Investigating Endothelial Dysfunction in a CRISPR/Cas9 Model of Fabry Disease

by

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For my family

In memory of the 1.5 million victims of the Armenian Genocide of 1915

This work is an example of the continued existence of the Armenian people.

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List of Abbreviations

BH2: dihydrobiopterin

BH4: tetrahydrobiopterin

Cas9: CRISPR associated protein

CRISPR: Clustered regularly interspaced short palindromic repeats

DHFR: dihydrofolate reductase

eNOS: endothelial nitric oxide synthase

ERT: enzyme replacement therapy

Gb3: globotriaosylceramide

Gb4: globotetroasylceramide

GLA: α-galactosidase A

GLA(-): GLA deficient

GlcCer: glucosylceramide

GlcSph: glucosylsphingosine

gRNA: guide RNA

GSL: glycosphingolipid

LacCer: lactosylceramide

NO: nitric oxide

NOS: nitric oxide synthase

scr-siRNA: scrambled siRNA control

TLC: thin layer chromatography

WT: wild type

Abstract

Fabry disease is a rare, X-linked lysosomal storage disease arising from deficiency of the lysosomal hydrolase, α-galactosidase A (GLA). Reduced GLA activity disrupts glycosphingolipid (GSL) catabolism, leading to widespread accumulation of neutral GSLs in cells resulting in clinical manifestations such as stroke, vasculopathy, cardiomyopathy, and renal failure. The link between GLA deficiency and accumulation of GSLs with vascular dysfunction is unclear. Past work in the Shayman lab has led to the formulation of an overarching hypothesis for the mechanism of the vasculopathy in Fabry disease, namely that GLA deficiency leads to uncoupling of endothelial nitric oxide synthase (eNOS) and formation of reactive oxidants resulting in protein nitrosylation. The primary cause of eNOS uncoupling in the setting of GLA deficiency is unknown.

In this thesis, I used the CRISPR/Cas9 method for gene editing to mutate *Gla* in the human endothelial cell line, EA.hy926, to obtain GLA-deficient cells. Neutral GSLs were extracted and analyzed with thin layer chromatography (TLC). NOS activity was measured by monitoring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. Contrary to what is observed with siRNA knockdown of GLA expression in EA.hy926 cells, GLA-deficient cells did not accumulate the primary substrate for GLA, globotriaosylceramide

(Gb3). On the other hand, further analysis showed that these GLA-deficient cells accumulate the downstream GSL, globotetraosylceramide (Gb4). However, both the siRNA and CRISPR/Cas9 models have elevated globotriaosylsphingosine (lyso-Gb3).

As observed with siRNA knockdown of GLA expression, CRISPR/Cas9 GLA-deficient cells had less NOS activity. Two different methods of reducing globo series GSLs had opposite effects on NOS activity. Restoring GLA activity in GLA-deficient cells with exogenous GLA treatment improved NOS activity. In contrast, treating cells with a glucosylceramide synthase inhibitor (inhibits the synthesis of downstream GSLs) decreased NOS activity. These results suggest that eNOS uncoupling is due to GLA deficiency, and not necessarily due to elevated GSLs. Other studies showed that NOS activity is improved in GLA-deficient cells by increasing the availability of the eNOS cofactor, tetrahydrobiopterin, suggesting that GLA-deficient cells have a limited supply of endogenous BH4. Finally, it was observed that lyso-Gb3 inhibits NOS activity.

