Generation and Correction of a Novel Mouse Model of Farber Disease

by

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Abstract

Farber disease is a fatal inherited disorder of lipid metabolism. It is characterized by a deficiency of the lysosomal enzyme acid ceramidase (AC), resulting in the buildup of the bio-effector molecule ceramide. A previous attempt to generate a Farber mouse model by Asah1 knockout was unsuccessful owing to embryonic lethality in homozygous embryos. Here, we successfully generated viable homozygotes that developed Farber disease by introducing a single-nucleotide human patient mutation into a conserved region of murine Asah1 (P361R). Indeed, homozygotes manifested Farber disease symptoms and died within 7-13 weeks. Furthermore, treatment of neonatal pups with intravenous injections of AC lentiviral vectors (LVs) yielded homozygotes with intermediate Farber phenotypes highlighted by reduced symptoms and increased longevity. Therefore, this first model of Farber disease can facilitate the advancement of experimental therapies and offer mechanistic insights into the integral roles of acid ceramidase, ceramide, and related sphingolipids in cell signaling, growth, and oncogenesis.
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List of Abbreviations

AC   Acid Ceramidase
ACER Alkaline Ceramidase
ASAH Acyl Sphingosine Amidohydrolase
BMT  Bone Marrow Transplantation
CD   Cluster of Differentiation
CNS  Central Nervous System
CT   Computed Topography
DAG  Diacylglycerol
DIG  Digoxiginen
ERT  Enzyme Replacement Therapy
ES Cell Embryonic Stem Cell
GFP  Green Fluorescent Protein
GVHD Graft Versus Host Disease
Het  Heterozygote
HIV  Human Immunodeficiency Virus
HPLC High Performance Liquid Chromatography
HSC  Hematopoietic Stem Cell
HSCT Hematopoietic Stem Cell Transplantation
LA   Long Arm
LSD  Lysosomal Storage Disorder
LV   Lentivirus
M6P  Mannose-6-Phosphate
<table>
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<tr>
<td>MCP</td>
<td>Monocyte Chemoattractant Protein</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>Neo</td>
<td>Neomycin</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-Human Primate</td>
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<td>NOD</td>
<td>Non-Obese Diabetic</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<td>S1P</td>
<td>Sphingosine-1-Phosphate</td>
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<tr>
<td>SA</td>
<td>Short Arm</td>
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<tr>
<td>SAP</td>
<td>Saposine Activator Protein</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SIN</td>
<td>Self-Inactivating</td>
</tr>
<tr>
<td>SphK</td>
<td>Sphingosine Kinase</td>
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<td>SPM</td>
<td>Sphingomyelin</td>
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<tr>
<td>SPP</td>
<td>Sphingosine Phosphatase</td>
</tr>
<tr>
<td>TCP</td>
<td>Toronto Centre for Phenogenomics</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VSV</td>
<td>Vesicular Stomatitis Virus</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus Post-transcriptional Regulatory Element</td>
</tr>
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<td>WT</td>
<td>Wild-Type</td>
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Chapter 1

INTRODUCTION

1.1 Farber Disease

1.1.1 Overview and Pathogenesis

Farber disease is a very rare inborn error of lipid metabolism. It is characterized by a deficiency of the lysosomal enzyme acid ceramidase (1). The disease was first described by Sidney Farber in 1957 (2). Until 2006, only 100 cases had been reported worldwide. Classically, the disease is characterized by a triad of symptoms – hoarseness, arthritis, and subcutaneous nodules.

Genetically determined, Farber disease follows the autosomal recessive pattern of Mendelian inheritance. It is caused by a mutation of ASAH1 (N-acylsphingosine amido hydrolase) gene encoding acid ceramidase (1). Deficiency of acid ceramidase activity results in the accumulation of lysosomal ceramide. The mechanisms through which acid ceramidase deficiency leads to the development of the phenotype remains poorly understood (3). Traditionally, ceramide accumulation has been thought to trigger the immune system leading to widespread infiltrations of monocytes into various tissues (4). Once inside the tissues, macrophages start to engulf the excess ceramide in an attempt to protect the tissues from its detrimental effects. Overwhelmed by the huge amounts of ceramide, macrophages (histiocytes) acquire a foamy appearance due to lipid accumulation. The cells fail to dispose ceramide from
the tissues, and granulomas eventually ensue in different organs. This process of ceramide accumulation, histiocytosis, and granuloma formation accounts for Farber disease symptoms: granulomatous infiltrations of the vocal cords lead to the hoarseness manifested in patients with Farber disease; histiocytic infiltrations of the joints result in chronic arthritis and joint deformities; and granulomas formed in the skin are manifested as subcutaneous nodules.

It remains unknown how ceramide accumulation is linked to the organ damage observed in Farber patients. The sequence of molecular mechanisms leading from a defect in ceramide metabolism to chronic granulomatous inflammation still needs to be elucidated (5). It has been suggested that the damage is mediated by leukocytes (4). Infiltrations of tissues by lipid-laden macrophages cause widespread destructions and eventually loss of function of the affected organs. However, whether this response results from the mere accumulation of ceramide or from its signaling properties or both remains to be fully understood.

Several theories have been developed to explain the pathogenesis of Farber disease. Classically, the reaction of immune cells towards ceramide accumulation resembles the reaction towards a foreign substance. This view may explain the granulomas formed in Farber patients which are similar to granulomas formed in response to chronic inflammation against foreign bodies. Supporting this hypothesis, injection of ceramide into rats produces lesions that resemble those observed in Farber disease patients (4). Another theory used to explain the pathogenesis of Farber disease implicates ceramide as a signaling molecule that triggers the immune-cell reaction in Farber disease. This theory emphasizes the signaling properties of ceramide rather than the mere storage of ceramide (6). It is supported by the finding that hematopoietic stem cell transplantation (HSCT) alleviates the symptoms in Farber patients, indicating that the immune-cell response seen in Farber patients is rather due to internal dysfunction of leukocytes caused by
ceramide signaling (6). Further investigations are needed to elucidate the exact mechanisms through which ceramide activates the immune system in Farber disease.

The association between acid ceramidase activity, ceramide storage, and the severity of Farber disease is not fully understood. The basic abnormality in Farber disease is acid ceramidase deficiency (7). The build-up of lysosomal ceramide follows, defining the hallmark of the disease. In 1983/1984, Conzelmann and Sandhoff theorized a model on the pathogenesis of sphingolipidosis. Their model linked the enzyme deficiency, the amount of substrate deficiency, and the severity of the disease to each other (8). In contrast, Moser et al. found that the enzyme defect is equally severe in the mild and severe cases of Farber disease (4). Later, Van-Echter et al. studied the correlation between ceramide levels and acid ceramidase activities in fibroblasts from seven different Farber patients (9). Their data showed significant differences in the accumulation of ceramide between individual Farber cell lines. However, no correlation existed between the residual activity of acid ceramidase and the level of ceramide storage. Their data also showed no correlation between the level of accumulated ceramide and the lifetime of patients. Levade et al. reported a metabolic study on twelve patients with Farber disease where cases were differentiated based on the residual ceramide turnover rates (8). Cells from patients were loaded with labeled sphingomyelin (SPM) to determine the rate of ceramide degradation in diseased cells compared to controls. Normal cells metabolized SPM to ceramide, which was subsequently hydrolyzed. Cells from Farber patients failed to degrade ceramide. In normal cells, the undegraded ceramide averaged 5.9% of SPM metabolic products, while in Farber cells it amounted to from 49% to 98% (8). Based on the rates of ceramide degradation, a significant inverse correlation between the level of ceramide and the survival of patients was found. The most severe case in the study died at 3 days old. She had the highest level of accumulated...
ceramide. On the other hand, the patient that lived up to 30 years had the lowest level of ceramide. Ceramide level was also inversely correlated to the age of disease onset in this study. The patient with the highest ceramide level manifested the disease in utero and developed hydrops fetalis while the patient with the lowest level of ceramide and prolonged survival started to manifest symptoms at the age of 20 months.

Despite the correlation described between the level of accumulated ceramide, the onset and severity of the disease, and the survival of patients, no correlation between acid ceramidase activity and the severity of the disease was found. This is probably due to the artificial in vitro conditions under which the acid ceramidase assay is performed. The use of detergents in acid ceramidase assays is thought to reduce the accessibility of the enzyme to the substrate making the measurement of the enzyme activity somewhat inaccurate. The lack of correlation between the enzyme activity and the severity of Farber disease makes the prediction of the natural history of the disease based on the level of the enzyme activity also rather inaccurate. More studies are needed to establish the relationship between acid ceramidase activity, ceramide levels, and the clinical course of Farber disease.

1.1.2 Clinical Features

Farber disease is a very severe disorder. It is one of 10 lysosomal storage disorders (LSDs) that may present with intra-uterine fetal death (10). It commonly presents early in childhood with a unique triad of symptoms: arthritis, subcutaneous nodules, and hoarseness. Granulomatous infiltrations of the nervous system and viscera are not uncommon, leading to psychomotor retardation, hepatosplenomegaly, and respiratory failure. Of 27 reported cases, 7 had hepatomegaly and 1 had splenomegaly (4). Patients succumb to the disease within the first
two years of life (4). However, milder types of the disease also exist. Patients with less severe illness are mildly affected and they live longer life spans. It is not fully understood why some patients manifest mild but others manifest severe disease. Some studies have shown that the level of accumulated ceramide is correlated to the severity of neurodegeneration and the life spans of patients (8).

1.1.2.1 Case reports

The clinical features of Farber disease are mostly described in very few case reports. Lijnschoten et al. reported a case of Farber disease in a fetus that died at a gestational age of 29 weeks (11). The fetus was the result of a complicated pregnancy. Ultrasound examination showed intrauterine growth retardation. The mother reported complete loss of fetal movement at 29 weeks of gestation after which delivery was induced. Macroscopic examination of the organs revealed an enlarged spleen and normal sized liver. Under light microscopy the spleen was found to be heavily infiltrated by foamy cells with eosinophilic cytoplasm. The cells were shown to be of histiocytic origin, as demonstrated by staining with CD68 and Ham56 antibodies. Foamy cells were also found in the bone marrow. Comma-shaped bodies were identified inside foamy cells under the electron microscope. Ceramide levels were also high in the liver, spleen and lungs.

Kattner et al. described a case of Farber disease presented as non-immune hydrops fetalis (10). The fetus was the result of an uneventful pregnancy until the 26th week of gestation when ultrasound examination showed an edematosus fetus with massive hepatosplenomegaly. The child died 3 days after a Cesarean-section delivery. Macroscopic nodules were seen on the peritoneal surfaces of the liver, spleen, and visceral pleura. Microscopically, storage macrophages were
identified in the liver, spleen, bone marrow, lymph nodes, thymus, thyroid gland, parotid gland, and adrenal medulla but not in the brain. Ceramide levels were also elevated.

Sana et al. reported a 1-month-old neonate with Farber disease (12). The first symptoms appeared 10 days after birth consisting of painful joints contractures. Erythematous nodules were observed at 1 month located at the occiput, elbows, heels, and interphalangeal joints. Hepatosplenomegaly was absent. Deficient activity of acid ceramidase in cultured leukocytes confirmed the diagnosis of Farber disease.

Antonarakis et al. reported two patients, a brother and sister, with features of Farber disease (13). The girl was 12-week old with the triad of symptoms and hepatosplenomegaly. She died at 6 months. The boy was 10-weeks old and presented with suspected malignant histiocytosis. He died at 12 weeks.

Pellissier et al. reported two siblings from a consanguineous marriage who showed a severe form of Farber disease (14). The central and peripheral nervous systems were involved. Low conduction velocity was observed, and macular cherry red spots were identified. The diagnosis was confirmed by evidence of acid ceramidase deficiency in cultured fibroblasts.

Zarbin et al. studied a 35-month girl who manifested macular red cherry spots (15). Diagnosis of Farber disease was confirmed by low acid ceramidase activity level. By 18 months, the patient was unable to talk, walk, or sit up. The patient also manifested subcutaneous nodules and a hoarse cry. She had an abnormal brain stem auditory evoked response, demonstrating pontomesencephalic dysfunction. A CT scan of the brain revealed prominent ventricles and sulci, suggestive of brain atrophy. The patient continued to deteriorate neurologically and died of pulmonary failure.
Chedrawi et al. identified two non-identical twins with Farber disease (16). The first patient was a 2.5-year old boy with multiple subcutaneous nodules and painful joint contractures. He had hypotonia with diffuse muscle atrophy. An MRI of the brain revealed loss of deep white matter volume and ventricular dilatation. Electro-encephalogram was suggestive of generalized myoclonic epilepsy. Nerve conduction studies revealed sensory and motor neuropathy. The younger sister was 9 months old. She presented with a triad of symptoms (subcutaneous nodules, hoarse cry, and joint contractures). She developed hypotonia and developmental delay. Nerve conduction studies were suggestive of demyelinating polyneuropathy. An MRI of the brain revealed diffuse brain atrophy. A novel mutation in the acid ceramidase gene was identified.

