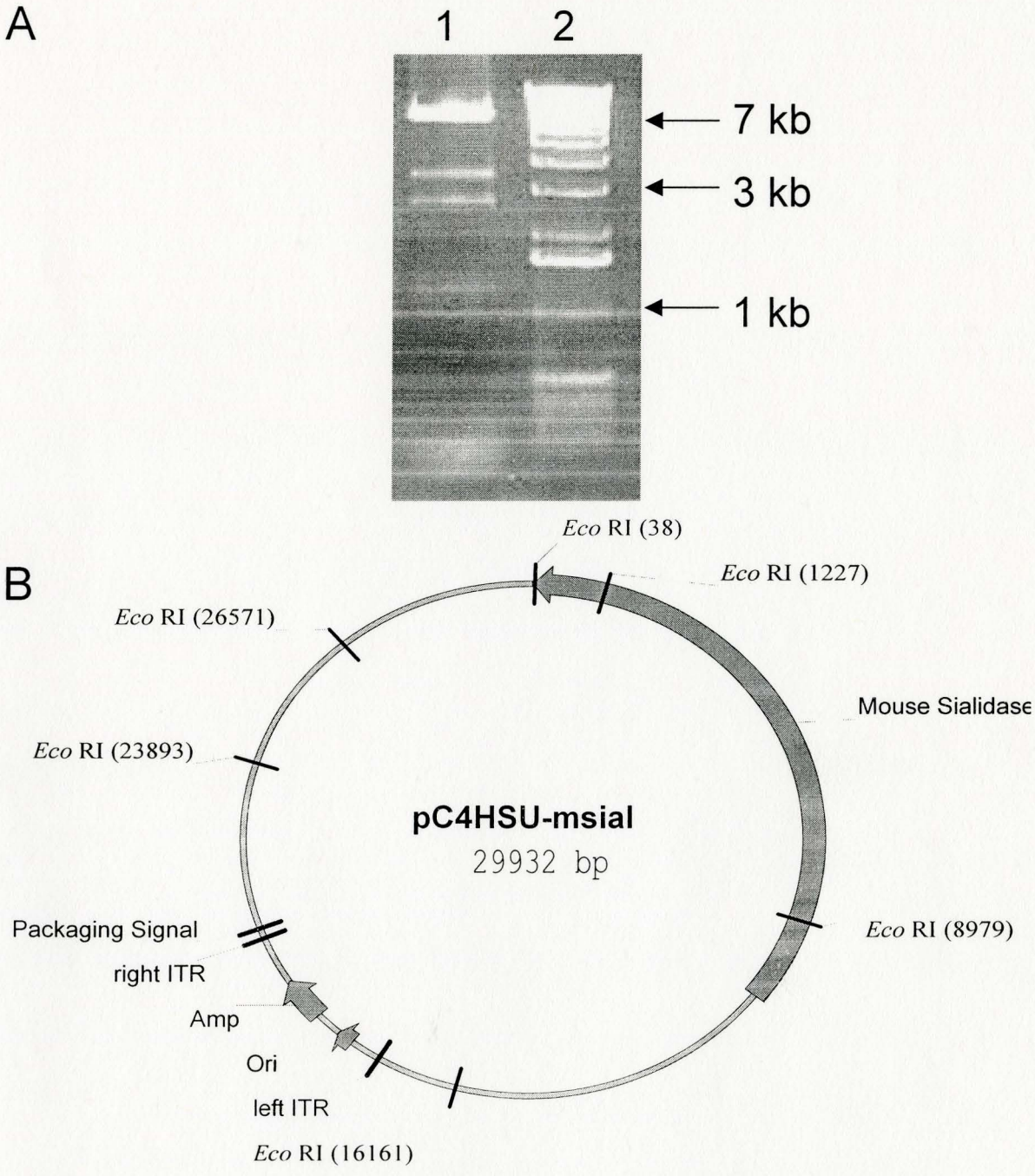

Figure 3.2 (A) Restriction analysis of pC4HSU-msial using *EcoRI* revealing the appropriate fragments following digestion (lane 1 pC4HSU-msial, lane 2· 1 kb plus ladder (B) Plasmid map displaying fragment sizes corresponding to an *EcoRI* digestion

3.2 Propagation of AdmsialHD by serially passaging through 293cre4 cells.

Following pC4HSU-msial verification, the plasmid was digested with *PmeI* to release the bacterial amplification components, ampicillin resistance and the origin of replication. 293cre4 cells were transfected with the digested pC4HSU-msial followed by helper virus (AdLC8Cluc) infection at an MOI of 5 the next day. CPE was reached in 48 hours and subsequent passages of 293cre4 cells were co-infected with both the lysate from the previous passage and AdLC8Cluc at an MOI of 1. Correct helper-dependent fragments following *HindIII* digestion are 5192, 4728, 3231, 2791, 2694, 2501, 2371, 1992, 815, 412, and 308 (Figure 3.3). AdLC8Cluc HindIII digestion would produce fragments of sizes 8010, 5322, 4597, 3307, 3274, 2937, 2871, 2081, 1918, 1005, and 75 bp. Passages 6 – 8 demonstrated proper AdmsialHD structure after *HindIII* digestion (Figure 3.4) despite several fragments being less visible than others. Figure 3.4 displays the faintness of the Hd-vectors 2501, 1992, and 815 bp fragments, which are all identifying fragments relative to the helper virus' restriction analysis pattern. It was also apparent that the 2937 and 2871 bp fragments disappear from passage 7 to passage 8, as well as the 2081 bp fragment. To further demonstrate that AdmsialHD was being amplified, passage 8 DNA was submitted for sequence analysis for the mouse lysosomal promoter and the results were positive, proving the existence of the mouse lysosomal sialidase promoter within the adenoviral genome. The adenoviral vector, AdmsialFG, was purified twice through CsCl before any *in vivo* experiments were performed.

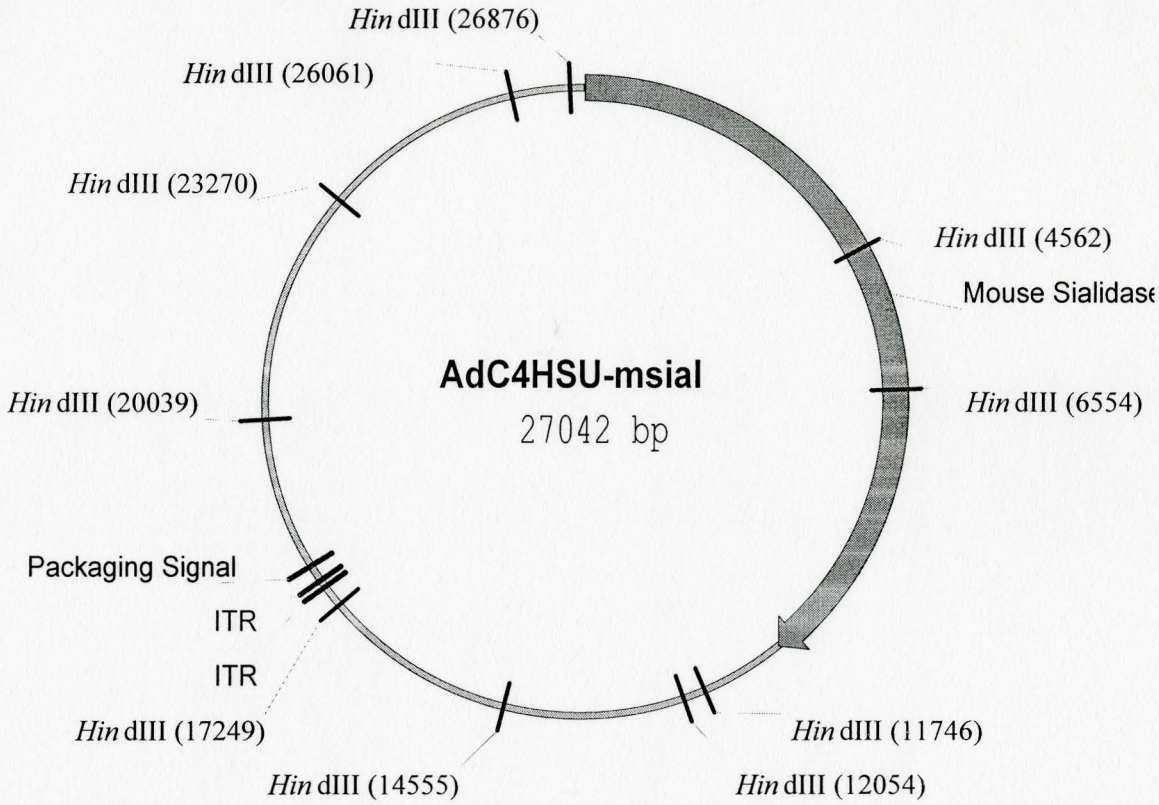

Figure 3.3 Plasmid map for AdmsialHD displaying the restriction sites for *HindIII* The two individual ITRs represent the left and right end of the linear viral genome respectively

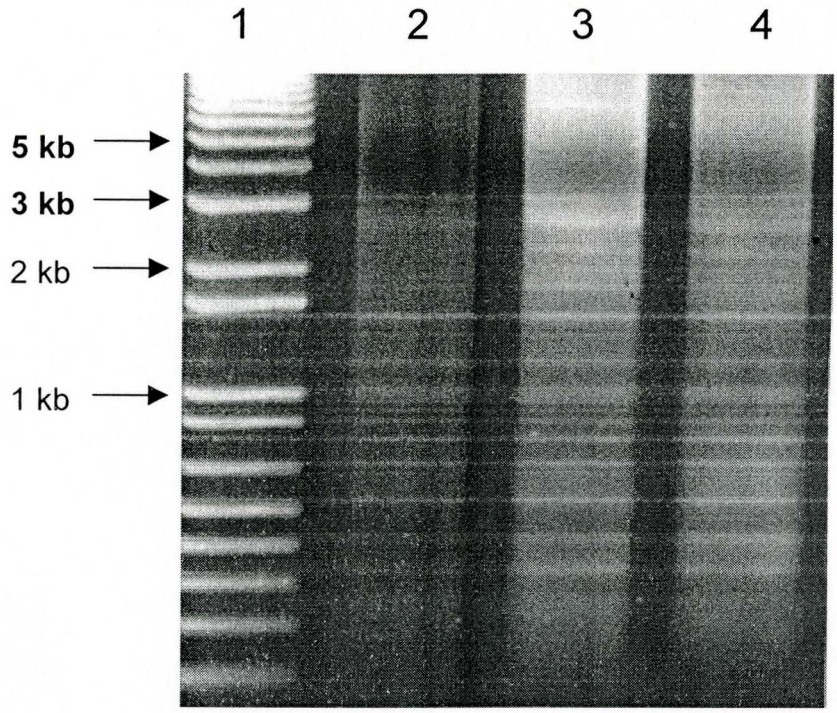

Figure 3.4 Restriction analysis of AdmsialHD DNA following *HindIII* digestion at passages 6 (lane 2), 7 (lane 3), and 8 (lane 4) of helper-dependent adenovirus propagation The distinct HD-vector fragments, 2501, 2371, 1992, and 815 bp are all visible despite the faintness in the image The doublets at the 2.7 and 3.2 kb range also noticeably disappear from passage 7 to 8, indicating the loss of helper virus during serial passaging Lane 1 is the 1 kb plus ladder used as the DNA standard

3.3 Generation of the first-generation adenovirus AdmsialFG.

The shuttle plasmid pDC312-msial was made through cloning 7.8 kb of the mouse lysosomal sialidase gene into pDC312 followed by purification through CsCl in preparation for transfection along with the genomic adenovirus plasmid pBHGlox(ΔE1, E3)Cre into 293 cells. The 7.8 kb mouse lysosomal sialidase fragment used for the first-generation adenovirus construction is 2.8 kb smaller than that used for the helper-dependent adenovirus as the genomic adenovirus plasmid pBHGlox(ΔE1, E3)Cre can only accommodate a transgene of 8.2 kb in length. The missing 2.8 kb portion is solely within the 3' untranslated region and does not include the poly-A signal or any portion of the exons. AdmsialFG was propagated in 293 cells after co-transfection of pDC312-msial and pBHGlox(ΔE1, E3)Cre, with adenoviral plaques forming around 5-6 days post-transfection. Several plaques were picked and used to infect 60-mm dishes of 293 cells to further amplify and characterize the plaques. The genomic map for AdmsialFg indicates that the expectant fragment sizes are 8010, 5322, 4670, 4597, 4470, 3012, 2937, 2081, 1992, 902, and 75 bp in length when digested with *HindIII* (Figure 3.5). Following viral DNA purification and subsequent digestion with *HindIII*, the proper genomic structure was verified (Figure 3.6). The faint fragments seen in Figure 3.6 around 1.8 kb are expected to be from the digestion of cellular DNA with *HindIII*. The resulting adenoviral vector, AdmsialFG, was propagated in 30 dishes (150-mm) of 293 cells and purified twice through CsCl before any *in vivo* experiments were performed.