Chapter 1: Introduction

Fabry Disease

Fabry disease (OMIM 301500) is a rare lysosomal storage disease caused by a deficiency in the lysosomal hydrolase, α-galactosidase A (GLA, EC 3.2.1.22). GLA cleaves terminal α-galactosyl sugar groups. The primary substrate for GLA is the neutral glycosphingolipid (GSL), globotriaosylceramide (Gb3). Loss of GLA function results in progressive accumulation of Gb3 in various cells and tissues. *Gla* is located on the X chromosome (Xq22.1), which results in the disease primary affecting males, although heterozygous females also experience complications (Anderson 1898, Fabry 1898, Brady, Gal et al. 1967). A simplified pathway of the globo series GSLs is shown in figure 1.1.
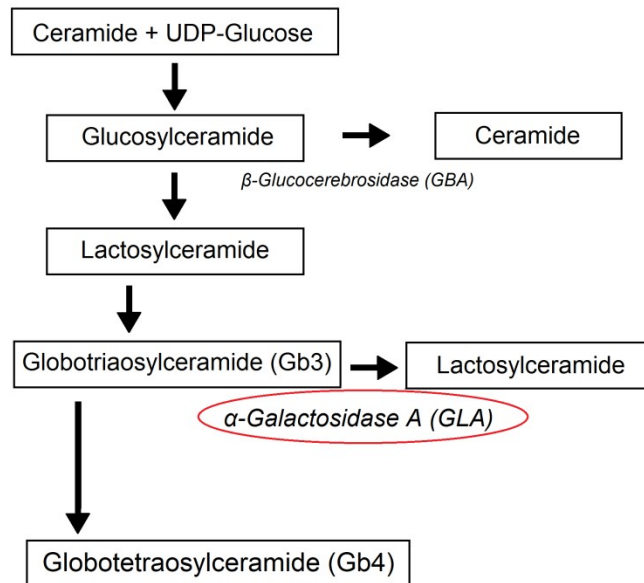
Figure 1.1: Synthesis and degradation of some globo-series GSLs. Fabry disease is caused by deficient GLA activity. GLA degrades Gb3 to LacCer. Deficient β-glucocerebrosidase (GBA) activity causes Gaucher disease, another lysosomal storage disease.

The pathogenesis of the cardiovascular complications of Fabry disease is not well understood. Past clinical studies (Moore, Scott et al. 2001, Moore, Ye et al. 2004) and mouse studies in the Shayman group (Shu, Park et al. 2009, Kang, Shu et al. 2014, Shu, Vivekanandan-Giri et al. 2014) have reported that GLA deficiency causes dysregulation of nitric oxide (NO) production and increased production of oxidant species. Nitrotyrosine, a product of protein nitrosylation, is elevated in patients with Fabry disease, GLA-deficient mice, and *in vitro* models of Fabry disease (Shu, Vivekanandan-Giri et al. 2014). Nitrotyrosine is a product of peroxynitrite, which is produced by uncoupling of endothelial nitric oxide synthase (eNOS) (Heinecke 2002). The link between GLA deficiency and eNOS uncoupling is not clear.

History and discovery

Fabry disease was first described as "angiokeratoma corporis diffusum" in 1898, by the two physicians, Johannes Fabry and William Anderson (Anderson 1898, Fabry 1898). It was originally described as a skin condition because the most prominent finding in patients was skin lesions on the hands and feet.

Thickening of blood vessel walls and enlargement and pronounced vacuolization of smooth muscle cells was observed in the first autopsy of a patient with Fabry disease, performed in 1945. It was suggested that the vasculopathy was due to accumulation of a material that could not be stained between muscle fibers. Autopsies of other patients a few years later showed accumulation of a lipoid-like substance in blood vessel walls, smooth muscle cells, cardiac muscle fibers, ganglion cells in the brain, and the peripheral nervous system (Ruiter 1957).

The main lipid that accumulates in Fabry disease was determined to be Gb3 (GL-3), and to a lesser extent, galabiosylceramide (GL-2) (Sweeley and Klionsky 1963). In 1965, GLA deficiency was reported to have X-linked inheritance (Opitz, Stiles et al. 1965). The enzymatic defect contributing to Gb3 accumulation was attributed to a deficiency of ceremidetrihexosidase (GLA) activity in 1967 (Brady, Gal et al. 1967).

The *Gla* gene is located on Xq22.1, is 12 kilobases long, and has 7 exons that translate to 429 amino acids (Bishop, Kornreich et al. 1988). The functioning GLA enzyme is a homodimer, with each monomer containing 398 residues (Garman and Garboczi 2004). GLA undergoes post-translational modification by N-glycosylation of three residues of each monomer (Matsuura, Ohta et al. 1998). The glycosylation pattern varies by tissue and affects the solubility (Ioannou, Zeidner et al. 1998) and stability of GLA (Lemansky,

Bishop et al. 1987), as well as trafficking of GLA to the lysosome, via the mannose-6-phosphate receptor (Chiba, Sakuraba et al. 2002).