Cvitanovic-Sojat et al. studied a case of late-onset and early death of a boy with Farber disease (17). The first symptoms appeared at 12 months as painful arthritis. Severe neurological deterioration began at the age of 18 months. Hoarseness of voice was manifested at an unusually late age of 25 months, and the first subcutaneous nodule appeared much later at 31.5 months. Hepatomegaly was noted, and macular red cherry spots were documented. The diagnosis of the disease was confirmed by demonstrating low levels of acid ceramidase activity in fibroblast lysates. Tracheostomy was eventually performed to alleviate respiratory distress, and death occurred at the age of 38.5 months. The history of the disease from the beginning of the symptoms to the death of the patient lasted 26.5 months. The neuronal dysfunction seemed to limit the duration of the disease.
1.1.2.2 Farber disease classes

Farber disease is traditionally classified into 6 subtypes differing on the severity of the disease, age of onset, and organ involvement. Type 1 is the classical and most severe form of the disease, which manifests as severe progressive arthritis (especially in the interphalangeal, metacarpal, ankle, wrist, knee, and elbow joints), subcutaneous nodules, and hoarseness of the voice. The nervous system and the viscera are usually involved, resulting in severe psychomotor retardation, mental retardation, and liver and spleen disorders (4). Symptoms usually appear early after birth. As the disease progresses, chronic arthritis leads to joint contractures and the limitation of movement, subcutaneous nodules increase in size and number, and hoarseness may eventually lead to aphonia. Respiratory functions deteriorate over time due to repeated episodes of pneumonia and disturbances in breathing due to granuloma formation in the larynx. Pulmonary failure is the major cause of death in patients with this classic form of Farber disease. Lymphadenopathy was reported in seven patients. Whereas the liver was enlarged in most patients with type 1 disease, enlargement of the spleen was reported in only one case. Six patients had cardiac murmurs due to granuloma formation over the cardiac valves. Patients with type 1 disease eventually succumb to their illness within the first two years of life (4).

Involvement of the nervous system in type 1 Farber disease is prominent. Severe and progressive psychomotor impairments occur in many patients. Seizures and mental retardations have also been reported. Hypotonia and muscle atrophy may be the result of storage in the anterior horn cells of the spinal cord or in peripheral nerves (4). The eye in Farber disease shows macular cherry-red spots without signs of visual impairment. Granulomatous nodules also develop on the conjunctiva.
Patients with type 2 and 3 have longer life span. One patient with a mild form of the disease lived to the third decade of life (8). Subcutaneous nodules, arthritis, and hoarseness of the voice are the major manifestations in these patients. The visceral organs are commonly spared. The nervous system is mildly or not affected. Death usually occurs due to pneumonia.

Type 4 patients present with visceral enlargement (hepatosplenomegaly) during the neonatal period. All patients with this type died by the age of 6 months. Histiocytic infiltrates are prominent in various visceral organs including the liver, spleen, thymus, and lungs.

Type 5, or neurologic progressive disease, is characterized by neuronal abnormalities in all patients. In contrast to Type 1, the viscera are spared in Type 5 Farber patients. The three reported patients - who belonged to two different families - developed psychomotor retardation beginning at age 1 to 2.5 years. The two girls from the first family showed prominent macular cherry-red spots. Progressive psychomotor manifestations appeared at age 1 year. Extensive neuronal loss was documented in autopsy. The third patient developed ataxia, seizures, and dementia at age 2.5 years. All three patients manifested subcutaneous nodules and arthritis.

Type 6 was a report on a patient with combined Farber disease and Sandhoff disease. The patient manifested a triad of symptoms (hoarseness, arthritis, and subcutaneous nodules) and macular cherry-red spots (4).

Type 7 Farber disease was a single patient with a deficiency in prosaposin, the precursor for saposins. Saposins are sphingolipid activator proteins involved in the activation of lysosomal enzymes that metabolize sphingolipids, including acid ceramidase (4). The patient developed a deficiency in glucocerebrosidase, galactocerebrosidase, and acid ceramidase (4).
1.1.3 Diagnosis

The diagnosis of Farber disease can be straightforward. The presentation of the triad of symptoms (hoarseness, subcutaneous nodules, and arthritis) is characteristic of Farber disease. However, the disease can be misdiagnosed as other conditions. The progressive arthritis presentation of Farber disease should be differentiated from other rheumatic diseases like rheumatoid arthritis. Infiltrations of the tissues with histiocytic cells should not be confused with other histiocytosis diseases including malignant histiocytosis and Erdheim-Chester disease.

The diagnosis of Farber disease can be approached at three levels: biochemical, histopathological, and ultrastructural. Acid ceramidase activity is reduced in all patients with Farber disease (4). Deficient activity can be demonstrated in various cells including skin fibroblasts, white blood cells, and amniocytes (4). The enzyme assay is available in very few laboratories around the world (18). This is attributed to the difficulty in running the assay and the use of a synthetic enzyme substrate that is unavailable commercially (4). Different methods were described to determine the activity of acid ceramidase. However, no standardized method has been developed as of yet.

He et al. described the use of two novel fluorescent ceramide substrates for the detection of acid ceramidase activity in vitro (19). Bodipy-or lissamine rhodamine-conjugated C12-ceramide was used a substrate for acid ceramidase. The hydrolysis of the substrate by acid ceramidase in an acidic environment released bodipy or lissamine-rhodamine dodecanoic acid, depending on the substrate used in the reaction. Separation of the product from the substrate was performed using high-performance liquid chromatography (HPLC). The activity of the enzyme was then determined based on the quantity of the product that was released during the reaction.
Using bodipy C12 ceramide, as low as 0.1pmol/mg protein/hour of acid ceramidase activity could be detected, indicating the high sensitivity of the assay (19).

Bedia et al. recently described a new method to determine acid ceramidase activity using a novel fluorogenic substrate (18). The new assay is less complex and faster than previous ones. It does not need separation techniques and can be performed in 96-well plates. This assay utilizes a newly synthesized ceramide analogue carrying a 2-oxo-2H-chromen-7-yloxy moiety in the CH3-terminal part of the sphingoid chain (RBM14-12). Umbelliferone is released after the hydrolysis of ceramide by acid ceramidase in an acidic environment. The activity of the enzyme is calculated based on the quantity of umbelliferone released in the reaction. The assay is very sensitive, exhibiting a detection limit of 50 pmoles. One advantage of this assay is the specificity of the substrate towards acid ceramidase (18). This specificity was indicated by the lack of substrate hydrolysis in fibroblasts from Farber disease patients. Another advantage of this assay is the absence of detergent in the reaction. It has been demonstrated that acid ceramidase activity is 20-fold higher without the addition of detergents (18). These properties make the assay useful for accurate measurement of acid ceramidase and the diagnosis of Farber disease.

Measurement of ceramide levels can also be used for the diagnosis of Farber disease, as accumulation of ceramide is primary characteristic of the disease (20). The demonstration of high ceramide levels in biopsies from subcutaneous nodules confirms the diagnosis. Quantification of ceramide can be done using HPLC (21) or by using *E. coli* diacylglycerol kinase and (γ-32P)-ATP that phosphorylates ceramide and DAG simultaneously (22).
A third diagnostic approach is the demonstration of histiocytic infiltrates in subcutaneous nodules or other tissues under the light microscope (4). The infiltrating cells are macrophages with lipid cytoplasmic inclusions. Full-blown granulomas can also be identified.

Under the electron microscope, Farber disease is associated with a number of different inclusions inside the cells. Zarbin et al. describe 5 abnormal structures observed in Farber disease (15): 1) Curvilinear structures commonly known as Farber bodies. These abnormal structures are tubular in shape and found inside vacuoles in the cells. They consist of two dark lines separated by a clear space. They are thought to represent ceramide molecules because adding ceramide to cultured fibroblasts from Farber patients produces similar structures. 2) Zebra bodies are found mainly in neurons and endothelial cells. They are likely to result from high ganglioside levels that are elevated in Farber disease. 3) Osmophilic inclusions that appear in hepatocytes and endothelial cells. 4) Oval or spindle-shaped inclusions in Schwann cells. 5) Cytosomes resembling swollen disrupted mitochondria in lymphocytes.

1.1.4 Treatment

Currently, there is no specific treatment for Farber disease (5). Management is only supportive. Treatment has been focused on pain therapy, anti-inflammatory medications to reduce the severity of arthritis, and cosmetic correction of subcutaneous nodules. Corticosteroids are effective as broad anti-inflammatory agents to relieve the peripheral symptoms. Surgical intervention to remove the granulomas can be attempted to provide relief from distressing symptoms that are refractory to medical treatment (23). Tracheostomy is needed to secure open airways in severe laryngeal involvement. Allogeneic bone marrow transplantation (BMT) has been shown to be effective in ameliorating the peripheral symptoms in patients with Farber
disease. This approach is justified based on the reasoning that peripheral blood cells with normal enzyme activity can work as vehicles to deliver the deficient enzyme and engulf/degrade tissue ceramide (24).

Ehlert et al. described four patients with a mild form of Farber disease (Type2/3) that received allogeneic BMT (5). All patients had the classical peripheral manifestations of Farber disease. The diagnosis of the disease was confirmed in all patients by demonstrating deficient activity of acid ceramidase. The four patients received busulfan and cyclophosphamide as a preparative regimen. In three patients, the bone marrow was the source of stem cells, while peripheral blood stem cells were used in one patient. Donor cell chimerism of >90% was reached in all patients. Patient #1 was a 3-year old female with severe joint contractures. She was almost wheel-chair bound and unable to walk. Post-transplant, the patient showed remarkable improvement. At the time of follow-up (1800 days post-transplantation), the number of joints with restricted mobility was reduced from 26 to 0. In addition, the subcutaneous nodules completely disappeared. A marked reduction in erythrocyte sedimentation rate (ESR) was also observed (5). The second patient was a 3-year old male and received peripheral blood stem cell transplantation. 1560 days post-treatment, his ESR normalized. His subcutaneous nodules disappeared and his deformed joints improved (5). Patient #3 was a 2-year old female, who responded favorably to the transplant. Prior to treatment, she had 18 visible subcutaneous nodules and 10 joints with restricted mobility. At the time of follow-up (990 days post-transplant), the mobility of her joints was restored, the subcutaneous nodules disappeared, and her ESR was normalized (5). The fourth patient was a 21-year old girl with a milder course of the disease compared to the other patients. Prior to treatment, she needed numerous surgeries to improve the mobility of her lower limbs. After transplant, all subcutaneous nodules disappeared
and no new ones appeared. Her joints remained affected but with observed improvement in the range of movement. No data on ESR was available (5).

In Farber patients where the nervous system is involved, BMT seems to have minimal benefits (25). Souillet et al. reported the first case of BMT in a case of Farber disease (26). The patient was 18 months old at the time of transplantation. Although the peripheral symptoms improved, the patient died with progressive neurological deterioration 6 months later. Yeager et al. described the long-term outcomes in an infant with Farber disease who underwent allogeneic BMT from a histocompatible sibling at age 9.5 months (25). The patient was a product of an uncomplicated delivery to healthy, unrelated parents. At age 4 months she started to have joint pain with active and passive movements. At age 7 months she developed progressive contractures of several joints. Subcutaneous nodules also appeared around the same age. The disease was confirmed by demonstration of low acid ceramidase activity (6% of normal). An MRI of the brain showed mild generalized volume loss. A repeat MRI at 9 months revealed diffuse volume loss and increased size of lateral ventricles and sulci (25). The patient received a pre-transplant conditioning regimen of oral busulphan and intravenous cyclophosphamide. The course of the transplant was uneventful. By 1 month post-transplant, the subcutaneous nodules substantially decreased in number and disappeared by 2 months post-transplant. Hoarseness had completely resolved, and the mobility of the joints was remarkably improved. By 6 months after BMT, the contractures and joint pains had completely resolved (25). Enzyme activity in leukocytes was increased to heterozygous levels (44%). However, donor chimerism was lost so that only 1% of donor DNA was found in the peripheral blood at 21 months after BMT. The patient’s neurological and developmental status deteriorated over time. She died 28 months after transplant at the age of 37.5 months (25).
It appears that allogeneic BMT has limited success in patients with central nervous system involvement. Whereas the peripheral manifestations were reduced, patients eventually die of deteriorating neurological impairments. The favorable outcome of BMT has been observed in patients with milder types of the disease (type1/2) where the central nervous system was intact. This is plausible knowing that the replacement of the microglia in the brain with donor-derived cells takes as long as one year to occur, in contrast to the peripheral tissues where replacement of original cells with donor-derived macrophages takes much shorter time (25). Thus sustained, high levels of chimerism may help to achieve correction in the brain similar to that seen in the peripheral tissues (25). Alternative therapeutic approaches remain an important target to be developed. Such therapeutics must be able to correct the enzyme deficiency in the central nervous system in order to achieve effective results. It is worth mentioning that enzyme replacement therapy (ERT) is currently unavailable for Farber disease given the small population of Farber patients and the high cost of development of this therapy.