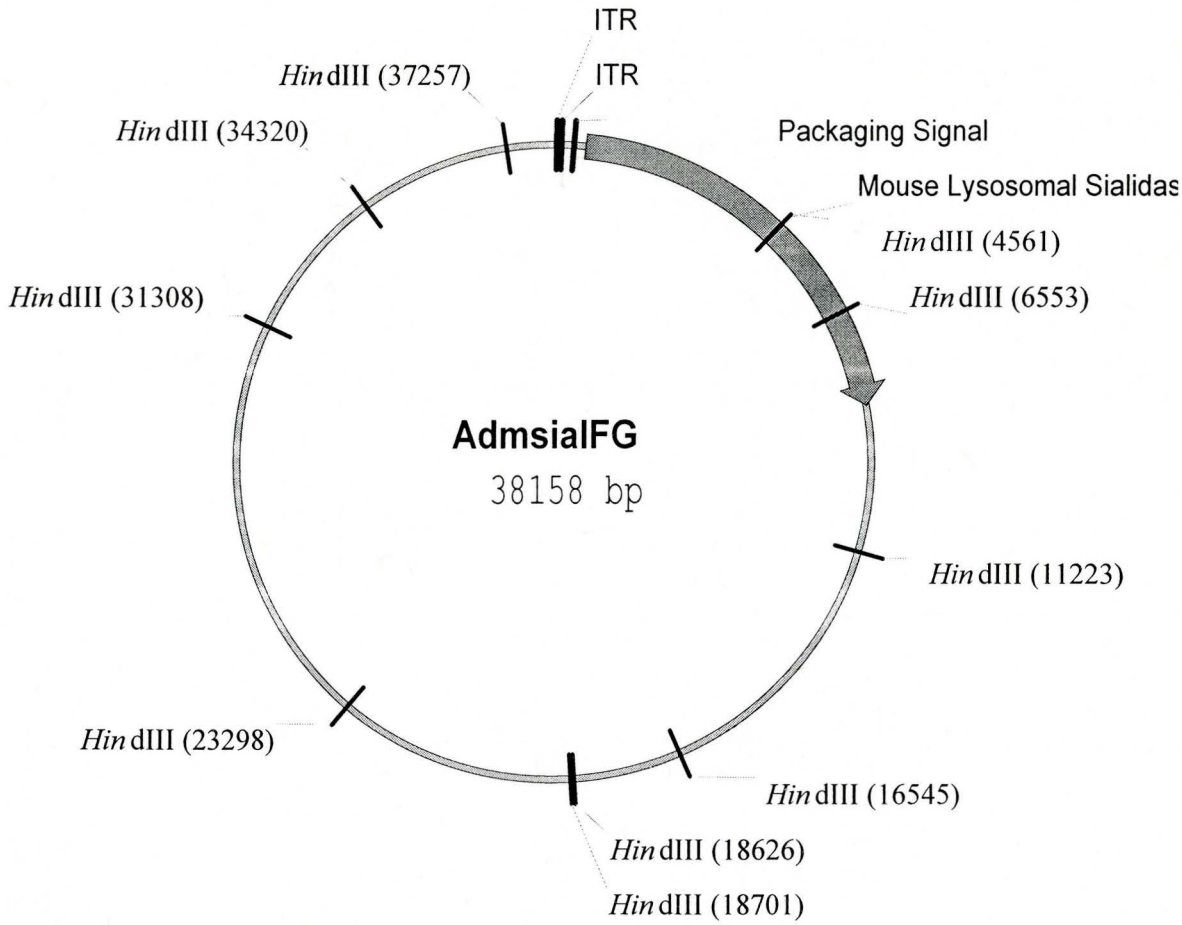

Figure 3.5 Plasmid map for AdmsialFG displaying the restriction sites for *HindIII* The two individual ITRs represent the left and right end of the linear viral genome respectively

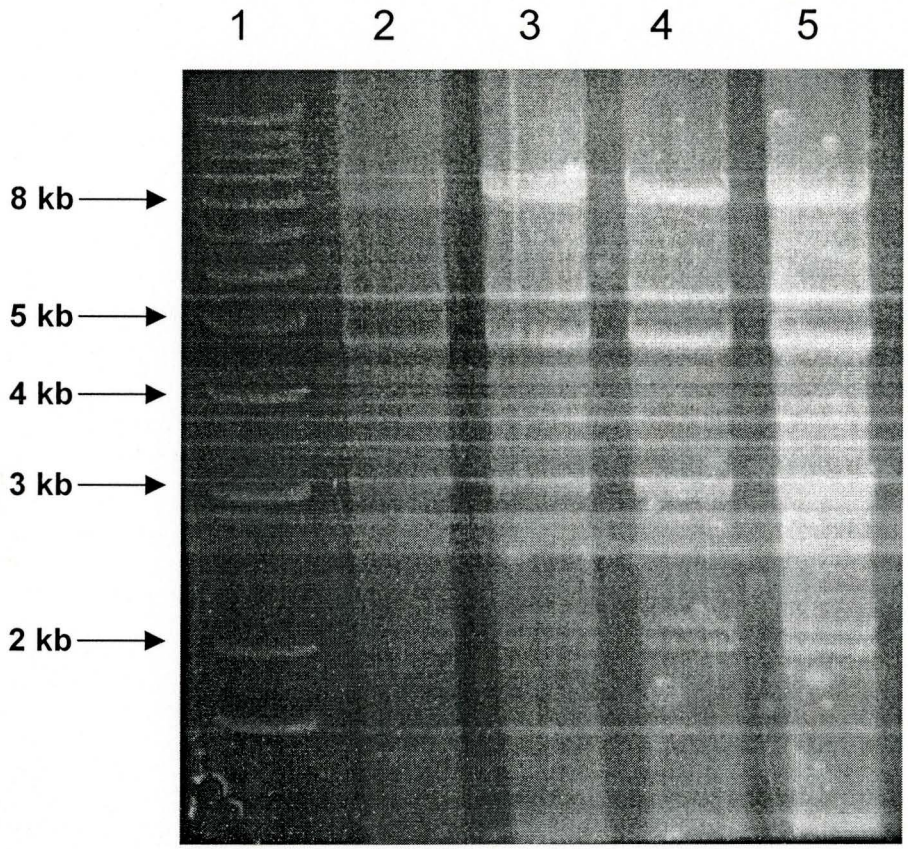

Figure 3.6 Restriction analysis of AdmsialFG DNA following *HindIII* digestion All fragments reveal correct viral genomic assembly in all viral plaques characterized (lanes 2-5) The faint fragment seen at approximately 1 8 kb represents digested cellular DNA. Lane 1 is the 1 kb plus ladder used as the DNA standard

3.4 Subcloning of human lysosomal sialidase gene into pCR2.1 vector.

The 8 kb human lysosomal sialidase gene was PCR amplified, gel purified (Figure 2.1) and cloned into the TA-cloning site of the pCR2.1 cloning vector. Since clones that correctly contain the human lysosomal sialidase gene will disrupt the LacZ gene in the pCR2.1 vector, positive clones were selected based on blue/white colonies. Several white colonies were picked and harvested for plasmid purification. After *HindIII* restriction digestion, fragment sizes of 2.9 and 9 kb were expected (Figure 3.7) and obtained in two of the 4 DNA preparations for pCRhsial (Figure 3.8). Subsequent cloning strategies towards inserting the human lysosomal sialidase gene from pCRhsial into the helper-dependent vector pC4HSU did not yield any positive clones and thus can be attempted in the future.

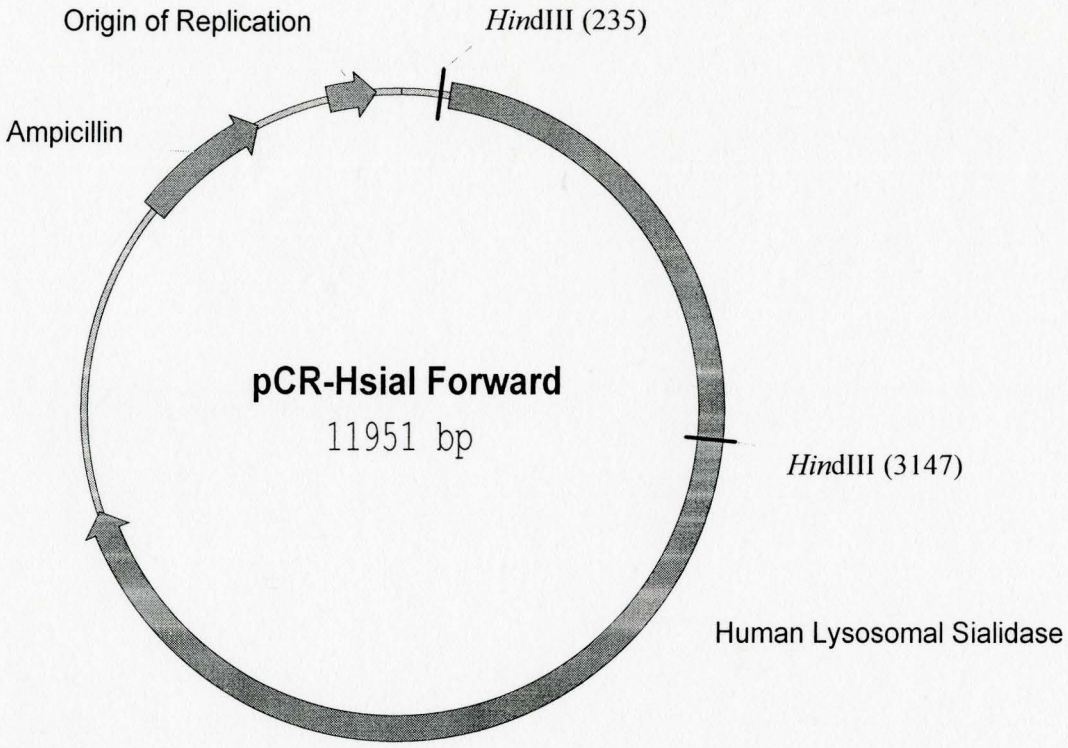

Figure 3.7 Plasmid map of the subclone pCRhsial containing the human lysosomal sialidase gene. The *HindIII* restriction sites produce 2 fragments of approximately 9 and 2.9 kb in size upon *HindIII* digestion.