Epidemiology, symptoms, and diagnosis

Reports of the occurrence of the disease vary, but some sources say Fabry disease is found in 43,000-117,000 males. One survey of newborns indicated that 1 in 3100 males have a GLA mutation (Spada, Pagliardini et al. 2006). Over 300 GLA mutations have been reported (Garman 2007). Because it is a rare disease, and presenting symptoms could be attributed to other causes, it is often not diagnosed until later in life. The prevalence may be underestimated (Colon, Ortolano et al. 2017). A study of 366 patients revealed a 13.7 and 16.3 year gap for males and females, respectively, between age of onset of symptoms and diagnosis of Fabry disease (Mehta, Ricci et al. 2004). A study of 1765 patients revealed that the median ages of diagnosis for males and females were 23 and 32, respectively, but the onset of symptoms were at age 9 and 13, respectively (Eng, Fletcher et al. 2007).

GLA deficiency results in an accumulation of neutral GSLs, leading to cellular and organ dysfunction. Poor perfusion due to accumulated GSLs may contribute to symptoms. Symptoms in early childhood include neurological pain, hyperhidrosis or hypohidrosis, angiokeratomas, gastrointestinal issues, and abnormal facial features. However, deaths usually arise from end-stage renal disease and premature stroke (Sims, Politei et al. 2009).

Fabry disease is diagnosed by measuring GLA activity in plasma or isolated leukocytes. This method is more reliable for males because heterozygous females have one functional copy of *Gla*, resulting in GLA activity in the normal range. Therefore, in

females, genetic testing is the preferred diagnostic method. Residual enzyme activity can slow the progression of the disease, resulting in the late-onset, cardiac and renal, variants (Spada, Pagliardini et al. 2006). As little as 5-10% of normal GLA enzyme activity may prevent accumulation of Gb3 and disease progression (Clarke 2007).

In males, early clinical symptoms of the disease include neuropathic pain (burning sensation in hands and feet), angiokeratomas (reddish-purple skin lesions), hypohidrosis, abdominal pain, gastrointestinal issues, and corneal opacities and cataracts in the eye. Later in life, as more Gb3 accumulates, patients experience renal disease and cardiovascular dysfunction (Desnick, Ioannou et al. 2001). By years 35-45, most male patients will require a kidney transplant or dialysis (Branton, Schiffmann et al. 2002, Thadhani, Wolf et al. 2002). Life expectancy in males is reduced by 20 years (MacDermot, Holmes et al. 2001), and in females by 15 years (MacDermot, Holmes et al. 2001).

Although Fabry disease has X-linked inheritance, heterozygous females can still develop symptoms of the disease. However, clinical manifestations are variable due to random X-chromosomal inactivation (Willard 2001, Morrone, Cavicchi et al. 2003, Dobrovolny, Dvorakova et al. 2005). Symptoms often present between ages 30 to 50. These include non-life-threatening (e.g. angiokeratomas) and life-threatening (e.g. left ventricular hypertrophy) manifestations (Kampmann, Baehner et al. 2002). In some female patients, the disease can be as severe as that seen in males (Desnick, Simmons et al. 1972).

Cardiac manifestations in patients include thickening of left heart valves, left ventricular hypertrophy, electrocardiographic abnormalities, and arrhythmias (Zarate and Hopkin

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2008). Gb3 accumulates in endothelial and cardiac muscle cells, which leads to vascular dysfunction and structural changes (Linhart and Elliott 2007). However, there are some conflicting reports regarding blood flow and peripheral endothelial function. Altarescu and colleagues reported that forearm blood flow and acetylcholine responses were elevated in patients with Fabry patients compared to control subjects (Altarescu, Moore et al. 2001). Several clinical studies report an increase in intima-media thickness and flow-mediated dilatation (Boutouyrie, Laurent et al. 2001, Boutouyrie, Laurent et al. 2002, Kalliokoski, Kalliokoski et al. 2006), while others report no change in radial artery wall thickness (Moore, Altarescu et al. 2002) and decreased flow-mediated dilatation (Kalliokoski, Kalliokoski et al. 2006). Increased cerebrovascular blood flow has been observed in Fabry patients (Moore, Altarescu et al. 2002). Autopsies of Fabry patients have shown no atherosclerotic lesions (Elleder 2003), or many atherosclerotic lesions (Schiffmann, Rapkiewicz et al. 2006). Clearly, Fabry disease has a complicated pathology that varies from person to person.