1.1.5 Mouse Models of Farber Disease

In 2002, Li et al. attempted to create the first mouse model of Farber disease by knocking out the murine acid ceramidase gene (Asah1) (27). They disrupted the Asah1 gene using a targeting vector that replaced exons 3 and 5 with a neomycin resistance cassette (27). No mice homozygous for the knockout gene were observed among the offspring of heterozygous intercrossings. These data suggested that homozygosity for Asah1 gene knockout was lethal during embryogenesis (27). Embryos were genotyped as early as 8.5 days of gestation and no homozygotes were identified. This finding indicated that homozygous lethality took place early during embryogenesis.
Further analyses revealed that homozygous embryos could actually be formed but underwent apoptotic death at the 2-cell stage (28). Importantly, embryos were partially rescued by adding soluble spingosine-1-phosphate (S1P). In the presence of S1P, embryos reached the 8-cell stage (28). These studies showed that acid ceramidase is an essential factor necessary for embryonic development, probably by removing ceramide and generating S1P. Notably acid ceramidase is one of the first proteins expressed during the 2-cell stage of development, and its activity is necessary for the subsequent expression of the normal developmental program (28). It may also be suggested from these studies that most human embryos with a severe deficiency of acid ceramidase die in utero. In fact, there are at least two reports of intrauterine fetal death in infants with acid ceramidase deficiency (10, 11).

While mice heterozygous for the *Asah1* gene knockout developed progressive lipid storage starting at 6 months, no clinical symptoms or other abnormalities were found (27). No other attempts to generate an animal model of Farber disease have been reported. Currently, there is no viable animal model (large or small) of Farber disease available.

1.2 Sphingolipids

1.2.1 Ceramide

1.2.1.1 Structure and Production

Ceramides are a family of closely related molecules that consist of sphingosine and a free fatty acid (Figure 1.1). There are three major groups of ceramide: unsaturated ceramide, dihydroceramide, and phytoceramide. Differences in sphingosine structure determine the type of
ceramide. Unsaturated ceramide contains sphingosine with a double bond at the C4-5 position in the sphigoid base backbone; dihydroceramide contains dihydrosphingosine which does not have a double bond; and phytoceramide contains phytosphingosine which has an additional hydroxyl group. Additional variation of ceramide structure originates from the free fatty acid component of its structure. The length and saturation of the fatty acid chain contributes to the diversity of ceramide molecules. To date, 200 ceramide subspecies have been described (29).

Ceramide can be generated via three different pathways (Figure 1.2): 1) Ceramide is synthesized de novo from palmitoyl CoA and serine; 2) Sphingosine and a free fatty acid are converted to ceramide by the reverse activity of acid ceramidase; 3) Sphingomyelin is hydrolyzed to ceramide by sphingomyelinases. The hydrolysis of sphingomyelin can take place at different subcellular localizations depending on the enzyme involved in the reaction. While neutral sphingomyelinase generates ceramide at the plasma membrane, acid sphingomyelinase hydrolyzes lysosomal sphingomyelin into ceramide.

1.2.1.2 Biological Effects of Ceramide

Apart from its structural roles, ceramide appears to play a role as a second messenger in mediating cellular response. In 1995, Jayadev et al. demonstrated that ceramide causes cell cycle arrest in Molt-4 cells (30). Another group in the same year showed that inhibition of glucosyleceramide synthase, which results in accumulation of ceramide, leads to cell cycle arrest in NIH 3T3 cells (31). Those findings and others suggest an anti-growth role for ceramide.

Numerous studies have shown that ceramide induces apoptosis in various cell types. The classical theory on the pathway of ceramide-induced cell death identifies ceramide as a second messenger mediating cellular response to stressful stimuli. For instance, exposure of endothelial
cells to ionizing radiation results in the generation of ceramide and the initiation of apoptosis (32). Another study showed similar effects of radiation on prostate cancer cells (33). Similar effects of stressful stimuli on ceramide generation and initiation of apoptosis have also been demonstrated in response to TNF-α, Fas ligand, and chemotherapeutic drugs (34, 35, 33). These effects of ceramide seem to be dependent on the certain species and the localization of ceramide. For instance, one group showed that C_{20}-C_{22} ceramides confer resistance to anoxia in *C. elegans* (36). In contrast, C_{24}-C_{26} ceramides promoted cell death (36).

Regarding subcellular localization, it remains controversial in which compartment ceramide produces its regulatory effects. It was shown that ceramide exhibits biomodulatory functions at the level of the plasma membrane and in the mitochondria (37, 38). Neutral ceramidase, which is localized to the plasma membrane, was found to be involved in ceramide-induced apoptosis. Moonsuk et al. showed that neutral ceramidase inhibition in mesangial cells leads to increased ceramide and apoptosis (39). In another study, however, neutral ceramidase inhibition in melanoma cells had no effect on cell survival (69).

In the lysosomal compartment, it has been suggested that ceramide has no roles in cell signaling. Chatelut et al. showed that natural ceramide is unable to escape the lysosome, in contrast to a fluorescent analogue (40). Segui et al. demonstrated that endolysosomal ceramide does not mediate stress-induced apoptosis (41). In this regard, cells from patients with Farber disease, where lysosomal ceramide accumulates, do not exhibit apoptotic features (42). Furthermore, skin fibroblasts from patients with Farber disease have no altered sensitivity to stress-induced apoptosis (41).
Controversy concerning the roles of lysosomal ceramide in signal transduction came from numerous studies that demonstrated altered sensitivity of various cell types in response to manipulation of the lysosomal enzyme acid ceramidase. For instance, upregulation of acid ceramidase confers resistance to FasL in head and neck squamous cell cancers (35), TNF-α in L292 cell line (34), dacarbazine in melanoma cells (69), and chemotherapy in prostate cancer cells (43). Similarly, inhibition of acid ceramidase sensitizes various cells to stressful stimuli (69). Those effects of acid ceramidase are likely mediated through ceramide and S1P. Thus the balance between ceramide and S1P is crucial in determining cell fate.

1.2.2 Sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) is a bioactive lipid that is involved in many cellular functions. The only pathway of S1P generation is the hydrolysis of ceramide into a free fatty acid and sphingosine, which is subsequently converted to S1P by the action of sphingosine kinases. There are at least two sphingosine kinases that have been identified: SphK1, and SphK2 (44). The two enzymes catalyze the same enzymatic reaction but differ in their subcellular localization. While SphK1 is located in the cytoplasm of the cell (45), SphK2 is occasionally found in the nucleus (46). Thus, S1P is mainly generated intracellularly. S1P can also exist extracellularly through the action of SphK1 released from the cell (47), or the release of S1P (47). Once generated, S1P can be dephosphorylated to sphingosine by the actions of two S1P phosphatases: SPP1 and SPP2 (48). S1P can also be metabolized to hexadecenal and phophoethanolamine by the action of S1P lyase (49).

The effects of S1P are mediated by a family of 5 S1P receptors (S1P₁-S1P₅) (48). The expression of those receptors differ according to the cell type, giving S1P diversity in its
biological effects. S1P was shown to regulate cell proliferation, migration, and differentiation (50). Overexpression of SphK1 has been reported in several types of cancers including lung, and breast cancers (48). Furthermore, staining for SphK1 in human colon cancer samples revealed high expression in 89% of cases (51). Inhibition of SphK1 was also demonstrated to sensitize prostate cancer cells to chemotherapy, while upregulation of SphK1 enhanced the resistance of prostate cancer cells to chemotherapeutic agents (42). Therefore, targeting the SphK1/S1P pathway is an attractive therapeutic strategy to develop new anticancer drugs.

In the blood, S1P plays a major role in homeostasis. S1P was found to be crucial for stabilizing and enhancing the endothelial cell-cell junction (53). Knocking out the S1P_1 receptor in mice resulted in a lethal embryonic phenotype due to hemorrhage (54). Concerning blood cells, S1P is a key molecule in regulating immune cell trafficking (55). It was shown that in erythrocytes the generation of S1P from sphingosine through the action of alkaline ceramidase contributes to the high plasma levels of S1P (62). This S1P is crucial for maintaining a gradient of high S1P plasma levels compared to interstitial tissues. Lymphocytes were found to migrate towards sites of high S1P levels (55).
Figure 1.1. Structure of ceramide. Illustration of the chemical structures of (A) ceramide, (B) dihydroceramide, and (C) phytoceramide.
Figure 1.2. Ceramide metabolism. Schematic representation of the pathways involved in ceramide metabolism showing the intermediate molecules and the enzymes involved.
1.3 Ceramide-Hydrolyzing Enzymes

1.3.1 Acid Ceramidase

Acid ceramidase (AC), also known as N-acylsphingosine amidohydrolase (ASAH1), is the lysosomal enzyme that catalyzes the hydrolysis of ceramide to sphingosine and free fatty acid (56). The enzyme is encoded by the \textit{ASAH1} gene that was mapped to the short arm of chromosome 8 (8p21.3 to 8p22) (57). The gene spans a region of around 30 kb and consists of 14 exons and 13 introns. The start codon is located in exon 1 and the stop codon is located in exon 14. The human and murine genes are very similar in structure, aside from minor differences (58). The first and the last exons are larger in the human than the corresponding murine exons. Intron 1 is larger in the murine gene (20 kb) compared to the corresponding human intron (9 kb). The murine \textit{Asah1} gene was also mapped to chromosome 8. The full mRNA is transcribed as 2.4 kb transcript (57). However, other variants of 1.7 and 1.2 kb also exist in some tissues (57). The full cDNA consists of a 17 bp 5’-untranslated sequence, a 1185 bp open reading frame encoding 395 amino acids, a 1110 bp 3’-untranslated sequence containing three polyadenylation sites, and a 17 bp poly(A) tail (58).

The acid ceramidase protein was first purified in human urine in 1995 (59). The enzyme is expressed as a single precursor of 53-55 kDa consisting of attached α and β subunits (60), of 13 and 40 kDa respectively (59). In the Golgi compartment, the protein is glycosylated with mannose-6-phosphate (M6P) in five out of six possible locations (60). All six glycosylation sites are located on the β subunit. Deglycosylation of the enzyme reduces the molecular mass of the β subunit to 28 kDa. The α subunit is devoid of carbohydrate residues. Improper formation of M6P residues prevents the correct targeting of lysosomal proteins (60). Cells that lack the ability to
glycosylate lysosomal proteins secrete large amounts of the enzymes into the culture medium (60). Inside the lysosome, the acid ceramidase precursor protein is autocleaved by the endoproteolytic activity of the precursor protein itself into a disulfide-linked heterodimeric protein consisting of α and β subunits. This maturation step can only take place in the acidic environment of the lysosome (60).

The mature acid ceramidase enzyme has a half-life of >20 hours. It has a Km of 149 µM and a Vmax of 136 nmol/h/mg (59). The optimum pH for the enzyme activity is 4.5. In vivo lysosomal cleavage of ceramide is achieved by the coordinate action of acid ceramidase and the membrane-active sphingolipid activator protein SAP-C or SAP-D, which presumably interacts with lipid bilayers in such a way that the amide bond of ceramide becomes more accessible to the active center of the enzyme (60). It was also shown that acid ceramidase catalyzes a reverse reaction that synthesizes ceramide from sphingosine and a free fatty acid at pH 5.5. (61). Indeed, acid ceramidase from Farber patients was also deficient in catalyzing the reverse reaction, in addition to having a deficiency in degrading ceramide (61).

It has long been accepted that acid ceramidase is localized to the lysosome. The appearance of lipid storage vacuoles in Farber disease patients that are reminiscent of lysosomes, as well as the enhanced in vitro activity of acid ceramidase at acidic pH support this. However, a recent study involving oocytes revealed that acid ceramidase also exists extra-lysosomally (28). Thus acid ceramidase may also contribute to the hydrolysis of non-lysosomal ceramide (28), or may even have diverse functions outside of the lysosome.
1.3.2 Mutations in Farber Disease

Several mutations of acid ceramidase have been reported in patients with Farber disease; the majority of which are point mutations where the enzyme maintains some residual activity (27). Mutations have been identified in both subunits of the enzyme. It has not been shown how those mutations change the characteristics of the enzyme. However, deficiency in acid ceramidase activity is the end result of all reported mutations, with most Farber patients having less than 6% of normal enzyme activity (4).

1.3.3 Neutral and Alkaline Ceramidases

Two other forms of ceramidases exist in addition to acid ceramidase: neutral ceramidase and alkaline ceramidase. The main function of all ceramidases is the hydrolysis of ceramide into sphingosine and a free fatty-acid. After the generation of sphingosine, its phosphorylation by sphingosine kinases leads to the formation of sphingosine-1-phosphate (S1P). This phosphorylation step is the only known source of S1P generation (62). Ceramidases are therefore essential not only for the hydrolysis of ceramide but also for the generation of S1P.

The human neutral ceramidase gene, Asah2, is located on the long arm of chromosome 10 (63). The enzyme was first identified in the mitochondria as a protein of 763 amino acids (64). A later study showed that the full length enzyme is 782 amino acids and that it is localized mainly at the plasma membrane (65). The enzyme is ubiquitously expressed, with the highest levels detected in the kidney (65). The optimum pH for the activity of the enzyme is between 7.5 and 9.5 (65). Substrate specificity studies showed that neutral ceramidase has a preference towards long-chain ceramides (66). It is unclear whether neutral ceramidase plays a major role in
regulating cellular responses. It was shown that the overexpression of neutral ceramidase in primary hepatocytes protected the cells from TNF-α-mediated apoptosis (67). Furthermore, neutral ceramidase was suggested to protect the mesangial cells against inflammatory stimuli (68). However, induction of neutral ceramidase overexpression was not associated with significant changes in melanocyte viability after treatment with cytotoxic drugs (69). A neutral ceramidase knockout mouse had a normal life span and did not show obvious abnormalities or major alterations in total ceramide levels in tissues (70). However, knockout mice were deficient in the intestinal degradation of ceramide suggesting a role of this enzyme in digesting dietary ceramide (70).