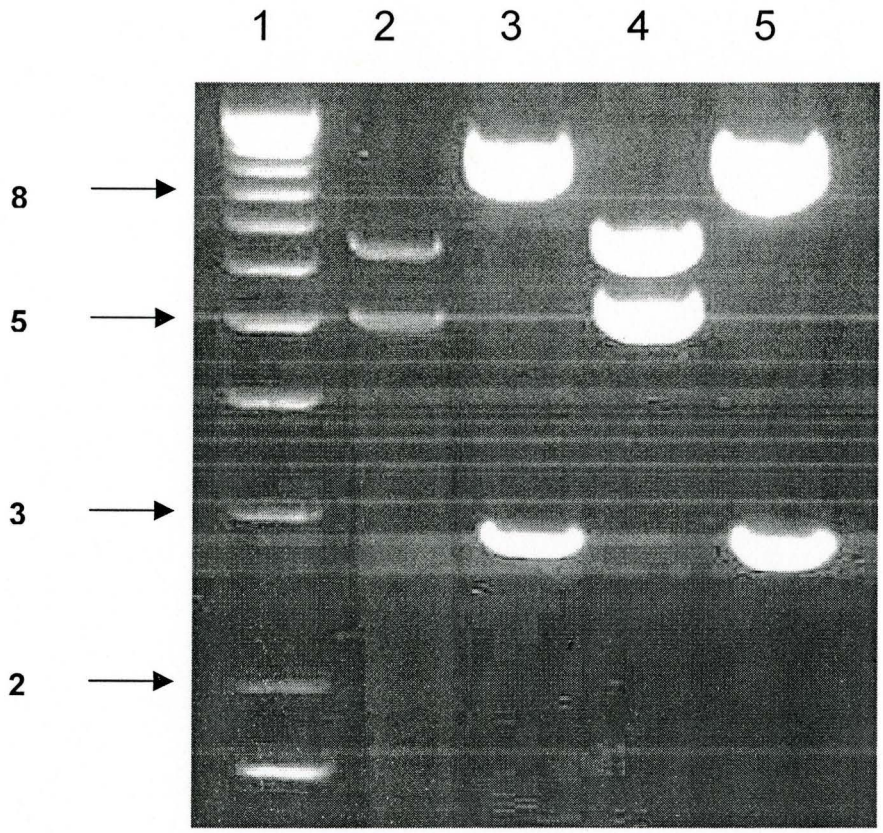

Figure 3.8 Restriction analysis of pCRhsial following *HindIII* digestion Lanes 3 and 5 display positive clones revealing the 9 and 2.9 kb fragments that are produced upon *HindIII* digestion while lanes 2 and 4 display negative clones. Lane 1 is the 1 kb plus ladder used as the DNA standard

3.5 Mouse lysosomal sialidase activity in sialidase-deficient cells (WG544) 96 hours post-infection with AdmsialHD and AdmsialFG.

Once the AdmsialHD was properly characterized and CsCl purified, the next step was to test the sialidase activity of infected cells. The assay for lysosomal sialidase activity requires the substrate 4-methylumbelliferyl-n-acetyl-α-D-neuramide (Mu-Nana) in an acidic environment, pH 4.8. Upon cleavage by lysosomal sialidase, 4-methyl-umbelliferone is released and can be detected using a fluorometer. This assay directly relates the amount of functional sialidase with the amount of sialidase activity in a sample reaction, making this a good method for determining the amount of functional lysosomal sialidase that the vector has produced. WG544 cells were cultured in 100-mm dishes until 80-90% confluency and were infected with different MOIs of AdmsialHD. The WG544 cell line is devoid of lysosomal sialidase activity, and thus provides a unique cell line where any increase in lysosomal sialidase activity can be detected. Individual dishes were infected at either an MOI of 50, 100, 500, or 1000 particles per cell or with 0.5 ml of PBS++ (Figure 3.9). The infection had an adsorption period of 1 hour followed by an incubation period of 96 hours to allow for sialidase expression. Due to the nature of the endogenous promoter, very high levels of lysosomal sialidase were not expected. After the 96 hour incubation, the cells were harvested and lysed accordingly to be assayed for lysosomal sialidase activity. A dose-response curve can be seen in Figure 3.9 with relatively little activity at an MOI of 50, and a steady, almost linear increase in sialidase activity

with increasing MOI (approx. 5-fold from MOI 50 to MOI 500 and 5-fold from MOI 100 to MOI 1000). The moderate increase in lysosomal sialidase activity was expected at the low MOIs due to the endogenous promoter activity. From these results, it is clear that AdmsialHD is a functional vector that is capable of producing mouse lysosomal sialidase at supraphysiological levels *in vitro*.

In a similar experiment, the AdmsialFG vector was compared to a first-generation adenoviral vector containing the cDNA of human lysosomal sialidase (AdS+) with a cytomegalovirus (CMV) promoter controlling its expression. The AdmsialFG was used at 100 particles per cell where the AdS+ was used at 10 PFU/cell (approximately 100 particles/cell) and the control was a mock-infected dish subjected to PBS++ alone. All treatments were adsorbed for 1 hour with a 96 hour incubation period prior to harvesting the cells for determination of sialidase activity. By utilizing the same procedures as before for determining enzyme activity, it was apparent that the AdS+ vector produced approximately 5-fold more functional enzyme relative to AdmsialFG-infected cells (Figure 3.10). This was to be expected due to the nature of the promoters driving expression of the two transgenes, with the CMV promoter in AdS+ and the endogenous promoter in AdmsialFG. Furthermore, the WG544 cell line is a human fibroblast cell line and AdmsialFG expresses a mouse protein whereas AdS+ expresses a human protein, which could also increase the variability seen between the two vectors. Despite the lower levels of functional lysosomal sialidase expressed

from AdmsialFG relative to AdS+, the levels of functional enzyme in AdmsialFG-infected cells remained well above those of the PBS++- infected cells, indicating that AdmsialFG is a functional vector, expressing sialidase.

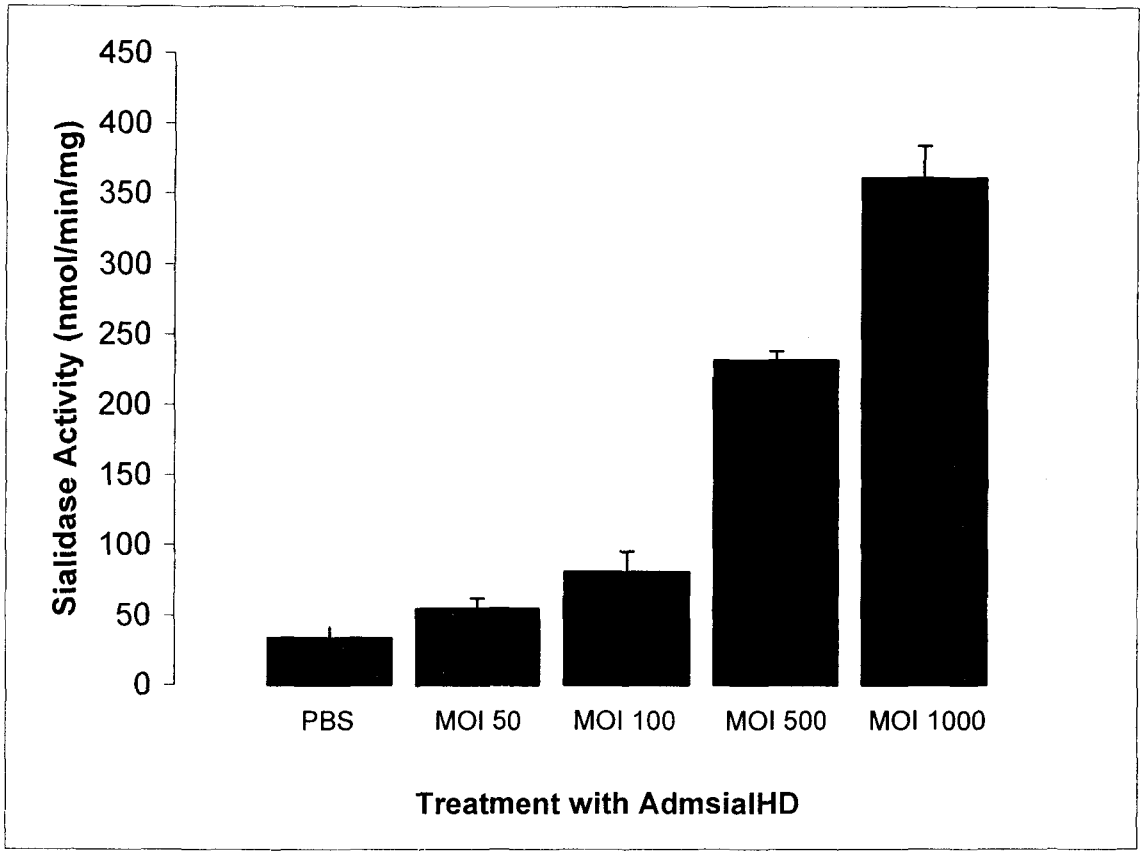

Figure 3.9 Sialidase activity from sialidase-deficient cells (WG544) that were infected with AdmsialHD at an MOI of 50, 100, 500, or 1000 particles/cell. Cells mock-infected with PBS++ alone served as controls. Enzyme assay was performed 96 hours post infection. Error bars are standard deviations as three assays were performed from a single sample from each infection variable.

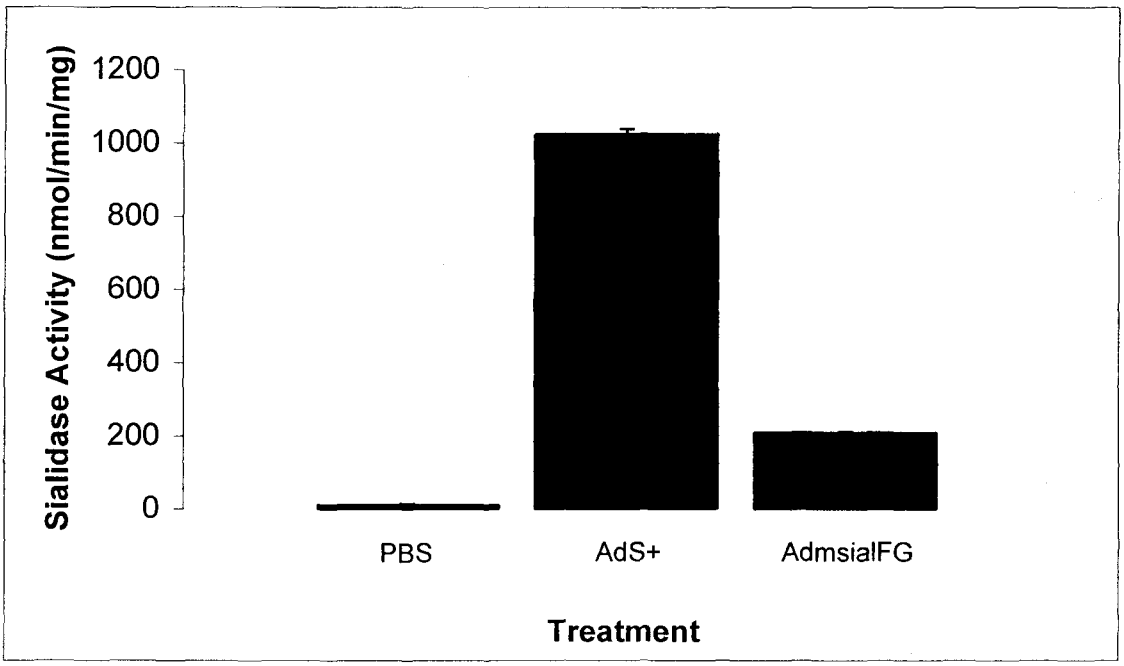

Figure 3.10 Sialidase activity from sialidase-deficient cells (WG544) that were infected with AdmsialFG or AdS+ at an MOI of 100 particles/cell or 10 PFU/cell respectively. Cells mock-infected with PBS++ alone served as controls. Enzyme assay was performed 96 hours post infection. Error bars are standard deviations as three assays were performed from a single sample from each infection variable.

3.6 Comparative mouse lysosomal sialidase activity 96 hours post-infection of sialidase-deficient cells (WG544) cells with AdmsialHD, AdmsialFG, or AdS+.

Based on the previous results that displayed the relative activities of AdmsialHD and AdmsialFG, it would appear that the AdmsialFG has higher expression levels of lysosomal sialidase, but no comparative study between the two had yet been performed. In this experiment, AdmsialFG was expected to have higher levels (approx. 5-fold) based on the previous human deficient experiments. The infections were carried out similarly to the previous experiments in 100-mm dishes, with AdS+ infecting at 10 PFU/cell, AdmsialFG infecting separate dishes at MOIs of 10, 100, 500, and 1000 particles/cell, AdmsialHD infecting separate dishes at MOIs of 10, 100, 500, and 1000 particles/cell and a mock-infected dish subjected to PBS++ alone. As before, following a 1 hour adsorption and a 96 hour incubation, the infected cells were harvested and lysed to determine their functional lysosomal sialidase activity. All of the samples were collected except for the AdmsialHD-infected plate with an MOI of 500 particles/cell due to contamination. As illustrated in Figure 3.11, all viral treatments produced an increase in lysosomal sialidase levels, except for the AdmsialFG at an MOI of 1000 particles/cell group. The AdmsialFG-infected cells at the MOIs of 10, 100, and 500 had approximately 2-fold greater enzyme activity than the PBS++ control while the AdmsialHD-infected cells at all MOIs had approximately 3-fold greater enzyme activity compared to the PBS++ control.