Treatment

Because Fabry disease is a multi-organ disease, managing symptoms is a part of treatment (Eng, Germain et al. 2006). In the United States, the only therapeutic to treat the underlying cause is enzyme replacement therapy (ERT) with recombinant GLA. Two forms of ERT are approved for use to treat Fabry disease, agalsidase alfa (Replagal™, Shire) and agalsidase beta (Fabrazyme™, Genzyme) (Vedder, Linthorst et al. 2007). ERT was FDA-approved in 2003 for use in the United States, and EMA-approved in 2001 for use in the European Union (Eng, Banikazemi et al. 2001, Eng, Guffon et al. 2001, Schiffmann, Kopp et al. 2001, Wilcox, Banikazemi et al. 2004). Based on clinical

trials, ERT is the standard of care as it has been shown to clear Gb3 from kidney cells (Thurberg, Rennke et al. 2002) and reduce renal, cardiac, and CNS events (Banikazemi, Bultas et al. 2007). ERT has been shown to improve neuropathic pain (Schiffmann, Kopp et al. 2001). Two studies have reported ERT decreases left ventricular mass (Weidemann, Breunig et al. 2003, Hughes, Elliott et al. 2008), while another has shown no improvement (Vedder, Linthorst et al. 2007). However, ERT does not lower the risk of cardiovascular events, unless ERT is initiated before any major event (Hopkin, Cabrera et al. 2016).

ERT has limitations in improving the clinical manifestations of Fabry disease. First, infusions are administered on a weekly or bi-weekly basis, which may be insufficient for Fabry patients to have normal, consistent, GLA activity (Schiffmann, Askari et al. 2007). Second, patients may develop an immune reaction to the infused enzyme, which can decrease the efficacy (Linthorst, Hollak et al. 2004, Rombach, Aerts et al. 2012). Third, when ERT is started after there has been organ damage, ERT does not halt disease progression. The earlier in life a patient starts ERT, the less likely they are to experience major complications from the disease (Cabrera, Politei et al. 2017), and the more likely they are to have reduction in globotriaosylsphingosine (lyso-Gb3), the deacylated metabolite of Gb3 (Arends, Wijburg et al. 2017), which will be discussed later. There is a need to better understand the pathology of GLA deficiency to develop a better treatment plan to improve the long-term effects of GLA deficiency that ERT alone cannot improve.

An alternative treatment, substrate reduction therapy, was proposed by Norman Radin in 1972 for treating lysosomal storage diseases (Radin, Arora et al. 1972). Inhibiting

sphingolipid synthesis can counteract the effect of a deficient catabolic enzyme. In Gaucher disease, the deficient lysosomal hydrolase is β-glucocerebrosidase, GBA (fig. 1.1), which degrades GlcCer to ceramide. Therefore, preventing synthesis of GlcCer by inhibiting GlcCer synthase would reduce the amount of accumulating GlcCer. An inhibitor for this enzyme, eliglustat tartrate (Cerdelga®, Genzyme), was developed in the Shayman lab, and was FDA approved in August 2014 for the treatment of Gaucher disease. It has since been approved for use in Europe, Australia, Japan, and Canada. Because at the time there was no suitable Gaucher mouse model, proof-of-concept studies with glucosylceramide synthase inhibitors were performed with the mouse model for Fabry disease (Abe, Arend et al. 2000, Abe, Gregory et al. 2000). GlcCer synthase inhibitors decrease the accumulation of lactosylceramide (LacCer), Gb3, and globotetraosylceramide (Gb4) (fig. 1.2), because GlcCer is a precursor for synthesis of these downstream GSLs. Treatment with the GlcCer synthase inhibitor, EtDO-P4, was able to reduce GlcCer and Gb3 content in cultured lymphocytes from Fabry patients, and in kidney, liver, and heart of GLA-deficient mice (Shayman and Larsen 2014).