Alkaline ceramidase exists in three different forms: ACER1, ACER2, and ACER3. The three enzymes are encoded by different genes and are mainly located at the endoplasmic reticulum and Golgi apparatus (71). The optimum pH of the activity of alkaline ceramidases is ~ 9.5 (62). Alkaline ceramidase activity was shown to be the only ceramidase activity in erythrocytes demonstrating its importance in maintaining S1P levels in the plasma (62). The generation of the three alkaline ceramidase knockout mice will further elaborate the roles of these enzymes in ceramide and sphingolipid metabolism. It is worth mentioning that alkaline and neutral ceramidase have not been associated with human diseases.
### Table 1.1: Reported mutations in *ASAHI* gene in patients with Farber disease

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th>Mutation in cDNA</th>
<th>Enzyme Subunit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P361R</td>
<td>Exon 13</td>
<td>1085C&gt;G</td>
<td>Beta</td>
<td>Li et al. 1999. Genomics (57)</td>
</tr>
<tr>
<td>V97E</td>
<td>Exon 4</td>
<td>290T&gt;A</td>
<td>Alpha</td>
<td>Muramatsu et al. 2002. J Inherit Metab Dis. (72)</td>
</tr>
<tr>
<td>W185R</td>
<td>Exon 8</td>
<td>533T&gt;C</td>
<td>Alpha</td>
<td>Al Jasmi et al. 2012. Brain Dev. (73)</td>
</tr>
<tr>
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<td>Exon 13</td>
<td>1144A&gt;C</td>
<td>Beta</td>
<td>Al Jasmi et al. 2012. Brain Dev. (73)</td>
</tr>
<tr>
<td>V182L</td>
<td>Exon 8</td>
<td>C&gt;G</td>
<td>Beta</td>
<td>Devi et al. 2006. J Hum Genet (74)</td>
</tr>
<tr>
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<td>Exon 9</td>
<td>703G&gt;C</td>
<td>Beta</td>
<td>Muramatsu et al. 2002. J Inherit Metab Dis (72)</td>
</tr>
<tr>
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<td>Exon/Intron</td>
<td>Change</td>
<td>Type</td>
<td>Reference</td>
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<td>-------------</td>
<td>-------------</td>
<td>------------</td>
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<td>--------------------------------</td>
</tr>
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<td>N/A</td>
<td>Alpha</td>
<td>Zhang et al. 2000. Mol Gen Met (3)</td>
</tr>
<tr>
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<td>N/A</td>
<td>Alpha</td>
<td>Zhang et al. 2000. Mol Gen Met (3)</td>
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<td>Exon 4</td>
<td>del 286-288</td>
<td>Alpha</td>
<td>Devi et al. 2006. J Hum Genet (74)</td>
</tr>
<tr>
<td>Stop402L</td>
<td>Exon 14</td>
<td>1204ins 1bpT</td>
<td>Beta</td>
<td>Zhang et al. 2000. Mol Gen Met (3)</td>
</tr>
</tbody>
</table>

N/A – no information available
1.4 Gene Therapy

1.4.1 Overview

Gene delivery represents a promising approach to treat both inherited and non-inherited diseases. Special interest has been given to correct monogenic diseases using gene delivery methods. This interest stems from the premise that delivering the normal gene into the host genome leads to expression of the normal gene product and amelioration of the disease. Several non-viral and viral delivery methods have been developed to achieve this goal. However, a number of factors must be considered in evaluating each method including the ability of the vector to deliver the gene efficiently into the targeted cells, the sustainability of gene expression for the required duration (long-term vs. transient), and the short- and long-term safety of the vector as well as the transgene. Among the various vectors developed for gene delivery, viral vectors have seemingly acquired a special interest over non-viral vectors. This is due to the high efficiency of viral vectors in delivering and maintaining the expression of the transgene.

Different kinds of engineered viral vectors are currently being used as gene delivery vehicles. The most common among them are adenoviruses and retroviruses (oncoretroviruses and lentiviruses). Adenoviruses are double-stranded DNA viruses that can infect dividing and non-dividing cells. They are non-integrating viral vectors that provide transient expression of the transgene (76). Retroviruses, on the other hand, are single-stranded RNA viruses that have the ability to integrate their genetic material into the host genome, providing long-term expression of the transgene (77). Different kinds of retroviruses are used for gene delivery depending on the approach. Oncoretroviruses were used in the early gene therapy clinical trial using integrating viral vectors conducted in France on patients with X-linked Severe Combined Immunodeficiency.
Syndrome (SCID-X1). However, it appeared later that 4 of 10 patients that received similar treatment developed clonal proliferation of T cells (78, 79). This disadvantage of oncoretroviruses has more recently shifted the focus from oncoretroviruses to lentiviruses, which are primarily HIV-derived viral vectors. In contrast to oncoretroviruses, lentiviruses do not appear to have preferable integration sites in the host genome. Furthermore, lentiviruses can infect dividing and non-dividing cells making them suitable to transduce various kinds of cells. This is in contrast to oncoretroviruses that infect mainly dividing cells (80, 81).

Different approaches can be utilized to deliver a transgene in vivo. Direct injection of viral vectors into the bloodstream ensures high bio-availability and distribution of the vectors into most tissues of the body. However, due to their large size, viral vectors are unable to cross the blood-brain barrier. This has an important implication when delivery of the vectors into the central nervous system (CNS) is necessary to correct the disease. Enhancement of vector delivery into the CNS can be achieved by transiently disrupting the blood-brain barrier, allowing the viral particles to enter the CNS (82). Pre-treatment with vascular endothelial growth factor (VEGF) has been reported to increase the permeability of the blood brain-barrier (82). Another option is to directly inject viral vectors into the cerebrospinal fluid, thereby bypassing the blood-brain barrier (83).

Hematopoietic stem cells (HSCs) are attractive targets in gene therapy. HSC transplantation is widely used in the treatment of genetic and non-genetic disorders. However, the challenge of finding an HLA-matched donor and the risk of developing graft-versus-host disease (GvHD) imposes challenges for this procedure. Utilizing gene therapy, autologous HSCs can be manipulated in vitro and transplanted back into the patient, thus circumventing the need for a donor and avoiding the risk of GvHD. The HSCs can be engineered in vitro to express the
transgene of interest. Once transplanted, HSCs will differentiate into different blood cell lines that circulate throughout the blood carrying the transgene. This is particularly important for the treatment of LSDs where the circulating blood cells become a source of the transgene product. Furthermore, macrophages, which are derived from the bone marrow, populate various tissues of the body - including the CNS - where they secrete the transgene product that can be taken by bystander cells. This finding makes HSC-based gene therapy an attractive approach to deliver the transgene product into the CNS.

1.4.2 Gene Therapy For Farber Disease

In 1999, Medin et al. showed for the first time that gene delivery is a feasible approach to correct acid ceramidase deficiency in Farber disease (84). A recombinant oncoretroviral vector was constructed and engineered to express the cDNA of human acid ceramidase. Skin fibroblasts from patients with Farber disease were then transduced with the vector. Increased levels of acid ceramidase activity to normal levels were observed. Expression of acid ceramidase was accompanied by the reduction of ceramide levels to normal. Importantly, the study demonstrated that transduced cells secreted enzyme that was taken up by bystander cells: incubation of deficient cells with the medium from transduced cells resulted in correction of the metabolic defect in a phenomenon known as metabolic cooperativity.

In 2008, Ramsubir et al. studied the effects of delivering AC-encoding viral vectors in vivo using two different approaches (85). Human CD34+ hematopoietic cells derived from umbilical cord blood were transduced with lentivectors encoding AC and CD25 as a cell surface marker. Transduced cells were then transplanted into sub-lethally irradiated NOD/SCID mice. Follow-up on the treated mice demonstrated that CD34+ cells transduced with lentivectors
encoding human acid ceramidase were able to reconstitute the recipient bone marrow and give rise to all lineages of HSCs. This finding suggested that acid ceramidase did not impair the repopulation ability of the transduced cells (85).

Ramsbir et al. also studied the effects of direct lentivector injection in vivo (85). Lentivectors encoding acid ceramidase and CD25 were injected via the temporal vein in wild-type neonatal mice. Treated animals developed normally without adverse effects. Seven weeks post-treatment, all mice showed high levels of CD25 in the plasma. In addition, livers from treated animals demonstrated higher than normal levels of acid ceramidase activity, suggesting distribution of the vectors into the animal tissues. This experiment showed the potential utility of gene therapy to treat Farber patients early after birth.

In 2011, Walia et al. reported a pre-clinical gene therapy study of Farber disease on non-human primates (NHP) (86). Mobilized peripheral blood cells from Rhesus Macaques were infected with lentivectors encoding acid ceramidase. Transduced cells were then transplanted back into the animals after full myeloablation. Supranormal levels of acid ceramidase activity were observed in the bone marrow, peripheral blood, spleens, and livers of treated animals. This increased activity of acid ceramidase was accompanied by reduction of ceramide below pretransplantation levels. Importantly, vector persistence was demonstrated until the end point of the experiment (1 year) without any pathological abnormalities. This study presented the first trial of Farber disease gene therapy on large animals. The results showed a promising potential use for lentivectors in the treatment of Farber disease.
1.5 Current Study Objectives

The current studies on Farber disease are limited to cell lines from patients with Farber disease. The scarcity of patients and the lack of an animal model of Farber disease imposes challenges on understanding the pathophysiology of Farber disease and developing new treatment modalities for this fatal inherited disorder. Despite the efforts to test new therapeutic options to treat Farber disease, including gene therapy, an animal model is required to fully evaluate the efficacy and safety of such therapies. An animal model that manifests acid ceramidase deficiency will also serve as a useful tool to study the biochemical aspects of the acid ceramidase enzyme and its roles in sphingolipid metabolism, cell signaling and cancer.

In this study we present the generation of the first viable animal model of Farber disease that manifests acid ceramidase deficiency. The approach we took to generate this model differs from the previous attempt by Li et al. to knock out the murine Asah1 gene. Their attempt showed that complete loss of acid ceramidase activity is lethal during early embryogenesis (27).

**Hypothesis:** Introducing a human patient mutation into a conserved region of the murine Asah1 gene will result in viable homozygotes that manifest Farber disease.

**Specific Aims:**

1. To generate an animal model of acid ceramidase deficiency that recapitulates Farber disease.

2. To begin explain the mechanism by which the build-up of ceramide leads to the phenotype of the disease.
3. To show as a proof-of-principle that lentivector-based gene therapy is an effective therapeutic modality to treat Farber disease
Chapter 2
MATERIALS AND METHODS

2.1 Targeting Vector Construction

Two arms of homology were subcloned into ploxPneo-1 plasmid that has neomycin resistance gene (neo) flanked by two loxP sites (Courtesy of Dr. Razq Hakem). The homologous arms were amplified by PCR using KOD Hot Start DNA Polymerase enzyme (Novagen). Genomic DNA of W4/129Sv mouse strain (Courtesy of Dr. Razq Hakem) was used as a template.

One arm is 4kb long (long arm) and consists of a genomic fraction extending from exon 8 to 12 with flanking intron sequences. This fragment was amplified using forward and reverse primers. The forward primer was designed to carry a Kpn1-restriction site at its 5’end:

5’- GGGGTACCCATAGT ACACTCAAGGCTCTGTTAAGGTTG -3’

The reverse primer was designed to carry an Xho1-restriction site at its 5’end:

5’- CCGCTCGAGGAATAGTGCGCTGACATTAATTTAAGGAGG - 3’.

The amplified fragment was then cloned into pGEMT-Easy Vector (Promega). Next the fragment was digested using Kpn1 and Xho1 restriction enzymes (New England Biolabs) and then eluted from the gel using Gel Extraction kit (Qiagen, Mississauga, Ontario). PloxPneo-1 was digested with Kpn1 and Xho1 and the long arm was ligated downstream to neo cassette by T4 DNA Ligase (New England Biolabs).
The second arm is 2.4kb long (short arm) and consists of a genomic fraction from exon13 and adjacent intronic region to exon14. This fragment was amplified by PCR using a forward primer designed to carry an EcoR1-restriction site at its 5’ end, and a reverse primer designed to carry a Sac1-restriction site at its 5’ end:

**Forward primer:**

5’- CCGGAATTCCCTCCTGAGTGCTTGGGATTAAAG -3’

**Reverse primer:**

5’- GAGCTCCTGACAGTGACTGTTTATTGCGCATCAC - 3’

The amplified fragment was then cloned into pGEMT-Easy Vector (Promega). Next the desired mutation was introduced into the short arm using single-primer PCR. This mutation involves the replacement of cytosine with guanine in the location that corresponds to position number 1082 of the mouse cDNA. This mutation was reported in a patient with a severe type of Farber disease. In addition, this mutation is located in a conserved region between the human and murine AC polypeptides. The primer used to create this mutation was designed to carry guanine instead of cytosine as indicated in bold in the following sequence:

5’- GCTACCATCTATGATGTCCTATCAACAAAAACGTGCCTCAAAACAGGTATG TCCTCAAC – 3’

Afterwards, the mutated short arm was isolated from pGEMT plasmid using EcoR1 and Sac1 restriction enzymes (New England Biolabs). The fragment was then eluted from the gel using Qiagen Gel Extraction kit (Qiagen, Missisauga, Ontario). At the same time, PloxPneo-1
carrying the long arm was digested with EcoR1 and Sac1 restriction enzymes (New England Biolabs) and the mutated short arm was then ligated into the digested plasmid upstream to neo\textsuperscript{R} cassette using T4 DNA Ligase (New England Biolabs). The final vector was confirmed by sequencing and internal digestion. Importantly, the two loxP sites sequences were confirmed and the mutated nucleotide was identified.