Since the last experiment with AdmsialFG was only carried out at an MOI of 100 particles/cell, perhaps the increased dose was toxic to the cells and resulted in a decrease in sialidase expression. Furthermore, the results here are the opposite from the previous two experiments, as the AdmsialHD-infected cells seemed to express similar levels of functional sialidase and even greater levels in the AdmsialHD 1000 particles/cell MOI group. The differences in Hd-vector and FG-vector expression levels were not the only difference from the last experiment (Figure 3.10), as the AdS+ infected group expressed approximately 5-fold lower lysosomal sialidase than in the last experiment, suggesting that perhaps the cells were not given the same amount of care when being cultured or that during the harvesting or lysing stage, some cells may have prematurely died resulting in loss of activity or that the cells were not lysed as well as in the previous experiments. A Western Blot was also performed on the cell lysates using the α-75 antibody (data not shown) with no specific band picked up that would represent the mouse lysosomal sialidase protein. Despite the discrepancies in the data, all three vectors produced functionally active lysosomal sialidase at levels greater than the PBS++ control.

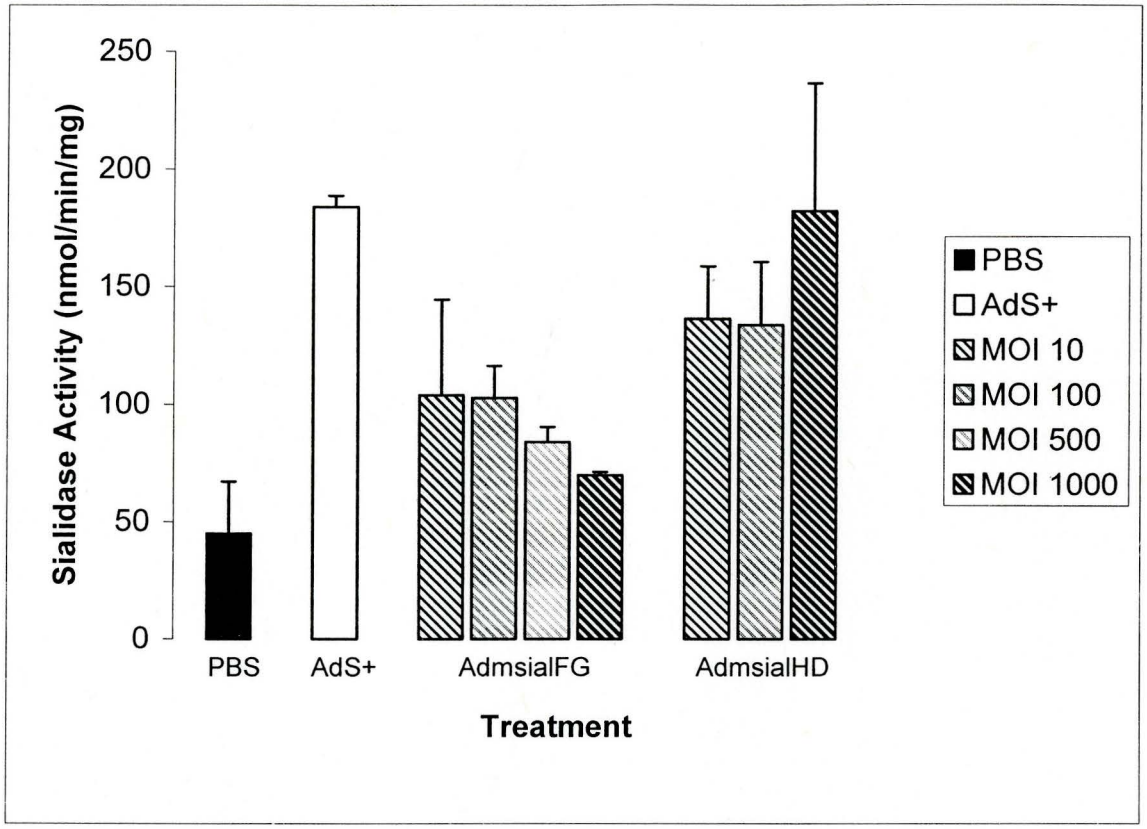

Figure 3.11 Sialidase activity from sialidase-deficient cells (WG544) that were infected with AdmsialFG or AdmsialHD at an MOI of 10, 100, 500 or 1000 particles/cell or AdS+ at an MOI of 10 PFU/cell Cells mock-infected with PBS++ alone served as controls. Enzyme assay was performed 96 hours post infection The AdmsialHD MOI 500 sample became contaminated and was discarded prior to enzyme assay Error bars are standard deviations as three assays were performed from a single sample from each infection variable.

3.7 Mouse lysosomal sialidase activity from isolated blood monocytes.

Since the lysosomal sialidase deficiency in the SM/J mouse has been linked with the immune system, it was necessary to determine if the sialidase activity assay could be performed on isolated blood monocytes from a mouse. Blood was collected via the retro-orbital bleed technique, followed by Tris-NH_4Cl lysing of red blood cells which after centrifugation isolated monocytes to be used for the enzyme activity assay. In this particular experiment, 150 µl of blood was collected for each C57/Bl6 mouse enzyme assay. In Figure 3.12, it can be seen that the sialidase activity assay was not sensitive enough to pick up enzyme activity from 150 µl of isolated blood monocytes. The level of sialidase activity, averaging 25 nmol/min/mg protein was well below that seen in the WG544 cell line, which is devoid of lysosomal sialidase (Figure 3.9 – 3.11 as examples). Additionally, the isolated monocytes in this particular experiment are from the C57/Bl6 mouse, which have no lysosomal sialidase defect, thus if the monocytes from the SM/J mouse have low levels of enzyme activity, then this protocol will not prove useful for future SM/J mouse experiments. Because of this, sialidase activity assays should be performed on mouse tissues such as the liver, kidney, and spleen rather than isolated blood monocytes, resulting in a one-time usage of each mouse.

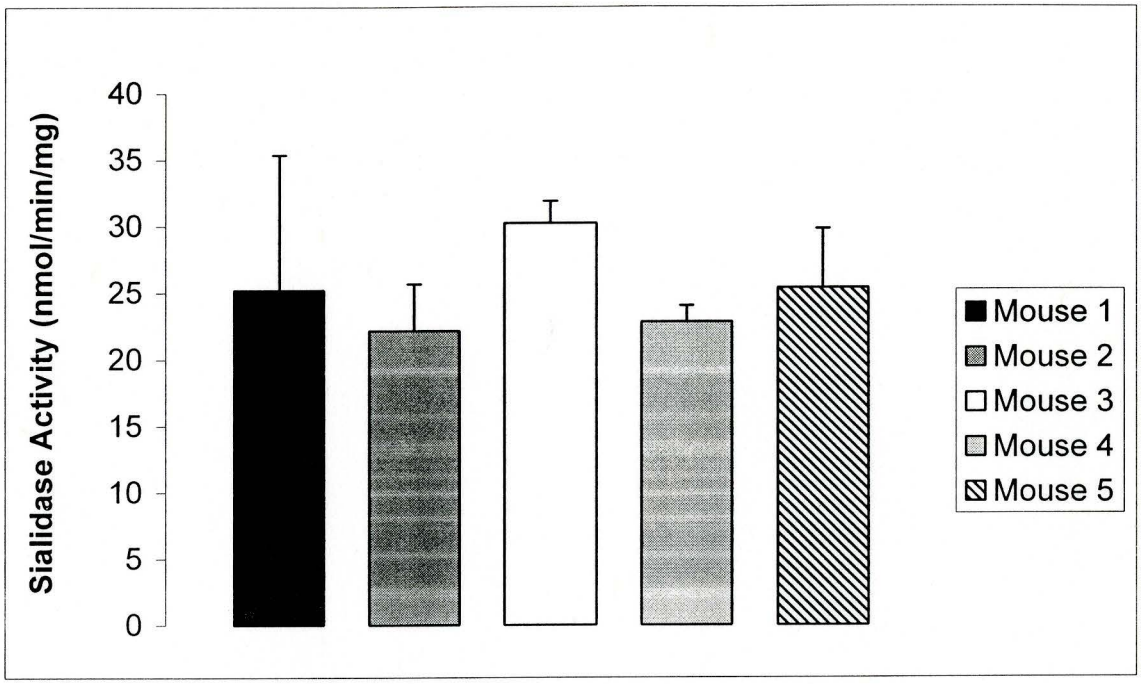

Figure 3.12 Sialidase activity from monocytes isolated from 150 µl C57/Bl.6 mouse blood Standard deviation represents pipetting error alone as three assays were performed from a single sample.

3.8 Comparative up-regulation of mouse lysosomal sialidase activity in mouse embryonic fibroblasts (MEF) by AdmsialFG and AdmsialHD.

With prior sialidase activity assays performed on the lysosomal sialidase-deficient cell line, WG544, there was no information on how these adenoviral vectors would perform therapeutically in normal mouse cells. The mouse embryonic fibroblast (MEF) cell line was chosen to test the hypothesis that both AdmsialFG and AdmsialHD should up-regulate sialidase activity beyond those of the PBS++ infected control. The experiment was performed exactly as before, with a 1 hour viral adsorption and a 96 hour incubation period. Cells were harvested and lysed accordingly in preparation for the sialidase activity assay. As shown in Figure 3.13, there exists a dose-response with the MEF cells infected with the AdmsialFG vector at the 3 different MOIs of 10, 100, and 1000 particles/cell. The AdmsialHD-infection groups did not display a dose related response as all three MOI treatments resulted in sialidase activity that was similar to the AdC4HSULacZ control and PBS++ mock-infected control. The potential reason for this could be that the MEF cells already express physiological levels of lysosomal sialidase and thus the enzyme could already be at saturating levels within the cell. Any increase copy number of the lysosomal sialidase gene may not increase expression due to saturation of the enzyme, especially when the additional genetic material is controlled by an endogenous promoter. AdmsialFG has additional viral promoters relative to the AdmsialHD as helper-dependent

adenoviruses are completely devoid of all viral genes, which could explain why the AdmsialFG-infected cells displayed the dose-related response.