Another therapeutic approach is chaperone therapy, which enhances residual enzyme activity by preventing misfolding and degradation of the mutated enzyme (Frustaci, Chimenti et al. 2001). Eligibility for this oral treatment is based on the specific *Gla* mutation a patient carries (Benjamin, Della Valle et al. 2017). The molecular chaperone migalastat (Galafold™, Amicus Therapeutics) was approved for use in Europe in 2016 (Germain, Hughes et al. 2016).

Globo series glycosphingolipids

Glycosphingolipids (GSLs) are molecules composed of a ceramide group and carbohydrate. A ceramide is made up of a fatty acid group with an amide linkage to a long-chain base, sphingosine. The carbohydrate has a glycosidic linkage to the primary hydroxyl group of the sphingosine. GSLs have both hydrophobic and hydrophilic properties. *De novo* formation of ceramide begins with the condensation of palmitoyl-CoA and serine by serine palmitoyltransferase (Brady and Koval 1958) and is followed by reduction and acylation enzymatic reactions, utilizing NADPH and acyl-CoAs (Sribney 1966). Ceramide is utilized by several catabolic pathways. It can be used to make phosphatidylcholine and sphingomyelin. Ceramide may be phosphorylated to form ceramide-1-phosphate. Another pathway ceramide is a precursor for is GSLs, resulting from glycosylation of ceramide. There are two classes of GSLs, neutral and acidic, based on the charge of the carbohydrate moiety. The following will further describe a class of neutral GSLs, the globo-series GSLs (structures shown in fig. 1.2).

The single-sugar GSLs, cerebrosides, are glucosylceramide (GlcCer) and galactosylceramide (GalCer). GlcCer is synthesized by UDP-glucose:ceramide glycosyltransferase from ceramide and UDP-glucose. GlcCer is further glycosylated to form lactosylceramide (LacCer, ceramide dihexoside, CDH), globotriaosylceramide (Gb3, ceramide trihexoside, CTH) (Makita and Yamakawa 1962, Makita 1964), and globotetraosylceramide (Gb4, globoside).

As previously described, the primary substrate for GLA is Gb3. Gb3 is a neutral glycosphingolipid that is a blood antigen. Gb3 is the receptor for verotoxin (shiga-like toxin), which is the toxin from *E. coli* causing hemolytic uremic syndrome (Karmali,

Petric et al. 1985, Lindberg, Brown et al. 1987). Gb3 is the blood group P^K antigen (Marcus, Naiki et al. 1976). Gb3 is comprised of a ceramide base, sphingosine, and the three sugar groups, glucose, galactose, and galactose. It is synthesized from LacCer by Gb3 synthase (Gb3/CD77 synthase, α1,4Gal-T) in the cytoplasm (Taga, Mangeney et al. 1995), and transported to the lysosome to be degraded by GLA. Although GLA is active in the lysosome, Gb3 accumulates outside of the lysosome in the cell membrane (Askari, Kaneski et al. 2007, Shu and Shayman 2007).

Although Gb3 is the primary substrate for GLA, it is not a reliable marker for severity of disease manifestations. Some Fabry patients do not have elevated Gb3 before beginning ERT (Young, Mills et al. 2005). There is no correlation between individual symptoms and urinary or plasma Gb3 levels of patients, or a decrease in plasma Gb3 after ERT, even with clinical improvement (Young, Mills et al. 2005, Vedder, Linthorst et al. 2007). However, in non-Fabry patients, there is a correlation between urinary Gb3 levels and risk of heart diseases (Schiffmann, Forni et al. 2014).

LacCer is formed by the degradation of Gb3 by GLA, or *de novo* synthesis by LacCer Synthase (β1,4-GalT family). LacCer synthase activity and LacCer levels are elevated in patients with familial hypercholesterolemia and atherosclerosis (Chatterjee, Sekerke et al. 1982, Chatterjee, Dey et al. 1997). LacCer has been shown to be a second messenger, and to stimulate the production of superoxide and other signaling mechanisms (Chatterjee 1998).