2.2 Transfection of Mouse ES Cells

ES cell transfection was carried out by Toronto Centre for Phenogenomics (TCP). The procedure is described elsewhere (86). Briefly, 20 ug of DNA (linearized targeting vector) was introduced to ES cells by electroporation. Around 10X10\textsuperscript{7} ES cells were electroporated for 8.7 milli second at 250 V. Afterwards, the cells were cultured and selected for G418 resistance at 166 ug/ml concentration. Surviving colonies were picked for screening.

2.3 ES Cells DNA Extraction

ES cells cultured in 96-well plates were lysed using TCP lysis protocol. Cells were allowed to grow to confluency. Afterwards, the media was aspirated and the cells were washed twice by PBS. Lysis buffer, containing 10mM Tris-HCL (pH 7.5); 10mM EDTA; 10mM NaCl; 0.5% sarcosyl; freshly added 1mg/ml proteinase K, was added to each well. The plates were sealed and incubated overnight at 37\textdegree C. Next day, 100ul of cold, freshly prepared, NaCl/Ethanol solution (150ul of 5M NaCl per 10ml of cold 100% ethanol) was added to each well. The plates were then incubated at room temperature for 2 hours. Before draining the fluid, the plates were spun for 15 minutes at 3000 rpm. The wells were then washed by 70% ethanol three times. Finally, DNA was stored in 70% ethanol at -20\textdegree C.
2.4 Southern Blot Screening of ES Cells

Ethanol was drained from the wells and the plates were left to air dry. Then 40ul of restriction digest mixture was added to each well. The mixture contained 0.5ul HindIII (100,000U/ml) Restriction Endonuclease (New England Biolabs), 4ul NEBuffer 2 (New England Biolabs), and 35.5ul ddH$_2$O. The DNA was resuspended in the mixture and incubated overnight at 37°C. Next day the samples were loaded onto 0.8% agarose gel and electrophoresis was allowed to run overnight at 30 Volts. The gel was then stained with ethidium bromide (200ml ddH$_2$O + 2ul of 10mg/ml EtBr) and the DNA was visualized under UV transillumination. Next the gel was washed in the following buffers: Depurination buffer (0.25M HCL) for 20 minute; Denaturation buffer (1.5M NaCl, 0.5M NaOH) for 30 minutes; and Neutralization buffer (1.5M NaCl, 0.5M Tris-HCl pH7.2, 10mM EDTA) for 30 minutes. After washing, the DNA was transferred in 10X SSC buffer to a positively-charged nylon membrane (Biorad) via capillary transfer. The DNA was cross-linked to the membrane by exposure to 1200mJ UV light for 50 seconds. The probes were synthesized using PCR DIG Probe Synthesis Kit (Roche). Two primers were used in the synthesis of each probe. The primers were as follows:

<table>
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<tr>
<th>Probe Type</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Downstream probe forward</td>
<td>5' - AGCAGTCATGCATGCTGACTGGG – 3’</td>
</tr>
<tr>
<td>Downstream probe reverse</td>
<td>5' - CCTAGGAGAGGCTCAAAAATGGGAAG – 3’</td>
</tr>
<tr>
<td>Upstream probe forward</td>
<td>5' - TCGGCCTTTATGAAAACATGATGGC – 3’</td>
</tr>
<tr>
<td>Upstream probe reverse</td>
<td>5' - AAATGTTACAGGCCCATGCTCTCAGTG – 3’</td>
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The cycling conditions used for the three probes were as follows:
- 1 cycle: 50°C for 2 minutes.
- 1 cycle: 95°C for 10 minutes
- 40 cycles: 95°C for 15 seconds; 59°C for 60 seconds; 72°C for 30 seconds.

2.5 Generation of Chimeric Animals

Outbred ICR (Harlan) were used as female host embryo donors. ICR females mated with vasectomized CD-1 (ICR) males were used as recipients for embryo transfer. Superovulation of donor females, embryo isolation, and transfer into uteri of pseudopregnant females were performed as previously described (87). Animals were housed in an accredited facility at TCP. All procedures were performed following Animal Use Protocols approved by the local UHN Animal Care Committee.

Embryos were collected at E2.5 from the oviducts of superovulated ICR females with M2 media (Millipore) and cultured in microdrops of KSOM media with amino acids (Millipore) under embryo-tested mineral oil (Millipore) at 37°C in 95% air/5% CO2. The details of aggregation with diploid embryos are described by Nagy et al. 2003 (87). In short, the zonae pellucidae of the embryos were removed by the treatment with acid Tyrodes solution (Sigma). Zona-free embryos were placed in depression wells made in the plastic dish in microdrops of
KSOM under mineral oil. ES cells were briefly trypsinized to form clumps of loosely connected cells and the clumps of 8-15 ES cells were aggregated with each embryo. After overnight culture in KSOM microdrops under oil, compacted morulae and blastocysts were transferred into the uteri of 2.5-day pseudopregnant recipients. Resulting chimeras were bred with ICR females and germline transmission was determined by the presence of black eyes and non-white coat colour.

2.6 Quantitation of Ceramide

After extraction of lipids, ceramide levels were determined by using *E. coli* diacylglycerol kinase and [γ32P]-ATP as previously described (22).

2.7 Acid Ceramidase Protein Expression In Various Organs

Western blots were performed to evaluate the expression of acid ceramidase in various mouse tissues. Briefly, kidneys, livers, and spleens were collected from wild-type, heterozygous, and homozygous mice and subsequently lysed via RIPA lysis buffer (Thermo Scientific) and sonication. Protein concentrations were quantified using BioRad DC Protein Assay (BioRad). 50µg of protein was loaded on 10% Sodium Dodecyl Sulphate (SDS) – PolyAcrylamide Gels. Following electrophoresis proteins were transferred onto Polvinylidene fluoride (PVDF) membrane using the BioRad Semi-Dry Electrophoretic Transfer Cell (BioRad). Following transfer, the membranes were blocked overnight at 4°C with 5% Non-fat dried milk (NFDM). Following this the membranes were incubated with antibodies. The following primary antibodies were used at the specified dilutions in 2.5% NFDM/TPBS: anti-β-actin (Millipore; 1:2000) and anti-ASAH1 (Sigma Aldrich, 1:1000). The following secondary antibodies were used in 2.5% NFDM/TPBS: anti-rabbit IgG- (Thermo Scientific; 1:10000) and anti-mouse IgG-HRP (GE
Membranes were developed using ECL reagents (Thermo Scientific Pierce) and exposed to autoradiograph film. Subsequent densitometry was performed using Image J v1.44 (National Institute of Health, USA).

2.8 Peripheral Blood and Cytokine Analyses

Mouse peripheral blood was collected from saphenous veins or cardiac punctures (at sacrifice) into EDTA-coated tubes. Peripheral blood counts including total leukocyte, neutrophil, monocyte, eosinophil, basophil, and lymphocytes were analyzed by Hemavet (Drew Scientific Group, Waterbury, CT, USA). Serum MCP-1 levels were analysed using Biorad multiplex cytokine magnetic plate assay (BioRad). Tissues for MCP-1 measurements were lysed using ultrasound and total protein levels were measured by Bradford protein assay (BioRad). MCP-1 levels were measured by ELISA (BioRad).

2.9 Histopathology and Ultrastructural Analyses

For histological analyses, WT, Het, and Asah1P361R/P361R mice were euthanized by CO₂ inhalation and tissues were collected and fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin (H&E). Specimens were examined and scored without advance knowledge of genotype or treatment.

2.10 Lentivirus (LV) Production and Neonatal Injections

LVs pseudotyped with vesicular stomatitis virus-glycoprotein (VSV-g) were generated from the transient co-transfection of the SIN transfer vector (pHR’-EGFP.WPRE or pHRC.D25.WPRE), second generation LV packaging construct pCMVΔ8.91, envelope plasmid
pMD.G, and pAdVantage™ vector (Promega) into HEK 293T cells (ATCC, Manassas, Virginia, USA) using polyethyleneimine (PEI) as described previously (88). Virus supernatants were harvested 48 hours and 72 hours after transfection, filtered, and sterilized using 0.22-µm filter (Nalgene), and concentrated at 50,000 X g for 2 hours using an Optima L-100 XP Ultracentrifuge (Beckman Coulter, Canada). LV pellets were re-suspended in serum-free RPMI-1640 medium (Sigma-Aldrich). Functional titers were determined by serially-diluted LV transductions of HEK 293T cells, followed by subsequent flow cytometry analysis. Typical functional titers of ultracentrifuge-concentrated LVs were in the range of 5x10⁸ to 1x10⁹ IU/mL. 1-3 day old mice were injected intravenously via the temporal vein as described elsewhere (89). All mice received 5x10⁷ IU of either LV/AC or LV/enGFP.

2.11 Statistics

Data are presented as mean results ± standard error of the mean, as per figure legends. Statistical analyses were performed using two-tailed, unpaired Student’s t-tests with unequal variances. Statistical analyses of survival data were performed using the Mantel-Cox log-rank test (GraphPad Prism, GraphPad Software, San Diego, CA.). P-values <0.05 were considered significant.
Chapter 3

RESULTS

3.1 Generation of $Asah1^{P361R/P361R}$ Mice

To generate mice that accumulate ceramide, we constructed a targeting vector carrying a single nucleotide mutation in the mouse acid ceramidase gene ($Asah1$) (Figure 3.1). At the DNA level, this mutation corresponds to the replacement of cytosine with guanine in position 1082 of the murine coding sequence, which is located in exon 13. This mutation leads to the replacement of proline with arginine at position 361 of the murine polypeptide, which corresponds to position 362 of the human polypeptide. The vector was designed so the neomycin-resistance ($neo^R$) cassette occupies an intronic region between exons 12 and 13. After embryonic stem (ES) cell transfection with the targeting vector plasmid, we screened 700 ES clones that survived antibiotic selection. One clone was found positive for homologous recombination by Southern blot using a 3’ probe. This result was further confirmed using a 5’ probe (Figure 3.2). Additional validation procedures included PCR amplification of the $neo^R$ amplicon in ES cells, sequencing of ES cell genomic DNA and tissue cDNA, and FISH analysis (Figure 3.3). The mutated ES-cell clone was aggregated with WT embryos to generate the chimeric mouse (Figure 3.4). Chimerism was determined based on the patchy coat of the mice (Figure 3.5). Breeding of three chimeric mice derived using the confirmed clone resulted in heterozygous progenies. Interbreeding of the heterozygotes generated individuals homozygous for the mutation ($Asah1^{P361R/P361R}$). Animals were born in near Mendelian ratios (Table 3.1).
Figure 3.1. Schematic representation of the targeting vector. Targeting vector consisting of a long-arm (LA) and a short-arm (SM) flanking region floxed neomycin resistance cassette (neo). The restriction sites used to clone the vector are shown.
Figure 3.2. Southern blot-mediated screening of the targeted ES-cell clone. DNA from ES-cell clones was digested with HindIII enzyme and screened A) using 3’ probe and B) using 5’ probe. Sample (1) is a WT control showing one band and sample (2) is a mutated clone showing two bands indicating heterozygosity.
Figure 3.3. FISH analysis. FISH analysis. Kidneys were harvested from 9-week old WT, Het, and Asah1\textsuperscript{P361R/P361R} mice. Samples were then analyzed using FISH for gene copy number. Green indicates LAMP-1 gene and red represents Asah1 gene.
Figure 3.4. Schematic representation of chimera generation. Aggregation procedure showing mutated ES-cells (red) and WT murine embryos (blue). The resulting blastocysts are implanted into pseudopregnant females and chimeric mice are born.
Figure 3.5. Chimeric mouse. A picture of a chimeric mouse showing patches of white and agouti coat color indicating chimerism.
Table 3.1: Mendelian ratio of *Asah1*^{P361R/P361R} mice.

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<tr>
<th>Genotype</th>
<th>Expected ratio</th>
<th>Generated ratio</th>
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<tr>
<td>Wild-type</td>
<td>0.25</td>
<td>0.175</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>0.50</td>
<td>0.554</td>
</tr>
<tr>
<td>Homozygous</td>
<td>0.25</td>
<td>0.268</td>
</tr>
</tbody>
</table>

Heterozygotes were intercrossed and ratios of WT, Het, and *Asah1*^{P361R/P361R} mice were calculated (n = 257 total).
3.2 \textit{Asah1}^{P361R/P361R} Mice Exhibited Signs Characteristic of Farber Disease

We first observed that \textit{Asah1}^{P361R/P361R} mice started to manifest growth retardation in comparison to their heterozygous and wild-type littermates as early as 3 weeks of age (Figure 3.6A, B). Monitoring growth over time, \textit{Asah1}^{P361R/P361R} mice reached a maximum size (measured in grams) at 4 weeks. Afterwards, \textit{Asah1}^{P361R/P361R} mice showed continuous weight loss (Figure 3.6B) punctuated by eventual death at approximately 7-13 weeks (Figure 3.7). Over the period of their illness, \textit{Asah1}^{P361R/P361R} mice manifested lethargy, general dystrophy, and weak forelimb grasp, which worsened with advancing age. Heterozygotes appeared phenotypically normal, showed growth patterns similar to their wild-type littermates, and were asymptomatic over the duration of their lifespan. Gross examination of the visceral organs revealed remarkably enlarged spleen, thymus, and lymph nodes (axillary, cervical, and inguinal) in \textit{Asah1}^{P361R/P361R} mice. Spleens had a firm texture and a pallor color (Figure 3.8A) and the ratio of the spleen weight to the total body weight was significantly increased (Figure 3.8B).