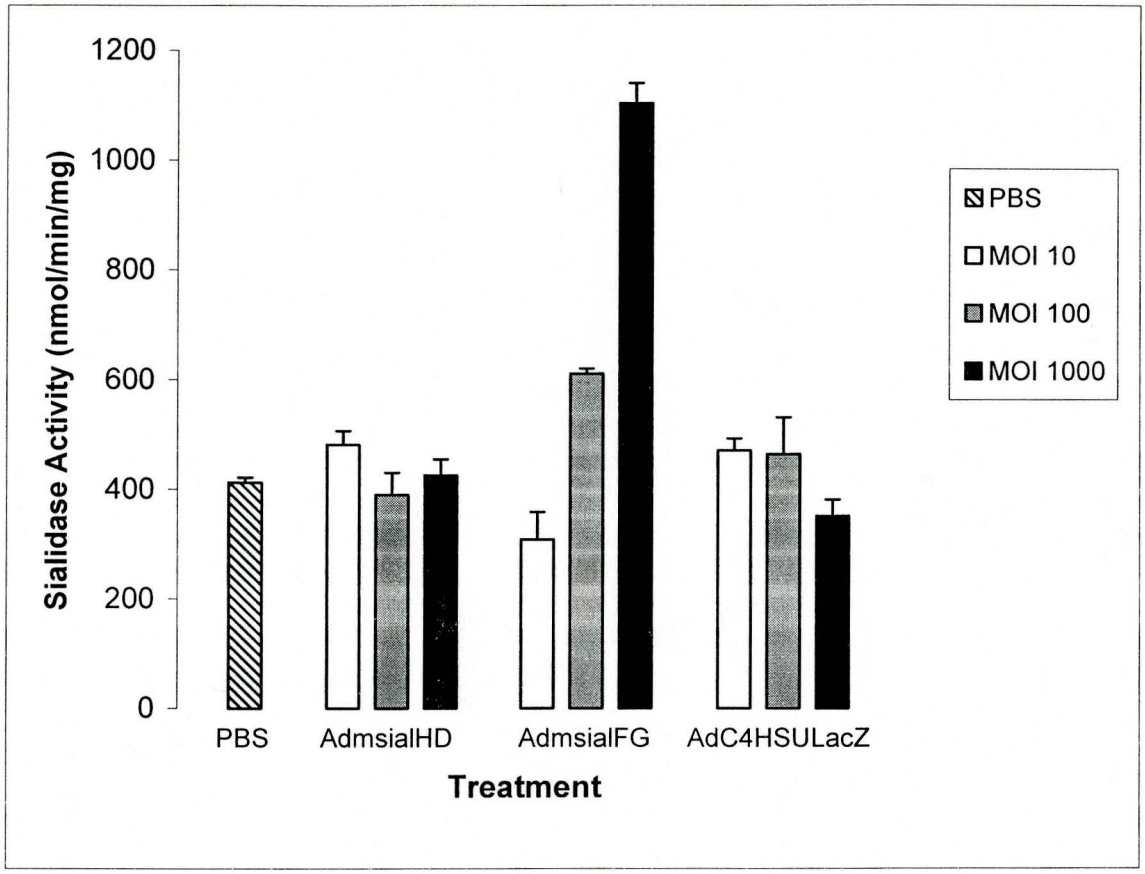

Figure 3.13 Sialidase activity from MEF cells that were infected with AdmsialHD, AdmsialFG or AdC4HSUlacZ at an MOI of 10 100 or 1000 particles/cell. Cells mock-infected with PBS++ alone served as controls. Enzyme assay was performed 96 hours post infection Error bars are standard deviations as three assays were performed from a single sample from each infection variable.

3.9 Rescuing of lysosomal sialidase defect in B6.SM -/- and +/- fibroblasts by the up-regulation of mouse lysosomal sialidase expression and activity through AdmsialFG and AdmsialHD infections.

Since the helper-dependent vector AdmsialHD did not display an upregulation of lysosomal sialidase in cells that express lysosomal sialidase at physiological levels (Figure 3.13), we were interested in seeing if AdmsialHD could upregulate lysosomal sialidase expression in mouse cells that are homozygous for the SM/J sialidase mutation to levels in the heterozygous cells. A moderate increase was expected due to the large increase seen in sialidase-deficient cells (Figure 3.9) and due to the reduced physiological levels of lysosomal sialidase in the homozygous cells. The B6.SM fibroblasts were prepared from sacrificed homozygous and heterozygous mice and infected with AdmsialFG, AdmsialHD, and AdC4HSULacZ at an MOI of 1000 particles/cell, or mock-infected with PBS++. Adsorption times were for 1 hour with a 96 hour incubation period following adsorption. Cells were harvested and lysed as previously stated with the cell lysate being used to determine lysosomal sialidase activity levels. Figure 3.14 displays the enzyme activity for all 4 treatments with the homozygous and heterozygous mock-infected controls showing a moderate difference in enzyme activity. It is visible that the AdmsialHD MOI of 1000 particles/cell and AdmsialFG MOI of 1000 particles/cell groups can increase lysosomal sialidase expression above levels seen in the heterozygous control cells, with AdmsialFG increasing the enzyme levels by approximately 100% and

AdmsialHD increasing enzyme levels by approximately 30% compared to those of the heterozygous control cells. A similar effect seen with AdmsialHD and MEF cells regarding cellular levels of sialidase and viral-induced sialidase activity (Figure 3.13) was also seen here as enzyme levels in B6.SM +/- cells increased 2-fold following AdmsialHD infection whereas enzyme levels in B6.SM -/- cells increased 3-fold following AdmsialHD infection. Interestingly, this is the first report demonstrating that B6.SM +/- and B6.SM -/- cells have significantly different enzyme activity (approximately 2-fold higher in B6.SM +/-) as seen when comparing both mock-infected and AdC4HSULacZ-infected controls between the 2 cell types.

RT-PCR analysis for mouse lysosomal sialidase transcripts following HD and FG-adenoviral infection is shown in Figure 3.15. The transcript levels (ratio of sialidase to mouse GAPDH) of mouse lysosomal sialidase were approximately 2.5-fold higher in AdmsialFG-infected B6.SM -/- cells when compared with that of PBS++-infected B6.SM +/- cells. Similarly, AdmsialHD increased transcript levels in B6.SM -/- cells approximately 2-fold higher than in PBS++-infected B6.SM +/- cells. The phenomenon seen with cellular levels of sialidase and its effect on HD-virally-induced sialidase activity was also shown at the transcript level. AdmsialHD-infected B6.SM +/- cells had transcript levels increased by less than 2-fold whereas the same treatment in B6.SM -/- cells resulted in transcript levels increased to almost 3-fold, relative to their respective PBS

controls. Transcript levels from both cell lines appear to be consistent with one another within the control groups which is consistent with the notion that the *Neu1* mutation only affects enzyme activity and not transcript production.

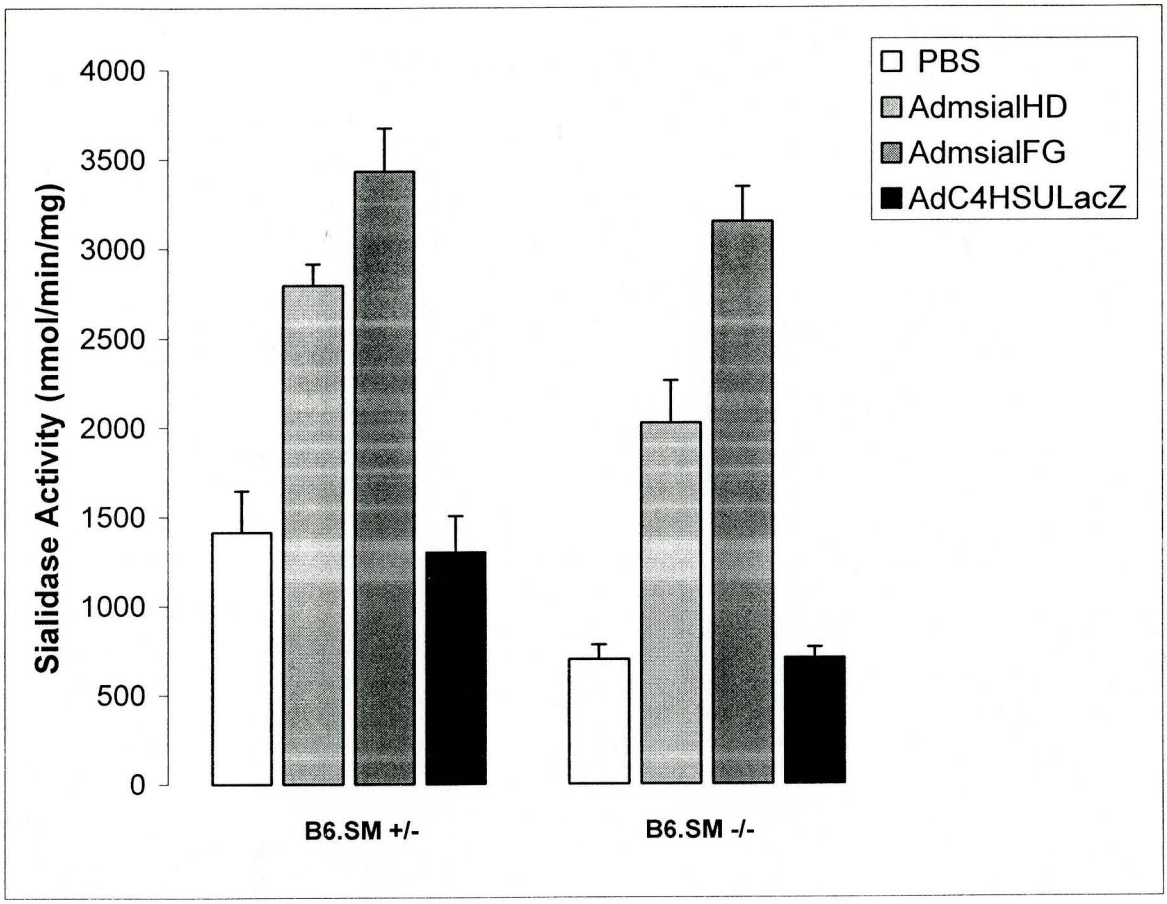

Figure 3.14 Sialidase activity from B6.SM +/- and B6.SM -/- cells that were infected with AdmsialHD, AdmsialFG or AdC4HSUlacZ at an MOI of 1000 particles/cell Cells mock-infected with PBS++ alone served as controls. Enzyme assay was performed 96 hours post infection Standard error is represented through a sample size of n=3.

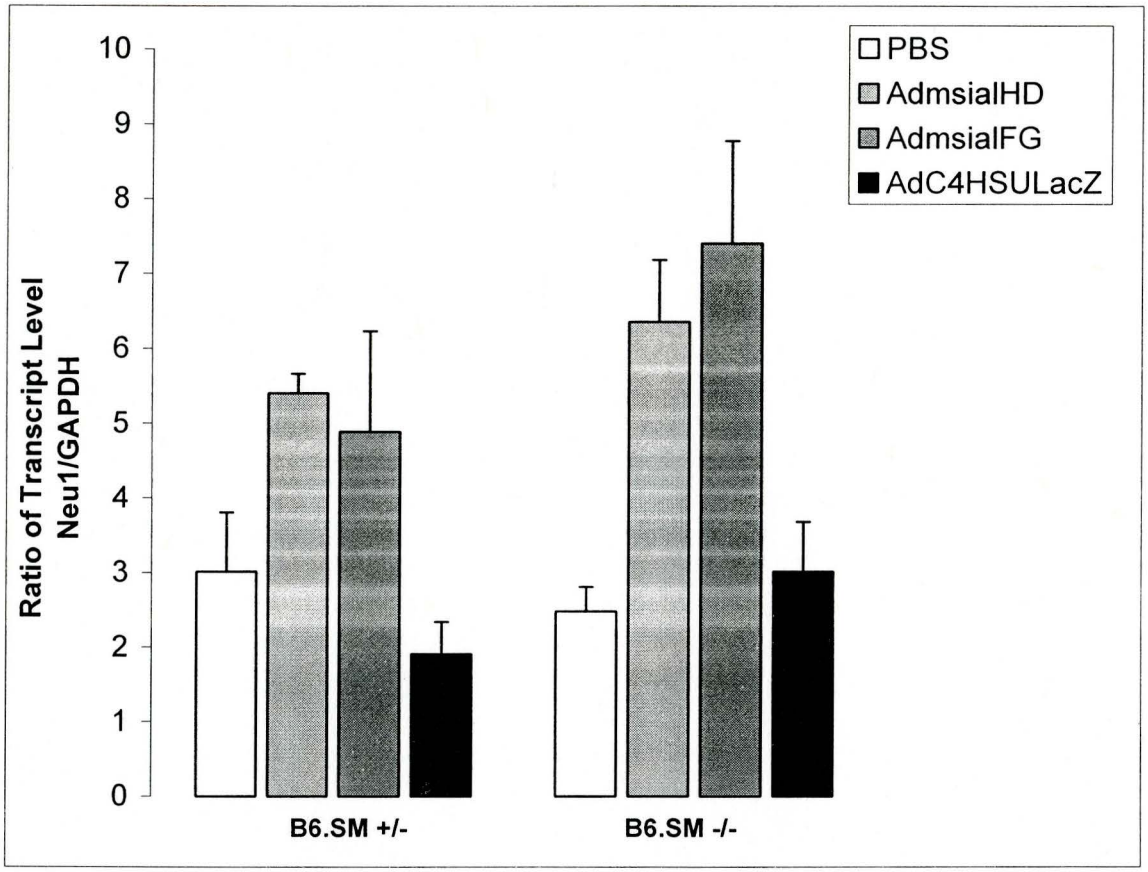

Figure 3.15 Comparative transcript ratio levels of mouse lysosomal sialidase to mouse GAPDH in B6.SM +/- and B6.SM -/- cells. Cells were infected at an MOI of 1000 particles/cell for 96 hours with AdmsialHD, AdmsialFG, AdC4HSULacZ or mock-infected with PBS++ alone prior to harvesting total cellular RNA for reverse transcription and ultimately RT-PCR amplification RT-PCR was performed using mouse lysosomal sialidase- and murine GAPDH-specific probes and primers. Standard error is represented through a sample size of n=3.