Gb4 is synthesized from Gb3 by *β*1,3-*N*-acetylgalactosaminyltransferase (*β*1,3GalNAc-T, Gb4 Synthase, Gb4S). Gb4 is the major neutral GSL of senescent erythrocytes, and is a major source for circulating Gb3 (Dawson and Sweeley 1970). Gb4 is the blood

group P antigen (Naiki and Marcus 1974, Naiki and Marcus 1975, Marcus, Naiki et al. 1976).

Lyso-Gb3 (globotriaosylsphingosine) is a deacylated form of Gb3. Acid ceramidase found in lysosomes metabolizes excess Gb3 to lyso-Gb3. Lyso-Gb3 is more water-soluble and is more easily excreted, providing a potential elimination pathway for Gb3 (Ferraz, Marques et al. 2016). Lyso-Gb3 is found in plasma of patients with Fabry disease (195-407 nM in affected males). Several studies suggest a link between lyso-Gb3 and Fabry disease, both as a biomarker and a cause for inflammation. In Fabry patients, a correlation exists between lyso-Gb3 (deacylated Gb3) and clinical manifestations (Rombach, Dekker et al. 2010). In many cases, heterozygous females have normal circulating levels of GLA, but still experience symptoms of the disease. Lyso-Gb3 may be a better marker for heterozygous females as it has been shown that symptomatic heterozygotes had elevated plasma lyso-Gb3 (22-76 nM) while lyso-Gb3 was not detected in an asymptomatic heterozygote. There have been similar findings in GLA-deficient mice (Aerts, Groener et al. 2008). Lyso-lipids found to be elevated in other lipid storage diseases, Krabbe disease (galactosylsphingosine), and Gaucher disease (glucosylsphingosine), have been shown to have toxic effects (Tanaka and Webster 1993, Schueler, Kolter et al. 2003).