X-ray imaging revealed no remarkable skeletal differences in \textit{Asah1}^{P361R/P361R} mice. No soft tissue lesions were observed (Figure 3.9A). Total-body MRI scans of two \textit{Asah1}^{P361R/P361R} mice showed massive hydrocephaly in one animal (Figure 3.9B).
**Figure 3.6. Physical phenotype analysis.** General assessment of $Asah^1_{P361R/P361R}$ mice. (A) A photograph of 10 week-old WT, Het, and $Asah^1_{P361R/P361R}$ mice. (B) Growth curves measured in weights versus age (n=10 of each genotype). $^{**}P < 0.005$, $^{*}P < 0.05$ comparing $Asah^1_{P361R/P361R}$ group to WT group.
Figure 3.7. Survival analysis. Kaplan-Meier survival curves for WT, Het, and Asah1<sup>P361R/P361R</sup> mice (n=16 for all genotypes) [\( P < 0.0001 \) for Asah1<sup>P361R/P361R</sup> group compared to WT and Het groups, log-rank (Mantel-Cox) test].
Figure 3.8. Spleen gross examination. A) A photograph of spleens from each genotype. B) Growth curves measured in weights versus age (n=10 of each genotype). **$P < 0.005$, *$P < 0.05$ comparing $Asah1^{P361R/P361R}$ group to WT group.
Figure 3.9. Radiological examinations. A) An X-ray image of an 8-week-old Asah1^{P361R/P361R} mouse. B) 10-week old Het and Asah1^{P361R/P361R} littermates were scanned by MRI. Hydrocephaly was detected in the Asah1^{P361R/P361R} mouse. Arrows indicate dilated brain ventricles.
3.3 \textit{Asah1}^{P361R/P361R} Mice Accumulated Ceramide in Various Organs

Total ceramide levels in various organs were measured. Two mice of each genotypic group were sacrificed at 8 weeks. In all tested organs (spleen, brain, heart, liver, lungs, and kidneys), total ceramide levels were elevated in \textit{Asah1}^{P361R/P361R} mice compared to heterozygotes and wild-type animals (Figure 3.10A). Importantly, heterozygotes did not build up ceramide in any of the organs tested.

Next, we determined the ratios of ceramide to diacylglycerol (DAG) in the same organs. Similar to the ceramide quantitation results, higher ceramide/DAG ratios were observed in \textit{Asah1}^{P361R/P361R} mice compared to heterozygote and wild-type mice (Figures 3.10B). Ceramide/DAG ratios were not different between heterozygous and wild-type mice. Moreover, the observed accumulation in ceramide did not appear to trigger any AC compensatory pathways and AC expression in \textit{Asah1}^{P361R/P361R} mice was unchanged compared to that seen in heterozygous and wild-type mice (Figure 3.11A, B).
Figure 3.10. Evaluation of ceramide levels. A) Samples from spleens, livers, brains, lungs, hearts, and kidneys were analyzed for total ceramide (n=2 for all genotypes). Results are represented as mean values. Error bars represent standard error of the mean.
Figure 3.10. B) Samples from spleens, livers, brains, lungs, hearts, and kidneys were analyzed for ceramide/DAG ratios (n=2 for all genotypes). Results are represented as mean values. Error bars represent standard error of the mean.
Figure 3.11. Western analysis of AC expression. Kidneys from WT, Het, and Asah1<sup>P361R/P361R</sup> mice were harvested at 7-9 weeks. A) The premature form of AC protein was detected. B) The densities of the bands were determined.
3.4 \textit{Asah1}^{P361R/P361R} Mice Exhibited Irregular Peripheral Blood Features

Leukocytes play a major role in the pathogenesis of Farber disease and the damage seen in tissues is largely produced by inflammatory immune cells. Therefore, we conducted peripheral blood analyses to determine the levels of various immune effector cells. Leukocytosis was seen in \textit{Asah1}^{P361R/P361R} mice and total white blood cell count was significantly elevated in \textit{Asah1}^{P361R/P361R} animals while levels remained unaltered in heterozygotes compared to the wild-types (Figure 3.12). Differential analyses revealed significantly higher levels of neutrophils and monocytes in \textit{Asah1}^{P361R/P361R} mice. Lymphocytes, eosinophils, and basophils did not show significant differences although a trend to higher levels in homozygotes was observed (Figure 3.12).

Red blood cells and hemoglobin levels were slightly increased above controls with significant differences observed between \textit{Asah1}^{P361R/P361R} and heterozygous animals (Figure 3.13).
Figure 3.12. Peripheral blood analyses. Blood samples were collected from all animals at 7-10 weeks. Total leukocytes, monocytes, neutrophils, eosinophils, basophils, and lymphocytes counts were determined in WT (n=10), heterozygous (n=7), and $Asah1^{P361R/P361R}$ mice (n=9). Bars represent mean values. Error bars represent standard errors of the mean. *$P<0.05$, **$<0.001$ indicate $Asah1^{P361R/P361R}$ group vs. WT group.
Figure 3.12. Blood samples were collected from all animals at 7-10 weeks. Total leukocytes, monocytes, neutrophils, eosinophils, basophils, and lymphocytes counts were determined in WT (n=10), heterozygous (n=7), and *Asah1*<sup>P361R/P361R</sup> mice (n=9). Bars represent mean values. Error bars represent standard errors of the mean. *P*<0.05, **P*<0.001 indicate *Asah1*<sup>P361R/P361R</sup> group vs. WT group.
Figure 3.13. Erythrocytes and hemoglobin analyses. Erythrocytes and hemoglobin levels were measured in WT (n=10), heterozygous (n=7), and $AsahI^{P361R/P361R}$ mice (n=9). Bars represent mean values. Error bars represent standard errors of the mean. *$P<0.05$ indicates $AsahI^{P361R/P361R}$ group vs. WT group.
3.5 Serum MCP-1 levels were elevated in \textit{Asah1}^{P361R/P361R} Mice

Serum samples from 3 WT, 5 Het, and 5 \textit{Asah1}^{P361R/P361R} animals were collected at 7-9 weeks. Multiplex analyses of cytokines and chemokines were conducted (Figure 3.14). Significant increases of MCP-1 levels in \textit{Asah1}^{P361R/P361R} animals were observed (Figure 3.14A). MCP-1 levels in heterozygotes remained relatively unchanged. Significant increases of IL-12 (P40) were also observed in \textit{Asah1}^{P361R/P361R} mice (Figure 3.14B). Elevated levels of MIP-1\(\beta\) and RANTES (that did not reach statistical significance) were also seen in \textit{Asah1}^{P361R/P361R} mice (Figure 3.14C, D). Measurements of TNF-\(\alpha\), INF-\(\gamma\), MIP-1\(\beta\), eotaxin, IL-13, IL-12 (P70), IL-10, IL-9, and IL-1\(\beta\) showed no difference between WT, Het, and \textit{Asah1}^{P361R/P361R} animals (Figure 3.14E, F, G, H, I, J, K, L, M).

**Figure 3.14. Serum cytokines analysis. A)** Serum was collected from 7-9 week old WT (n=3), Het (n=5), and \textit{Asah1}^{P361R/P361R} mice (n=5). MCP-1 levels were determined. Bars represent mean values. Error bars represent standard errors of the mean. *\(P<0.05\) indicates \textit{Asah1}^{P361R/P361R} group vs. WT group.
Figure 3.14. Serum was collected from 7-9 week old WT (n=3), Het (n=5), and Asah1\textsuperscript{P361R/P361R} mice (n=5). Levels of (B) IL-12 (P40), (C) MIP-1α, (D) RANTES, (E) TNF-α, (F) INF-γ, and (G) MIP-1β were determined. Bars represent mean values. Error bars represent standard errors of the mean. *P<0.05 indicates Asah1\textsuperscript{P361R/P361R} group vs. WT group.
Figure 3.14. Serum was collected from 7-9 week old WT (n=3), Het (n=5), and Asah1P361R/P361R mice (n=5). Levels of (H) Eotaxin, (I) IL-13, (J) IL-12(P70), (K) IL-10, (L) IL-9, and (M) IL-1β were determined. Bars represent mean values. Error bars represent standard errors of the mean. *P<0.05 indicates Asah1P361R/P361R group vs. WT group.
3.6 Microscopic and Ultrastructural Examinations of $Asah1^{P361R/P361R}$ Mice Revealed Diagnostic Farber Bodies

Histopathological examinations were carried out on H&E-stained tissue sections. Moderate-to-marked histiocytic infiltrations into the spleen, thymus, and liver of $Asah1^{P361R/P361R}$ animals were observed, with marked expansion and disruption of the normal parenchyma (Figure 3.15A, B, C). Histiocytes had abundant pale-foamy to finely-granular eosinophilic cytoplasms, with the nucleus displaced off to the side in most cases. Histiocytic infiltrates were accompanied by mild-to-moderate neutrophilic infiltrates. Patchy single-cell necrosis to random, multifocal areas of acute hepatocellular necrosis were also present. Similar histiocytic infiltrations were also observed in the bone marrow, lymph nodes, and the dermal layer of the skin. In longitudinal sections of the sciatic nerve, random, multifocal histiocytic aggregates were noted intercalating between axons, with disruption of myelin and accumulation of cellular debris (Figure 3.15D). Similar changes were noted in sagittal sections of spinal cord, with disruption of neuronal tracts (Figure 3.15F). Ganglia appeared unaffected. Mild pulmonary changes were also seen in homozygous animals, including mild pulmonary edema with scattered foamy alveolar macrophages and mild increases in neutrophils within alveolar septae (Figure 3.15E). No tissue changes were noted in sections of kidneys, testes, bone, joints, or skeletal muscles in $Asah1^{P361R/P361R}$ mice. Rare scattered vacuolation of cardiac myofibres were interpreted to be within normal limits. No intermediate phenotype was present in heterozygous animals. There was also no evidence noted of infectious disease in any of the animals.
Electron microscopy examinations revealed curvilinear tubular structures within cells from hepatic and peripheral nerve sections from $Asah1^{P361R/P361R}$ mice, which are exclusively found in Farber patients and are thought to derive from ceramide molecules (4) (Figure 3.16).
Figure 3.15. Histological examinations. A) Spleens were harvested from 7-10 week-old WT and $Asah1^{P361R/P361R}$ mice. H&E staining showed infiltrations of macrophages in tissues from $Asah1^{P361R/P361R}$ mice.
Figure 3.15. B) Thymuses were harvested from 7-10 week-old WT and $AsahI^{P361R/P361R}$ mice. H&E staining showed infiltrations of macrophages in tissues from $AsahI^{P361R/P361R}$ mice.
Figure 3.15. C) Livers were harvested from 7-10 week-old WT and Asah1<sup>P361R/P361R</sup> mice. H&E staining showed infiltrations of macrophages in tissues from Asah1<sup>P361R/P361R</sup> mice.
Figure 3.15. D) Sciatic nerves were harvested from 7-10 week-old WT and $Asah1^{P361R/P361R}$ mice. H&E staining showed infiltrations of macrophages in tissues from $Asah1^{P361R/P361R}$ mice resulting in interruption of nerve axons.
Figure 3.15. E) Lungs were harvested from 7-10 week-old WT and $Asah1^{P361R/P361R}$ mice. H&E staining showed infiltrations of macrophages in tissues from $Asah1^{P361R/P361R}$ mice accompanied by pulmonary edema.
Figure 3.15. F) Spinal cords were harvested from 7-10 week-old WT and Asah1<sup>P361R/P361R</sup> mice. H&E staining showed infiltrations of macrophages in tissues from Asah1<sup>P361R/P361R</sup> mice. Arrows indicate foamy macrophages.
Figure 3.16. Electron microscopy examination. Electron microscopy images of a hepatic section from 8-week old $Asah^P_{361R/P361R}$ animal. Arrows indicate Farber bodies.
3.7 \textit{Asah1}^{P361R/P361R} Mice Demonstrated Increased Apoptosis in The Spleen and Thymus