3.10 Rescuing of mouse lysosomal sialidase and body weight in the SM/J mouse through AdmsialFG and AdmsialHD gene therapy.

The SM/J mouse strain contains a mutation in the neu1 locus causing the lysosomal sialidase defect, which leads to several phenotypic manifestations. Among them is the specific deficiency of the enzyme in the liver. A 16 mouse experiment involving 4 different infection treatments (AdmsialFG, AdmsialHD, AdC4HSULacZ, and PBS) with 4 mice per treatment was carried out over a 30 day period. The purpose of this experiment was to test if lysosomal sialidase activity in the liver and monocytes could be restored through intravenous injections as well as increasing the SM/J's low body weight during its first 3 months of life and potentially decrease the risk of forming atherosclerotic plaques. Every mouse had its left top (LT), left bottom (LB), right top (RT), or right bottom (RB) ear clipped for identification purposes and each mouse was weighed prior to adenoviral injection and every day for 2 weeks and every 3-4 days after until the 30 day point of the experiment when the mice were terminated for blood and tissue samples. Adenovirus diluted in PBS (100 µl) was given to each mouse into the venous plexus underneath the eye. AdmsialFG and AdC4HSULacZ were given to each mouse at a dose of 5×10^9 total particles while AdmsialHD was given at a dose of 5×10^8 total particles. AdmsialHD was originally given to 4 mice at the same dose as the other treatments but all of the mice became sick and died within 48 hours, so the 10-fold lower dose was thought to be acceptable to continue the study. Along with the 4 AdmsialHD

mice that died, 2 AdmsialFG mice, 1 AdC4HSULacZ, and 1 PBS infected mouse also died within the first 48 hours, suggesting that there could have been technical error upon injecting the mice or some other unexplained factor. Within the final week of the experiment, 1 AdmsialHD at the lower dose and 1 more PBS injected mouse died from unknown causes.

The enzyme activity data received from the tissues did not show an increase in sialidase activity. Figures 3.16 through 3.18 display the lysosomal sialidase activity levels from the liver, kidney, and spleen respectively. All three figures show a general trend of increased activity with the PBS-infected mice compared to the rest, while the AdmsialFG and AdC4HSULacZ-infected groups were relatively the same level. Not too surprisingly, the AdmsialHD-infected group displayed the lowest levels of sialidase activity. Reasons being that they were infected at a lower dose of the therapeutic adenovirus and that the *in vitro* experiments only demonstrated a significant enzymatic increase at the 1000 particles/cell dose and in cells that were devoid of lysosomal sialidase. The SM/J mouse still retains some endogenous lysosomal sialidase activity, thus a mild dose contributing a mild increase of enzymatic activity would not be seen. Furthermore, there would have been an immune response significant enough to further reduce the levels of AdmsialHD in the blood. The AdmsialFG results could be explained by the fact that it is a first-generation adenovirus which is known to have a shortened lifespan in the host due to immunogenic adenoviral

proteins continually expressed by the vector, thus 29 days may have been too far past the lifespan of the vector within the host. A FACS analysis was performed on the monocytes that were isolated from the blood that was collected from the mice just prior to termination with the hopes of discovering that the glycoconjugates on the cell surface were being cleaved by the upregulated sialidase. The monocytes were labeled with PE-lectins and FITC-lectins, which bind to the cell surface glycoconjugates. The data received from this FACS analysis displayed mixed results due to low cell counts and thus was not included in this report.

Figure 3.19 displays the increase in body weight as a percentage of their experimental starting weight. It is evident that FG-LB had one of the highest increases over the 29 day period, but LZ-RT had almost similar results, suggesting that there are no differences in weight gain between the various infected groups. All of the other mice increased approximately 120 – 140% whereas FG-LB and LZ-RT had increases of around 155%. The extent to which FG-LB and LZ-RT grew could be attributed to the fact that they were much smaller initially when compared to the rest of the mice, so similar increases in weight will result in a higher percentage increase in the smaller mice when compared to the rest of the group.

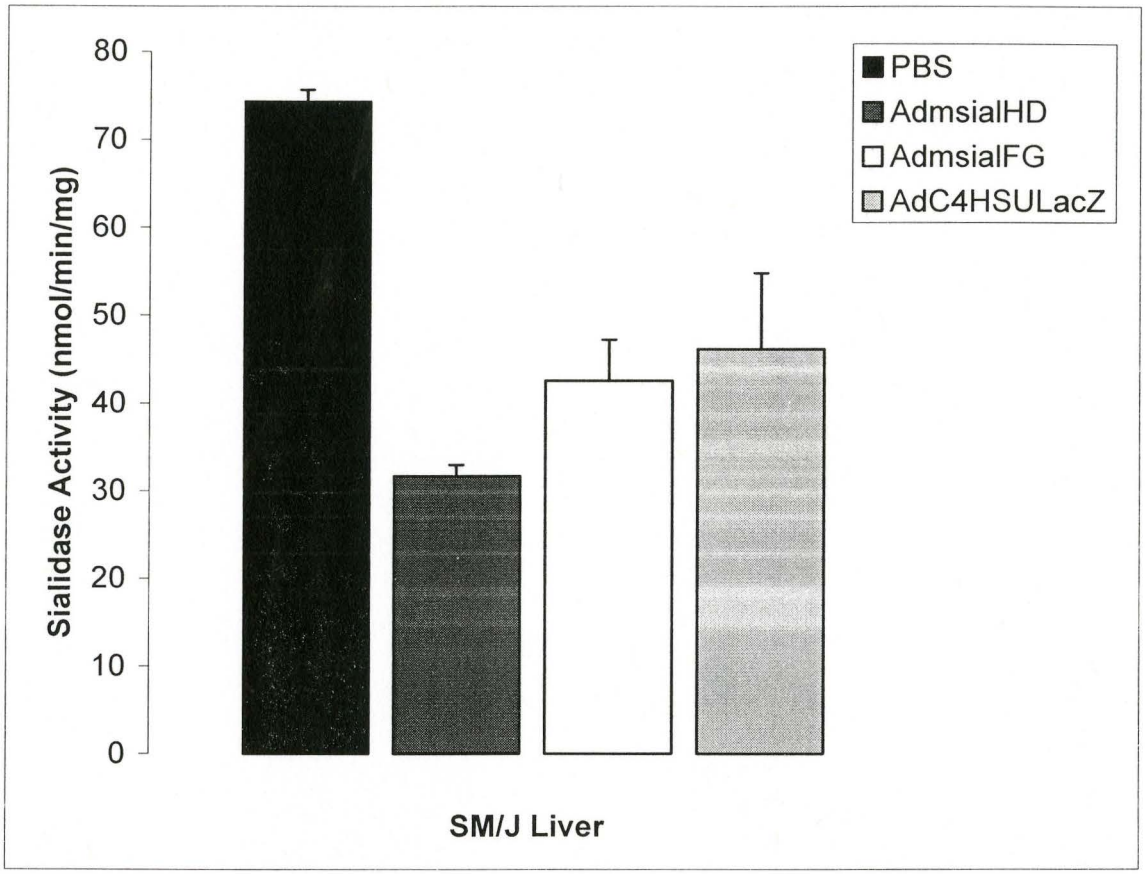

Figure 3.16 Sialidase activity from homogenized SM/J liver sections 29 days following adenoviral injection. 5 x 10^9 particles of AdmsialFG or AdC4HSULacZ was used for in vivo injection while only 5 x 10^8 particles of AdmsialHD was used for in vivo injection. PBS was injected at the same volume (100 µl) as with the adenoviral injections to act as the control Standard error is represented through a sample size of n=2 for both PBS and AdmsialFG-injected mice and n=3 for both AdmsialHD and AdC4HSULacZ-injected mice.

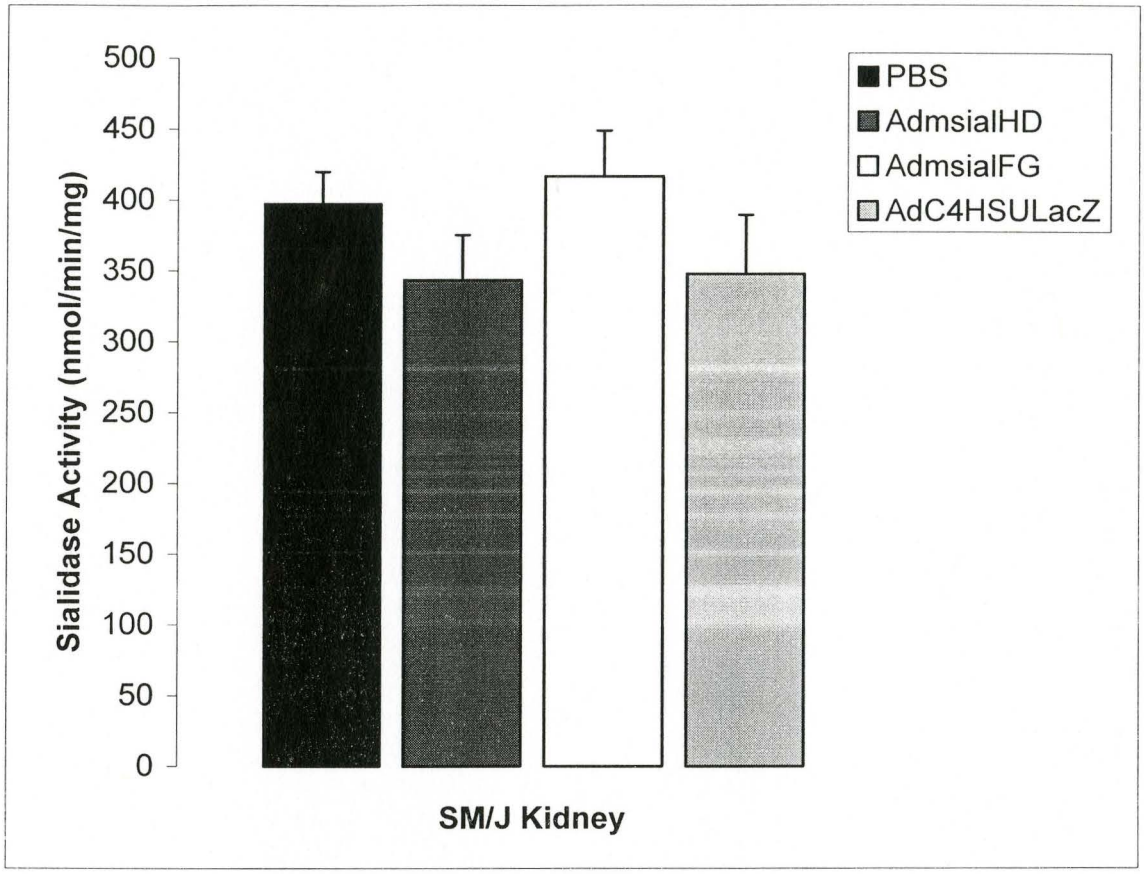

Figure 3.17 Sialidase activity from homogenized SM/J kidney sections 29 days following adenoviral injection 5×10^9 particles of AdmsialFG or AdC4HSULacZ was used for in vivo injection while only 5×10^8 particles of AdmsialHD was used for in vivo injection. PBS was injected at the same volume (100 µl) as with the adenoviral injections to act as the control. Standard error is represented through a sample size of n=2 for both PBS and AdmsialFG-injected mice and n=3 for both AdmsialHD and AdC4HSULacZ-injected mice.

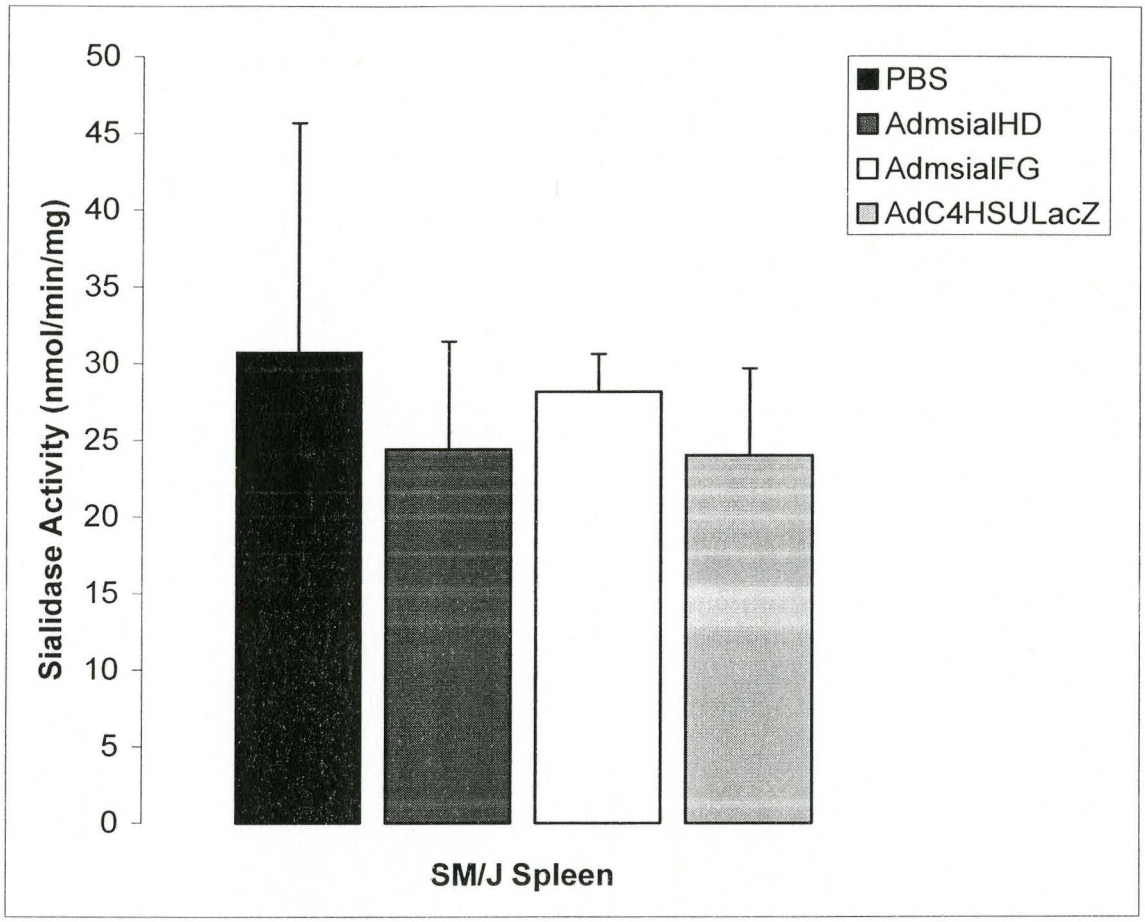

Figure 3.18 Sialidase activity from homogenized SM/J spleen sections 29 days following adenoviral injection 5×10^9 particles of AdmsialFG or AdC4HSULacZ was used for in vivo injection while only 5×10^8 particles of AdmsialHD was used for in vivo injection. PBS was injected at the same volume (100 µl) as with the adenoviral injections to act as the control. Standard error is represented through a sample size of n=2 for both PBS and AdmsialFG-injected mice and n=3 for both AdmsialHD and AdC4HSULacZ-injected mice

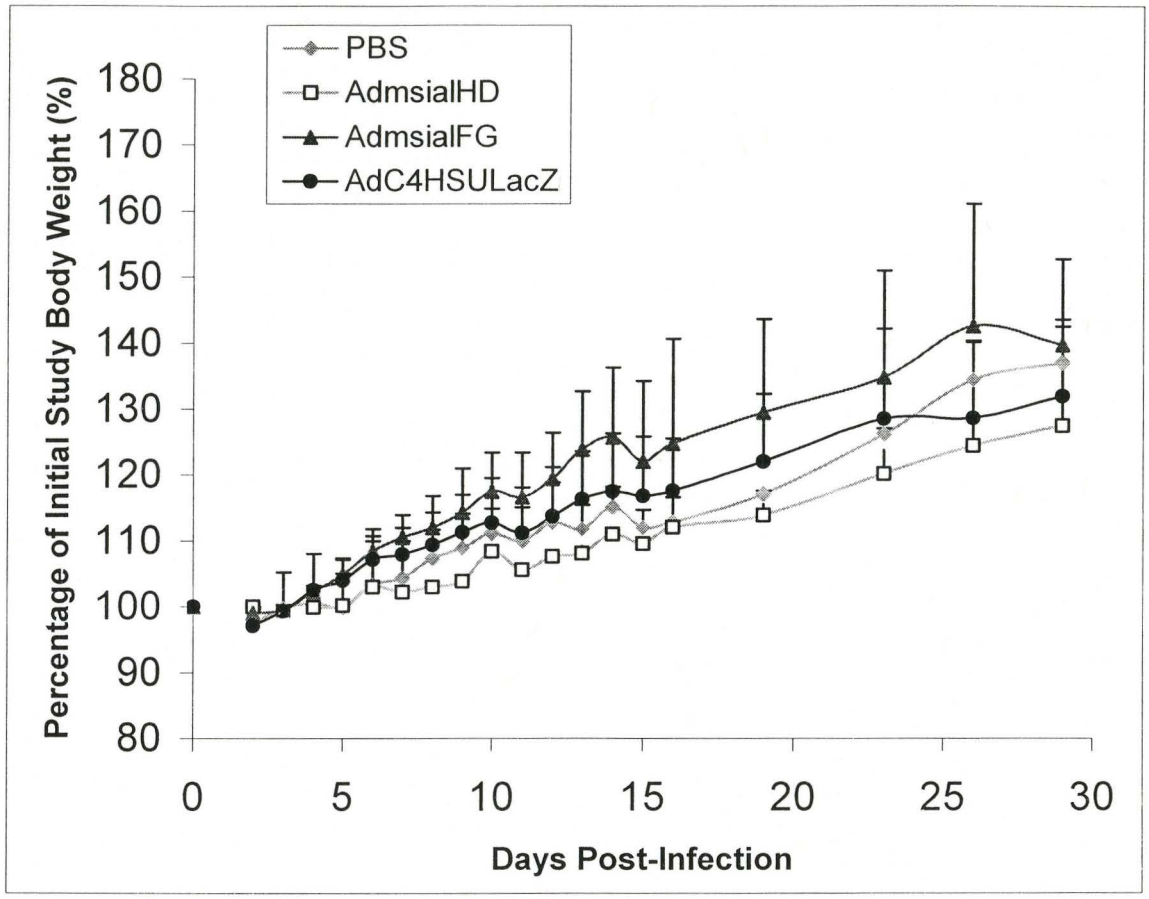

Figure 3.19 SM/J body weight as a percentage of initial study weight during the 29 day period following in vivo administration of AdmsialHD, AdmsialFG, or AdC4HSULacZ. PBS was used as the mock-infection control. Standard error is represented through a sample size of n=2 for both PBS and AdmsialFG-injected mice and n=3 for both AdmsialHD and AdC4HSULacZ-injected mice.

Chapter 4

Discussion

4.1 Generation and Activity of AdmsialFG and AdmsialHD in the Lysosomal Sialidase-Deficient Cell Line WG544.

The ability of the helper-dependent adenovirus to produce lifelong transgene expression (Morsy et al., 1998, Morral et al., 1999, Kim et al., 2001) has been well established over the past five years. Additionally, the helper-dependent adenovirus induces a reduced immune response compared with its first-generation counterpart (Morral et al., 1998). This is a direct consequence of the gutless helper-dependent vector, which is devoid of all adenoviral genes (Parks et al., 1996).

The helper-dependent system could thus be used as a potential tool for introducing the lysosomal enzyme, sialidase, into human patients with the autosomal recessive disorder sialidosis. Patients with sialidosis type I can exhibit phenotypical manifestations at a juvenile onset including the cherry red spot, myoclonus, mild mental retardation, seizures, and neuropathy (Achyuthan & Achyuthan, 2001). Type II patients exhibit symptoms similar to type I patients in addition to major mental retardation, somatic mutations, and death before the age of ten (Achyuthan & Achyuthan, 2001). Sialidosis is generally caused by missense mutations in the lysosomal sialidase gene, located on chromosome 6p21 within the major histocompatibility complex (Pshezetsky et al., 1997). Sialidase is only active at an acidic pH and when in a multi-enzyme complex

associated with the protective-protein cathepsin A and β-galactosidase (d'Azzo et al., 1982).

The diseased state of sialidosis is actually caused by the accumulation of the ganglioside GM3, sialyloligosaccharides, sialylglycoproteins and glycolipids, which is most pronounced in the liver with vacuolation in various organs including the liver, kidney, brain, and parts of the endocrine system (Sergi et al., 2001). Gene therapy utilizing a helper-dependent adenovirus containing the lysosomal sialidase gene could provide lifelong *in trans* lysosomal sialidase expression and rescue sialidosis patients from their diseased state. There exist several reports of helper-dependent adenoviruses reaching the liver (Reddy et al., 2003), kidney (Kojima et al. 2003), and even the brain (Zou et al., 2001) through intravenous or intraventricular injections. In fact, Zou et al. (2001) reported similar transgene expression in the brain following either intraventricular or intrahippocampal injection, demonstrating the potential for brain tissue to be targeted without intruding the brain cavity. These findings were encouraging towards the use of a helper-dependent adenovirus for the treatment of sialidosis.

Past results with helper-dependent adenoviruses promoted the production of a HD-vector containing the mouse lysosomal sialidase gene. The mouse form was chosen based on the principal that murine experiments on the SM/J mouse, which exhibit a lysosomal sialidase deficiency (Rottier, Bonten, & d'Azzo, 1997),

should be performed prior to any human experiments. The complete mouse lysosomal sialidase gene was cloned into the helper-dependent vector, pC4HSU-msial (Figures 3.1 and 3.2), and propagated as AdmsialHD to a high titre of 3.56 x 10^{11} particles/ml. In addition, the complete mouse lysosomal sialidase gene with 2.8 kb of the 3' UTR cut out was cloned and rescued into a first-generation adenoviral vector, AdmsialFG, and also propagated to a high titre of 3.08 x 10^{11} particles/ml.

Upon vector characterization through restriction analysis, it was apparent that both AdmsialHD (Figures 3.3 and 3.4) and AdmsialFG (Figures 3.5 and 3.6) possessed correct genomic structure. Interestingly, the AdmsialHD fragments were of varying intensity, with the fragments that were unique to the helper-dependent vector (relative to the helper virus, AdLC8Cluc) either bright or very dim relative to the non-unique fragments. Specifically, the unique fragments at 2371 and 815 bp were easily notified whereas the 1992 and 2501 bp bands were faintly visible, probably due to incomplete digestion. The final genome size of AdmsialHD is 27.2 kb, which is slightly lower than the 27.7 kb genome size that Parks & Graham, (1997) noted as the lower limit for efficient adenoviral packaging without causing vector genome rearrangements. Despite AdmsialHD having its genome 500 bp smaller than that noted for avoiding vector rearrangements, AdmsialHD appears to have the correct structure. DNA sequencing of AdmsialHD for the mouse lysosomal sialidase promoter further

revealed that the promoter was in fact cloned into the vector properly. AdmsialFG was much easier to characterize as all of the necessary fragments following digestion were produced at increasing intensity with increasing fragment size. Attempts were made to clone the complete human lysosomal sialidase gene into the helper-dependent adenovirus vector, pC4HSU, resulting in only the plasmid pCRhsial (Figures 3.7 and 3.8). Future cloning steps can be made to produce the desired HD-vector for viral propagation and potential human experiments.