Ceramide is thought to exhibit apoptotic effects on cells (90). We examined if the build-up of ceramide in \textit{Asah1}^{P361R/P361R} mice was accompanied by increased apoptotic death in organs. Spleens and thymuses were harvested from WT, Het, and \textit{Asah1}^{P361R/P361R} animals at 7-8 weeks. Tissues were stained for apoptosis using TUNEL stain. Significant increases in apoptosis were observed in both the liver and thymus from \textit{Asah1}^{P361R/P361R} mice compared to WT and Het animals (Figure 3.17A, B). The distribution of apoptosis in the spleen demonstrated a certain pattern. Increased staining was observed in the white pulp of the splenic tissue saving the germinal centers. Analyses of proliferation were also carried out. Same tissues from spleens and thymuses of WT, Het, and \textit{Asah1}^{P361R/P361R} mice were stained for increased proliferation using Ki67 assay (Figure 3.17C, D). A trend towards increased proliferation in the spleen from \textit{Asah1}^{P361R/P361R} mice was observed compared to WT, and Het controls. Thymuses from \textit{Asah1}^{P361R/P361R} mice showed significantly lower proliferation indicated by Ki67 staining.
Figure 3.17. Apoptosis and proliferation analyses. A) TUNEL assay on spleens from 7-9 week old WT, Het, and $Asah1^{P361R/P361R}$ animals. Bars represent mean values. Error bars represent standard errors of the mean. *$P<0.05$ indicates $Asah1^{P361R/P361R}$ group (n=5) vs. WT (n=3) and Het group (n=5).
Figure 3.17. B) TUNEL assay on thymuses from 7-9 week old WT, Het, and $Asah1^{P361R/P361R}$ animals. Bars represent mean values. Error bars represent standard errors of the mean. *$P<0.05$ indicates $Asah1^{P361R/P361R}$ group (n=5) vs. WT (n=3) and Het group (n=5).
Figure 3.17. C) Ki67 assay on spleens from 7-9 week old WT, Het, and Asah1P361R/P361R animals. Bars represent mean values. Error bars represent standard errors of the mean. *P<0.05 indicates Asah1P361R/P361R group (n=5) vs. WT (n=3) and Het group (n=5).
Figure 3.17. D) Ki67 assay on thymuses from 7-9 week old WT, Het, and $Asah1^{P361R/P361R}$ animals. Bars represent mean values. Error bars represent standard errors of the mean. *$P<0.05$ indicates $Asah1^{P361R/P361R}$ group (n=5) vs. WT (n=3) and Het group (n=5).
3.8 Treatment of Neonatal Mice With Acid Ceramidase Recombinant LV (LV/AC) Yielded \( Asah1^{P361R/P361R} \) Mice With Intermediate Phenotype

To further validate the association of the observed phenotype of our novel mouse model with a deficient activity of AC enzyme and to develop an initial therapeutic platform for the treatment of Farber disease, we delivered the wild-type human \( ASAH1 \) cDNA \textit{in vivo}. We injected a total of 84 1-3 day-old mice via the temporal vein with LVs that engineered expression of either human acid ceramidase (LV/AC) or enGFP (LV/enGFP) as a control. Fifty-one neonates each received \( 5 \times 10^7 \) IU of LV/AC. Genotyping after three weeks identified 9 \( Asah1^{P361R/P361R} \) recipients among the 51 LV/AC injected animals. Eight out of 33 mice treated with \( 5 \times 10^7 \) IU of LV/enGFP were also identified as \( Asah1^{P361R/P361R} \). Two \( Asah1^{P361R/P361R} \) treated animals, one heterozygote, and one wild-type animal in the LV/AC-treated group demonstrated seizures at 3 weeks and were excluded from the initial growth curve analysis. Physiologically, \( Asah1^{P361R/P361R} \) mice treated with LV/AC manifested intermediate phenotypes (Figure 3.18A) and generally appeared healthier. Their growth was comparable to mice from the heterozygous and wild-type groups until 5 weeks of age. Afterwards, they started to lose weight at a rate similar to the LV/enGFP-treated \( Asah1^{P361R/P361R} \) group. That said, their body weights remained significantly higher than the weights of \( Asah1^{P361R/P361R} \) mice treated with LV/enGFP (Figure 3.18B).

Importantly, neonatal treatment with LV/AC extended the lifespans of \( Asah1^{P361R/P361R} \) individuals. All \( Asah1^{P361R/P361R} \) mice treated with LV/enGFP died by the age of 11 weeks while 7 out of 9 LV/AC-treated mice lived beyond 11 weeks and up to 16.5 weeks (Figure 3.19). Two
Asahl<sup>P361R/P361R</sup> mice from the therapeutic group were euthanized at 10 weeks due to pelvic prolapse and inability to urinate.

Peripheral blood analyses 10-12 weeks post-treatment revealed that LV/AC treatment also had profound effects on peripheral blood cell counts in Asahl<sup>P361R/P361R</sup> mice. As expected in the LV/enGFP-treated group, total leukocytes, neutrophils, monocytes, eosinophils, and basophils in Asahl<sup>P361R/P361R</sup> mice were elevated compared to heterozygous and wild-type mice. In comparison, a reduction of total leukocyte counts to normal levels was observed in LV/AC-treated Asahl<sup>P361R/P361R</sup> animals (Figure 3.20). LV/AC gene therapy also significantly altered the differential white blood cell profile in Asahl<sup>P361R/P361R</sup> mice versus controls. Eosinophil counts showed significantly reduced levels while we only observed a trend towards lower counts of monocytes, neutrophils, basophils, and lymphocytes in Asahl<sup>P361R/P361R</sup> mice treated with LV/AC as compared to Asahl<sup>P361R/P361R</sup> mice treated with LV/enGFP (Figure 3.20). Interestingly, we observed a similar trend to increased cell counts in wild-type mice treated with LV/AC compared to wild-type mice treated with LV/enGFP.

Ceramide levels were strikingly reduced in the spleen and liver of Asahl<sup>P361R/P361R</sup> mice that received a single LV/AC injection (Figure 3.21A, B). Tissues from two 10-week old LV/enGFP-WT, LV/enGFP-Asahl<sup>P361R/P361R</sup>, and LV/AC-Asahl<sup>P361R/P361R</sup> animals, and two 14-16-week old LV/AC-Asahl<sup>P361R/P361R</sup> mice were harvested for total ceramide and ceramide/DAG ratio measurements. Total ceramide as well as ceramide/DAG ratios were remarkably reduced in the spleen and liver in the LV/AC-treated animals while ceramide levels in the brain remained elevated (Figure 3.21A, B). Microscopic examinations demonstrated reduction in macrophage infiltrations into mainly the liver and spleen (Figure 3.22A, B). Mild reduction was also noted in the brain. Infiltrations were still observed in the spinal cord, sciatic nerve, thymus, lymph nodes,
bone marrow, lung, and skin in \textit{Asahi}\textsuperscript{P361R/P361R} mice, however, despite systemic delivery of LV/AC.
Figure 3.18. Evaluations of \(AsahI^{P361R/P361R}\) mice response to therapy. (A) A representative picture of 10 week-old LV/enGFP-WT, LV/enGFP-\(AsahI^{P361R/P361R}\), and LV/AC-\(AsahI^{P361R/P361R}\) mice. (B) Growth curves measured in weights versus age for: LV/enGFP-WT (n=8); LV/AC-WT (n=7); LV/enGFP-Het and LV/AC-Het (n=10); LV/enGFP-\(AsahI^{P361R/P361R}\) (n=8); and LV/AC-\(AsahI^{P361R/P361R}\) (n=7). \(* P < 0.05, ** P < 0.001\), for groups indicated vs. LV/enGFP-\(AsahI^{P361R/P361R}\).
Figure 3.19. Effects of therapy on survival. Kaplan-Meier survival analysis for all treated genotypes: LV/enGFP-WT (n=8); LV/AC-WT (n=8); LV/enGFP-Het (16); LV/AC-Het (n=34); LV/enGFP-Asah1^{P361R/P361R} (n=8); and LV/AC-Asah1^{P361R/P361R} (n=9). P < 0.05 for LV/AC-Asah1^{P361R/P361R} vs. LV/enGFP-Asah1^{P361R/P361R}.
Figure 3.20. Effects of therapy on peripheral blood cells. Blood samples were collected from all animals at 10-12 weeks. Total leukocytes and differential counts were analyzed in all control and experimental genotype groups: LV/enGFP-WT (n=8); LV/AC-WT (n=7); LV/enGFP-Het (n=8); LV/AC-Het (n=8); LV/enGFP-AsahI^{P361R/P361R} (n=7); and LV/AC-AsahI^{P361R/P361R} (n=9). Bars represent mean values. Error bars represent standard errors of the mean. *P < 0.05 for groups indicated vs. the corresponding control group.
Figure 3.20. Blood samples were collected from all animals at 10-12 weeks. Total leukocytes and differential counts were analyzed in all control and experimental genotype groups: LV/enGFP-WT (n=8); LV/AC-WT (n=7); LV/enGFP-Het (n=8); LV/AC-Het (n=8); LV/enGFP-Asah1P361R/P361R (n=7); and LV/AC-Asah1P361R/P361R (n=9). Bars represent mean values. Error bars represent standard errors of the mean. *P < 0.05 for groups indicated vs. the corresponding control group.
Figure 3.21. Effects of treatment on ceramide levels. A) Samples from spleens, livers, and brains of 10-week-old LV/enGFP-WT, LV/AC-WT, LV/enGFP-Asah1P361R/P361R, and LV/AC-Asah1P361R/P361R mice, and 14-16-week old LV/AC-Asah1P361R/P361R mice were analyzed for total ceramide (n=2 for all groups). Bars represent mean values. Error bars represent standard errors of the mean.
Figure 3.21. B) Samples from spleens, livers, and brains of 10-week-old LV/enGFP-WT, LV/AC-WT, LV/enGFP-Asah1<sup>P361R/P361R</sup>, and LV/AC-Asah1<sup>P361R/P361R</sup> mice, and 14-16-week old LV/AC-Asah1<sup>P361R/P361R</sup> mice were analyzed for ceramide/DAG ratios (n=2 for all groups). Bars represent mean values. Error bars represent standard errors of the mean.
Figure 3.22. Effects of therapy on macrophage infiltrations. A) Spleens from 10-week-old LV/enGFP-WT, LV/enGFP-Asahi$^{P361R/P361R}$, and LV/AC-Asahi$^{P361R/P361R}$ mice were stained with H&E. Arrows indicate foamy macrophages.
Figure 3.22. B) Livers from 10-week-old LV/enGFP-WT, LV/enGFP-$Asah1^{P361R/P361R}$, and LV/AC-$Asah1^{P361R/P361R}$ mice were stained with H&E. Arrows indicate foamy macrophages.
3.9 MCP-1 Levels Were Reduced In Organs From \textit{Asah1}^{P361R/P361R}

Mice Treated With LV/AC

Livers from 2 LV/enGFP-WT (10 weeks), 3 LV/enGFP-P361R/P361R (10 weeks), and 4 LV/AC-\textit{Asah1}^{P361R/P361R} mice (10-16 weeks) were analyzed for MCP-1 protein by ELISA. Above-normal levels of MCP-1 protein were observed in LV/enGFP-\textit{Asah1}^{P361R/P361R} mice compared to LV/enGFP-WT. MCP-1 levels were significantly reduced to WT levels in livers from \textit{Asah1}^{P361R/P361R} animals after treatment with LV/AC (Figure 3.23).
Figure 3.23. MCP-1 levels in livers from treated mice. MCP-1 levels in livers from LV/enGFP-WT (n=2), LV/enGFP-AsahI_{P361R/P361R} (n=3), and LV/AC-AsahI_{P361R/P361R} mice (n=4). Bars represent mean values. Error bars represent standard errors of the mean. *P < 0.05 for group indicated vs. LV/enGFP- AsahI_{P361R/P361R} group.
Chapter 4

DISCUSSION

Acid ceramidase (AC) has recently garnered much attention in cancer biology owing to its role in cell growth and proliferation and its association with malignant transformations (90). To this end, AC, its substrate ceramide, and phosphorylated metabolic product sphingosine-1-phosphate (S1P) play integral roles in the tight homeostatic regulation that ultimately determines cell-fate (90). Such sphingolipid balance is particularly important during embryogenesis. Previously, AC knockout mouse embryos were shown to not live beyond the two-cell stage (28). While this property partially explained the rarity of Farber disease, we and others were still challenged with the conundrum of generating a model that accurately recapitulated the AC-deficient phenotype to allow us to better study Farber disease progression, the role of lysosomal ceramide in vivo, and evaluate experimental therapies. To generate a model of AC deficiency, we introduced a single nucleotide mutation into the murine Asah1 coding sequence via genomic recombination (“knock-in”). We selected the mutation that was reported in a patient with a severe type of Farber disease (P362R). Given the one amino acid difference in length between the human and murine polypeptides, this mutation is located in position number 361 of the mouse polypeptide (P361R), which resides on the beta subunit of the enzyme. Among all reported Farber patient mutations, this mutation is located in the most conserved region between the human and murine polypeptides. Furthermore, amino acid substitutions from arginine to proline normally result in dramatic alterations in enzyme biochemistry; thus, we reasoned that this change could provide sufficient disruption to enzyme function while preserving the low residual activity required for embryonic development.
Patients with classic Farber disease manifest symptoms early after birth (4). Most patients exhibit hoarseness, arthritis, and subcutaneous nodules. Enlargements of the liver and spleen are not uncommon and neurological manifestations are severe in the majority of patients. Given the insidious nature of the disease, patients develop growth retardation and eventually die within the first two years of life. In our study, disruption of AC in mice resulted in a physical phenotype similar to that seen in patients with Farber disease. Homozygote animals (Asah1<sup>P361R/P361R</sup>) started to manifest growth retardation – relative to their heterozygous and wild-type littermates – as early as 3 weeks after birth. They reached a peak size between 4 and 5 weeks and then started to exhibit irreversible deterioration. All Asah1<sup>P361R/P361R</sup> mice had shortened lifespans and died by the age of ~ 12 weeks. It is likely that this rapid and irreversible deterioration is due to CNS involvement. This is seen in patients where the neurological impairments are thought to cause the short duration of the illness rather than the general dystrophy (17). Detailed neurological characterizations of the Asah1<sup>P361R/P361R</sup> mice are now underway. Lastly, progressive hydrocephaly was previously reported in one patient affected with Farber disease (91). It was interesting to see that AC deficiency in mice also led to the development of hydrocephaly in Asah1<sup>P361R/P361R</sup> mice as revealed by MRI. Imperatively, further investigations are needed to determine the cause of this pathology in Asah1<sup>P361R/P361R</sup> mice.