AdmsialFG and AdmsialHD were both tested *in vitro* for lysosomal sialidase activity using Mu-Nana as the substrate. The human cell line WG544 was used for preliminary experiments. Since both AdmsialFG and AdmsialHD contained the endogenous lysosomal sialidase promoter, extremely high levels of enzyme activity were not expected, especially in a human cell line. Figures 3.9 and 3.10 display the preliminary AdmsialHD and AdmsialFG activity respectively. It is evident from these data that both vectors adequately produce functional lysosomal sialidase. Also, in Figure 3.10, it is clear that the first-generation adenovirus, AdS+, which contains the human lysosomal sialidase cDNA controlled by a CMV promoter, produces much more functional enzyme. This clearly illustrates the differences between having a CMV promoter controlling expression relative to having an endogenous promoter regulating expression.

With both AdmsialFG and AdmsialHD infections displaying increased expression of mouse lysosomal sialidase in the human cell line, WG544, it was necessary to perform an experiment involving both vectors to compare their relative activities in parallel. The WG544 cell line was used once again as it was previously shown to be quite permissive to infection by both viruses. Figure 3.11 demonstrated the relative enzyme activities of the mouse lysosomal sialidase vectors as well as the first-generation human lysosomal sialidase vector, AdS+. In human cells that are deficient in lysosomal sialidase, it appears that AdmsialHD causes a greater increase in enzyme activity than AdmsialFG at most MOIs. In addition, AdmsialHD displayed activity similar to that of AdS+ at its highest MOI of 1000 particles/cell. This was not expected as the AdS+ vector was used at an MOI of 10 PFU/cell, which roughly corresponds to 100 – 500 particles/cell, and also carries a CMV promoter for the regulation of the human sialidase cDNA.

4.2 Activity of AdmsialFG and AdmsialHD in the Mouse Embryonic Fibroblast (MEF) Cell Line.

The constructed adenoviral vectors, AdmsialFG and AdmsialHD both contain the mouse lysosomal sialidase gene. The previously mentioned experiments were performed on human cells that were deficient in lysosomal sialidase. Since these vectors were going to be used to treat a murine model of reduced lysosomal sialidase, the SM/J mouse, it was necessary to see if these

vectors could increase mouse lysosomal sialidase activity in a murine cell line with normal sialidase activity, MEF cells. Previous reports have shown that murine gene therapy on human and mouse cells is possible (Dkhissi et al., 2003 & Sanchez et al., 2003), each displaying impressive therapeutic results. With this in mind, MEF cells were infected with AdmsialFG and AdmsialHD to determine if the helper-dependent vector increases lysosomal sialidase levels to greater levels than its first-generation counterpart, AdmsialFG, in a murine culture model. Interestingly, in a murine model where lysosomal sialidase is present at physiological levels, the first-generation vector increased enzyme activity significantly higher than the helper-dependent vector at the MOIs of 100 and 1000 particles/cell (Figure 3.13). In contrast to previous results obtained in human cells deficient in sialidase (Figure 3.11), it appears that only the first-generation vector can push the enzyme levels past physiological levels in normal cells. A group of researchers (Gilbert et al., 2001) also discovered this phenomenom with the HD vectors. Gilbert et al., (2001) found comparatively that the first-generation vector encoding a minidystrophin gene had significantly higher transgene expression *in vitro* and *in vivo* relative to the HD vector that encoded the full human dystrophin cDNA. Gilbert et al., (2001) went on to discover that the E4 region of the adenovirus was responsible somehow for the enhancement of transgene expression. This can relate to AdmsialFG as it is based on an E1/E3 deleted adenovirus vector, but still contains the E4 region. It is suggested that the remaining adenoviral gene products produced by

AdmsialFG could be acting *in trans* on the endogenous promoters to up-regulate lysosomal sialidase expression (Grave et al., 2000 & Gilbert et al., 2001). Despite these similar findings, it is still unclear how AdmsialHD produced greater levels of enzyme activity in human sialidase-deficient cells relative to AdmsialFG (Figures 3.9 & 3.10). Perhaps the endogenous levels of sialidase present in the cell lines prior to infection play an important role in regulating transgene expression following infection. Since both vectors contain endogenous promoters, this could mean that cells which normally produce endogenous levels of a specified transgene will not be expressed beyond that level when the expression of additional copies of a transgene is controlled endogenously. In cells that are devoid of a particular transgene, there may not be a system in place to arrest expression at endogenous levels.

4.3 Rescuing of Lysosomal Sialidase Deficiency in Cultured Fibroblasts from SM/C57BL6 Mice (B6.SM) that were Homozygous or Heterozygous for Lysosomal Sialidase Mutation

While the previous experiments carried out proved that both AdmsialFG and AdmsialHD both increase mouse lysosomal sialidase expression *in vitro*, the results were mixed with respect to which vector increases enzyme activity at a greater level and in which cell line. To determine if both vectors would be suitable to treat the lysosomal sialidase deficiency in the SM/J mouse (Rottier,

Bonten, & d'Azzo, 1997), *in vitro* experiments needed to be performed on cells containing the lysosomal sialidase mutation. The B6.SM mouse was produced by backcrossing SM/J mice to C57/BL6 mice and was convenient to use as there were B6.SM mice that were homozygous (-/-) or heterozygous (+/-) for the lysosomal sialidase mutation. Since past AdmsialHD experiments resulted in high sialidase expression with WG544 cells (Figure 3.9) and low sialidase expression in MEF cells (Figure 3.13), it was unknown how much sialidase up-regulation would occur in cells that have only reduced lysosomal sialidase expression. As some patients with sialidosis still maintain residual sialidase activity (Bonten et al. 2000), this experiment could provide useful information on whether the HD-vector could be used to treat patients with low sialidase activity. From the past experiments, it was expected that AdmsialHD would increase lysosomal sialidase activity greater than in MEF cells, but lower than that seen in WG544 cells. As predicted, AdmsialHD increased sialidase activity in B6.SM -/- cells to well above that seen in the B6.SM +/- control cells (Figure 3.14), indicating that the HD-vector is capable of rescuing lysosomal sialidase activity *in vitro*. AdmsialFG was similar (Figure 3.14) except that it upregulated sialidase to greater levels than AdmsialHD at the same MOI of 1000 particles/cell. Lysosomal sialidase transcript levels were also found to be increased in both AdmsialHD and AdmsialFG-infected groups compared to controls (Figure 3.15) proving that the increased enzyme expression is correlated with the increased enzyme activity. Sialidase transcript levels were similar in both AdmsialHD and

AdmsialFG-infected B6.SM cells due to the fact that the *NEU1* mutation from the SM/J mouse only affects enzyme activity and not transcript production (Rottier, Bonten, & d'Azzo, 1998).

4.4 Comparative Rescuing of Lysosomal Sialidase Deficiency and Low Body Weight in the SM/J Mouse by AdmsialHD and AdmsialFG

With the therapeutic success that helper-dependent adenoviruses have seen recently *in vivo* (Belalcazar et al. 2003, Kojima et al. 2003 & Reddy et al. 2003), and the previous experiments using AdmsialHD *in vitro*, it was deemed appropriate to attempt to rescue the lysosomal sialidase deficiency and the low body weight associated with the SM/J mouse. There does exist a sialidosis mouse model (de Geest et al., 2002), but the deficiency in lysosomal sialidase in the SM/J mouse is sufficient to acknowledge any significant enzyme increase following adenoviral therapy. In addition to the reduced sialidase activity in the SM/J mouse, it was found in SM/C57 males that their infantile body weight was lower than those of the control C57/BL6 mice, indicating that the lysosomal sialidase mutation possibly has an effect on body weight in the SM/J mouse (Champigny, unpublished results).

The venous plexus behind the eye was chosen as a suitable site of viral entry to gain access to the blood as the sialidase deficiency was noted to be in

white blood cells (Chen et al. 1997) and the liver (Potier, Yan, & Womack, 1979). The amount of adenovirus chosen to infect was 5×10^9 particles as several researchers found high transgene activity with helper-dependent vectors at 10 to 100-fold higher doses than this (Schiedner et al., 1998, Kim et al., 2001 & Reddy et al., 2003), but our goal was to rescue the phenotype without increasing lysosomal sialidase to supraphysiological levels. Unfortunately, all mice receiving AdmsialHD died within 48 hours prompting additional injections at a lower dose of 5×10^8 particles, which was not our desired level due to having an endogenous promoter regulating transgene expression. Probably causes for the deaths were technical error, as a saline control mouse also died following injection, systemic shock due to adenovirus load, or glycerol content. The glycerol content would not have been too much of a concern as the viruses were diluted approximately 10-fold with each mouse received approximately 1 µl of glycerol. After the injections, each mouse was weighed every day for 2 weeks (except day 1 as several mice died) and every 3-4 days up to 30 days. No significant percentage body weight increase was seen, with one mouse from both the AdmsialFG and AdC4HUSLacZ groups showing the largest increase in weight (Figure 3.19). The two mice with the largest percentage increase were also the mice with the lowest starting weight, which could account for their larger percentage increase.

The lysosomal sialidase activity assay performed 2 weeks post-infection from isolated blood monocytes. Due to the SM/J mouse's small body weight, only a small amount of blood could be extracted every 2 weeks (5% total blood volume), and the amount of monocytes isolated could not provide enough enzyme activity to be recorded within the sensitivity range of the assay. This was supported by results using blood from C57/BL6 mice, which have no sialidase deficiency. Approximately 10% of the total blood volume was extracted with the monocytes isolated and assayed for sialidase activity. Even at that volume, enzyme levels were detectable at levels equivalent to levels seen in WG544 cells (Figure 3.12).

Despite this setback, tissue samples could be used to provide sialidase activity data, but only upon termination of the study. At day 30, sections of the liver, kidney, and spleen were extracted and analyzed for sialidase activity. In all three organs, it was discovered that in both AdmsialHD and AdmsialFG-infected mice, no increase in sialidase could be found relative to controls (Figures 3.16-3.18). In particular, the saline control mice had greater enzyme activity than the infected mice in the liver and similar to greater levels in the kidney and the spleen. These results were further supported by FACS analysis showing no reduction in cell surface sialylation (data not shown). These results suggest that the amount of AdmsialHD and AdmsialFG injected into the SM/J mice was not sufficient to rescue the SM/J lysosomal sialidase deficiency as well as decrease

the amount of cell surface sialylation and increase the infantile body weight past control SM/J levels. AdmsialFG may have lost sialidase expression based on the fact that first-generation adenoviruses lose transgene expression quickly with some reporting transgene expression anywhere from 66 days to 3 months (Zou et al., 2000 & Reddy et al., 2003), but this experiment lasted only 30 days and the transgene was endogenous to the mouse. Based on previous results reported in this thesis, another explanation for the lack of sialidase expression could be due to the existing levels of lysosomal sialidase in the SM/J mouse. As stated before, AdmsialHD did not demonstrate increased expression of sialidase in cells with endogenous levels of sialidase but did display an increase in cells devoid of sialidase activity. However, this possibility only exists with AdmsialHD as AdmsialFG increased enzyme activity in all cell lines tested. The most probable cause is the low levels of adenoviral particles used to infect in conjunction with the endogenous promoter controlling the expression of the transgene, mouse lysosomal sialidase. Schiedner et al., (1998) also used a transgene with its endogenous promoter but infected mice into the tail vein with 2 x 10^{10} particles, which was 40-fold greater than the amount of AdmsialHD that was ultimately used to infect the SM/J mice.

4.5 Future Directions and Applications

The helper-dependent system has proven itself over the past few years and in no way should these results discourage its future applications for gene therapy. The most obvious experiment that needs to be performed is one in which B6.SM -/- mice are infected with AdmsialHD and AdmsialFG at higher doses, 10^{10} or 10^{11} particles, and compared with B6.SM +/- controls with respect to body weight, sialidase activity, and cell surface sialylation for at least a period of 3 months. Additionally, Gilbert et al., (2001) found that co-infection with a FG-vector and a HD-vector significantly increased transgene expression, thus a similar experiment involving the co-infection of AdmsialFG and AdmsialHD could be beneficial for improving AdmsialHD sialidase expression. Lastly, the production of a human lysosomal sialidase HD-vector is the ultimate goal for eventual human therapy for sialidosis patients, as no current therapy exists for patients with this morbid disease.

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