We approached the characterization of this novel Farber model at three levels: biochemical, histopathological, and ultrastructural. The P362R mutation in humans results in normal levels of enzyme expression but reduced activity (30). Ceramide subsequently accumulates in various tissues of the body. Our analysis of total ceramide levels in brains, livers, spleens, kidneys, hearts, and lungs showed high levels of total ceramide in Asah1<sup>P361R/P361R</sup> mice but normal levels in heterozygotes and wild-types. This finding was further confirmed when we
observed higher ceramide/DAG ratios in $Asahi^{P361R/P361R}$ animals. Importantly, AC enzyme levels were normal as indicated by Western blot analyses of livers and kidneys. Unexpectedly, in vitro AC activity measurements showed no differences in enzyme activities between $Asahi^{P361R/P361R}$ mice and wild-type and heterozygous animals (unpublished data). We are currently working on optimizing the enzyme assay to delve into this question further. That being said, this finding further indicates that the mutation does not lead to lower protein expression or enhanced enzyme degradation.

Under light microscopy, examinations demonstrated that the $Asahi^{P361R/P361R}$ animals recapitulated the histopathological features of Farber patients. Moderate-to-marked histiocytic infiltrates characterized by the presence of lipid-laden macrophages were seen in livers, lungs, spleens, thymuses, lymph nodes, brains, spinal cords, and sciatic nerves of $Asahi^{P361R/P361R}$ mice. These cells were positive for Mac-2 staining supporting that the infiltrates are mainly formed by macrophages (unpublished data). The histiocytic infiltrates within these organs caused marked expansion and destruction of the parenchyma without formation of granulomas. The lack of granulomas was previously reported in a patient with Farber disease (92). Under the electron microscope, we identified Farber bodies within vacuoles inside cells in hepatic and peripheral nerve sections from $Asahi^{P361R/P361R}$ animals. This finding is pathognomonic of Farber disease (93).

Ceramide is known to have apoptotic effects (90). However, the role of lysosomal ceramide in cell signaling is still controversial. It was shown that fibroblasts from patients with Farber disease do not manifest increased apoptosis. Our analyses using TUNEL assay indicated increased apoptosis in the spleens and livers from $Asahi^{P361R/P361R}$ mice. The distribution of TUNEL staining suggests that apoptosis might be taking place in certain cell populations. Here,
it is likely that these apoptotic, TUNEL-positive cells are infiltrating foamy macrophages. Macrophages invading the tissues phagocytose external ceramide in an attempt to dispose the excess ceramide. During the phagocytosis process, it is possible that engulfed ceramide comes in contact with the cell membrane where ceramide signaling is thought to take place. Therefore, external ceramide is likely to be the key player that exhibits biological effects in Farber disease rather than intrinsic ceramide accumulated in the lysosomes. This proposal supports the compartmentalization theory that distinguishes the roles of ceramide depending on its subcellular compartment. Whether ceramide signaling effects are contributing to the pathogenesis of Farber disease remains to be fully understood.

Currently, there is no effective therapy for Farber disease. Management is limited to anti-inflammatory medications and surgical corrections of joint deformities and subcutaneous nodules. Allogeneic bone marrow transplantation has shown success in reducing the peripheral symptoms (5); however, neurological symptoms and patient lifespans remained unaltered. Here, we showed that gene therapy is a conceptually-viable treatment option for Farber disease. Previously, we demonstrated that recombinant oncoretroviral vectors could efficiently correct the enzymatic defect \textit{in vitro} in fibroblast cells (84). Furthermore, corrected cells expressing high levels of AC also secreted the enzyme that could be taken up and used functionally by uncorrected bystander cells. We later showed that recombinant LVs are also very efficient at generating expression of AC both \textit{in vitro} and \textit{in vivo} (85). We have also completed initial experiments in enzymatically-normal non-human primates (NHPs; rhesus macaques) involving autologous transplantation of LV/AC-transduced hematopoietic cells. There, we observed supranormal levels of AC-specific activity in the bone marrow, peripheral blood mononuclear
cells, spleen, and liver. Reductions in ceramide levels were also observed in those enzymatically-normal NHPs injected with modified hematopoietic cells (86).

To further confirm the phenotype of the novel mouse model we generated, we delivered human AC cDNA in an attempt to correct the disease symptoms. We used LVs engineered to express either human AC or enGFP as a control. In a previous study, we demonstrated that we can effectively transfer genes in vivo in neonatal mice via direct LV temporal vein injections (94). Impressively, gene therapy resulted in an intermediate phenotype in Asah1P361R/P361R mice treated with LV/AC compared to Asah1P361R/P361R controls treated with LV/enGFP as highlighted by enhanced growth, prolonged survival, reduced leukocyte counts, and lower ceramide levels. However, all treated mice eventually succumbed to the disease and subsequent declines in weights after 5 weeks are likely due to neurological impairments. It is also possible that human AC is not optimally functional in catabolizing murine ceramide in Asah1P361R/P361R mice.

The pathophysiology of Farber disease remains unknown. It is unclear how the build-up of lysosomal ceramide is linked to the phenotype of the disease. Heavy infiltrations of tissues with foamy macrophages are thought to mediate the damage seen in Farber patients. However, the sequence of events that starts with the accumulation of ceramide and leads to widespread tissue destructions by lipid-laden macrophages is to be determined. We proposed a model that lays out this sequence to explain the downstream events that follow the build-up of ceramide. We hypothesized that the infiltration of tissues with macrophages is mediated by specific chemokines. Indeed, serum analysis in Asah1P361R/P361R mice revealed high levels of MCP-1 protein compared to wild-type and heterozygous littermates. MCP-1 is a strong chemo-attractant for monocytes (95). High levels of MCP-1 drive monocytes from the blood circulation into sites of MCP-1 overexpression (96). Ceramide seems to be directly correlated with MCP-1 level.
While ceramide was shown to induce the expression of MCP-1 protein (97), the inhibition of de novo ceramide synthesis led to reductions in MCP-1 levels (98). Therefore, the accumulation of ceramide prompts the release of a call signal (MCP-1) that recruits circulating monocytes to help dispose the excess of ceramide from tissues (Figure 4.1). It is also likely that other cytokines including MIP-1α are involved the recruitment of macrophages and granulocytes into organs in Asah1P361R/P361R mice. Being deficient of AC activity themselves, recruited macrophages fail to degrade ceramide they engulf resulting in more storage, giving them the foamy appearance characteristic of Farber disease. This model explains why bone marrow transplantation is effective in relieving the peripheral symptoms of Farber disease. Having a sufficient enzyme activity, macrophages originating from the donor bone marrow are able to metabolize ceramide that accumulates in tissues, possibly leading to reductions in MCP-1 levels, thus resulting in less macrophage infiltrations and resolution of granulomas. This mechanism is in contrast to a previous model that suggested intrinsic dysregulation of leukocytes to be responsible for their abnormal behavior in Farber disease (6).

Our proposed model puts the pathophysiology of Farber disease – a very rare disorder - in the context of a common mechanism that was shown to contribute to the pathogenesis of atherosclerosis, obesity complications, and diabetic nephropathy (99, 100, 101). This new understanding of the disease process has impact on future therapeutics of Farber disease. Every level at the ceramide, the MCP-1, and the macrophage axis can be explored as a potential treatment modality for Farber disease.
Figure 4.1. Proposed model of the pathophysiology of Farber disease. Illustration of the mechanism underlying tissue infiltrations with macrophages due to acid ceramidase deficiency and accumulation of ceramide.
4.1 Concluding Remarks and Future Directions

In this thesis, we have generated a mouse model of Farber disease by introducing a single-nucleotide mutation into the murine *Asah1* gene. We have shown that our novel animal model of AC deficiency accurately recapitulated the Farber disease phenotype and can be utilized to evaluate novel experimental therapies. We also provided supporting evidence that the chemokine, MCP-1, plays a role in contributing to the damage that results from ceramide accumulation in our mouse model. This new mechanistic insight helps to explain the disease process and provides a platform for developing new treatment modalities. Here, we also showed that neonatal gene therapy is a feasible and effective experimental therapy that can offer additional options for future Farber patients. Moreover, this model of AC deficiency may also be utilized to examine sphingolipid metabolism and signaling and their roles in cancer biology and hematopoiesis.

More elaborations on the mechanisms underlying the pathophysiology of Farber disease can be undertaken. The new insight on the disease process that was presented in this thesis opens the door for new levels of the research on Farber disease. Further studies involving the ceramide, the MCP-1, and the macrophage axis can provide more information on the pathophysiology of the disease. This approach can be studied at three different levels:

1. Ceramide reduction: we have shown in this thesis that systemic delivery of acid ceramidase resulted in reduction of ceramide in peripheral tissues and extension of lifespan. Other approaches to deliver acid ceramidase can be tested in the future. Correction of the deficiency in the CNS appears to be crucial for the effective treatment of Farber disease. Every successful study in the future should consider...
enhancing the delivery of the enzyme into the CNS. For example, administration of VEGF increases the permeability of the blood brain barrier, allowing larger particles to enter the CNS. Another approach that can be tested in the future is HSC-based gene therapy. Here, transduced bone marrow cells can differentiate into blood cell lines where circulating cells can function as a source of normal, functional enzyme. Importantly, bone-marrow-derived microglia cells in the CNS can work as vehicles to deliver the enzyme into the brain and the spinal cord. Other treatment options that reduce ceramide levels can also be tested. This includes enzyme replacement and substrate reduction therapies.

2. MCP-1 targeting: MCP-1 appears to be the link between ceramide accumulation and macrophage infiltrations that cause the organ damage in Farber disease. Targeting this molecule helps to establish the role of this protein in the pathogenesis of Farber disease and determine how much MCP-1 is contributing to macrophage infiltrations in this disorder. In the future, a valuable model can be generated by crossing Farber heterozygous with MCP-1 knockout mice. Other approaches to target MCP-1 can also be used. Antibodies against MCP-1 can present an alternative therapy to steroids for the treatment of Farber disease. Targeting MCP-1 is a specific approach compared to steroids which have broad anti-inflammatory effects and a wide spectrum of side effects. To further explore the disease process, a future study targeting the production of ceramide-1-phosphate (C1P) should be undertaken. It was recently shown that C1P induces the expression of MCP-1 protein (102). It is likely that MCP-1 overexpression in *Asah1<sup>P361R/P361R</sup>* mice is mediated by C1P rather than ceramide. The
abundance of ceramide may induce upregulation of ceramide kinase that leads to overproduction of C1P and therefore induction of MCP-1 overexpression.

3. Macrophage depletion: the end result of ceramide accumulation is infiltration of tissues with foamy macrophages. The damage seen in tissues from Asah1<sup>P361R/P361R</sup> animals and in Farber patients seems to be produced by macrophages. Clodrosome<sup>®</sup> is a clodronate-containing liposome that can be specifically phagocytosed by macrophages. The release of clodronate inside macrophages leads to their death. This drug can be used to deplete macrophages <em>in vivo</em> (103). Administration of Clodrosome to Asah1<sup>P361R/P361R</sup> mice is a feasible approach to correct the disease phenotype. Depletion of circulating monocytes will help to distinguish whether macrophage infiltrates in Asah1<sup>P361R/P361R</sup> mice are derived from pro-inflammatory monocytes or anti-inflammatory resident macrophages. The former is likely to be the case in Asah1<sup>P361R/P361R</sup> mice knowing that MCP-1 recruits pro-inflammatory monocytes into sites of its overexpression. Depletion of macrophages using Clodrosome will also provide an important insight about the role of intrinsic accumulation of ceramide in the pathophysiology of Farber disease. Protecting the tissues from the damage generated by macrophages also enables us to understand the direct effects of lysosomal ceramide accumulation on tissues. Finally, the roles of lysosomal ceramide in cell signaling can be investigated using this animal model. Cells from Asah1<sup>P361R/P361R</sup> mice can be examined to see if they exhibit any signs of increased apoptosis. The response of those cells to stressful stimuli demonstrates whether lysosomal storage of ceramide is involved in signaling. In this regard, foamy macrophages from Asah1<sup>P361R/P361R</sup> mice are unique. In addition to building up
intrinsic ceramide, foamy macrophages from $Asah1^{P361R/P361R}$ animals are loaded with phagocytosed ceramide. During the phagocytosis process, the engulfed ceramide may come in contact with the plasma membrane receptors where ceramide signaling is thought to take place. Studying the effects of external ceramide on those cells may help answer some of the controversial topics in ceramide compartmentalization.
References