Lyso-Gb3 has been shown to promote smooth muscle cell proliferation, which may contribute to the increased intima-media thickening seen in patients; Gb3 and lactosylsphingosine do not exhibit the same effect. ERT can reduce lyso-Gb3 in plasma (Aerts, Groener et al. 2008, Rombach, Dekker et al. 2010, van Breemen, Rombach et al. 2011, Smid, van der Tol et al. 2015).
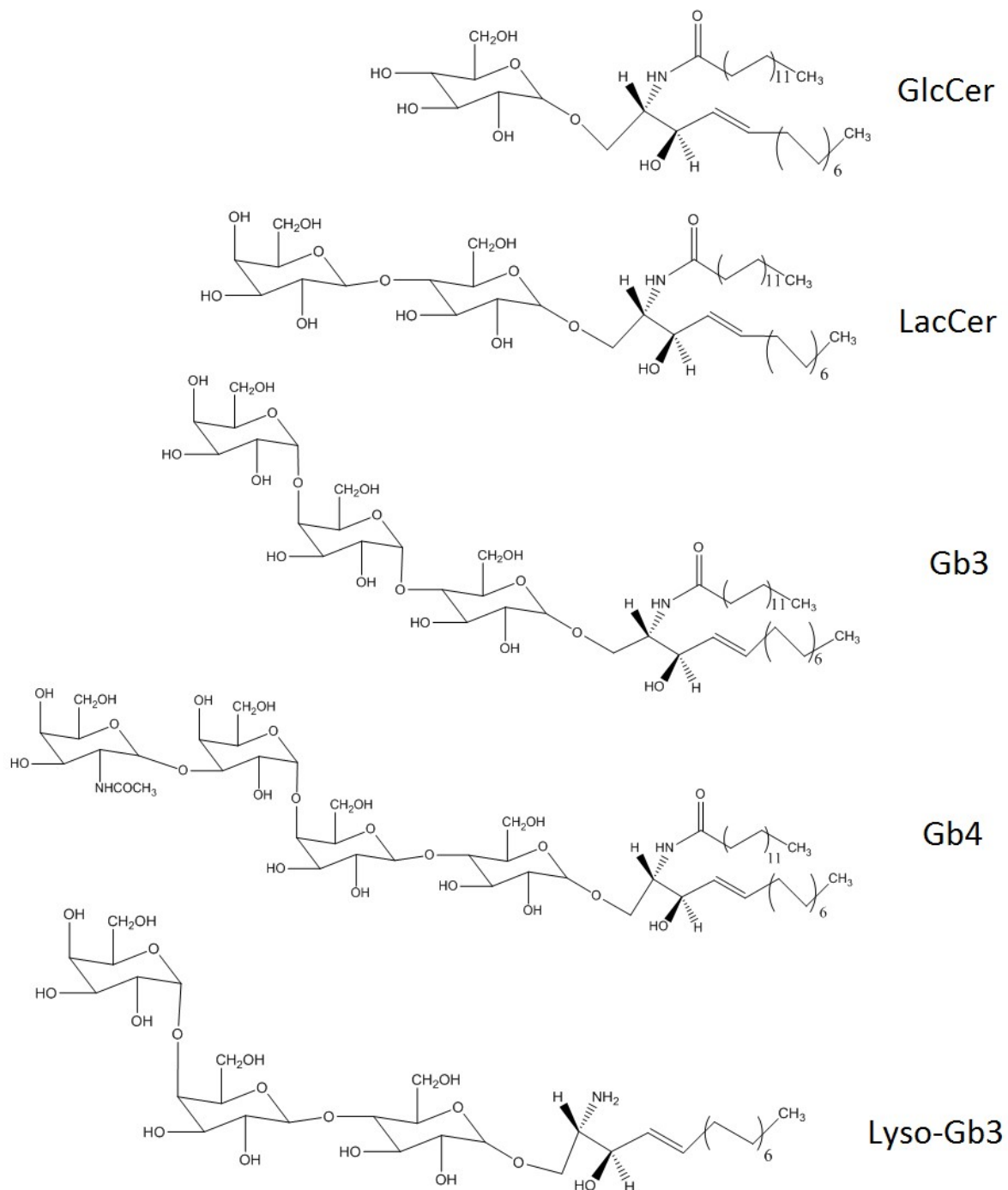
Figure 1.2: Structures of the globo-series GSLs. GSLs are composed of a carbohydrate and ceramide. Ceramide is made up of a fatty acid group with an amide linkage to a long-chain base, sphingosine. The length of the fatty acid acyl chain varies between 16 and 24 carbons. glucosylceramide (GlcCer); lactosylceramide (LacCer); globotriaosylceramide (Gb3); globotetraosylceramide (Gb4); globotriaosylsphingosine (Lyso-Gb3).

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In vivo Fabry disease model: *Gla*-knockout Mouse

In order to elucidate pathological mechanisms behind the clinical manifestations of Fabry disease, both *in vivo* and *in vitro* models of GLA-deficiency have been used.

The *Gla* knockout mouse (Ohshima, Murray et al. 1997) exhibits accumulation of lipids with terminal α-D-galactosyl moieties, mainly Gb3, in the liver and kidneys. GSL accumulation is also seen in cultured fibroblasts, and is corrected upon transfection with human GLA cDNA. The mice do not show any phenotypic changes before 10 weeks of age. Despite the accumulation of neutral GSLs in organs, the GLA-deficient mice differ from human Fabry patients in that they do not display renal failure until 80 weeks of age, nor do they have a spontaneous vascular phenotype. Red blood cells in GLA-deficient mice and humans show elevated amounts of Gb3 and Gb4 (Ohshima, Schiffmann et al. 1999).

Despite accumulating Gb3 in the vasculature, *Gla*-knockout mice do not display an obvious vascular abnormality. Previous studies in the Shayman group have utilized inducible models of vasculopathy to uncover defects in vascular function in *Gla*-knockout mice, specifically that deficiency of GLA leads to increased incidence of arterial thrombosis (Eitzman, Bodary et al. 2003), accelerated atherogenesis (Bodary, Shen et al. 2005), impaired vasorelaxation in both large and small arteries (Park, Whitesall et al. 2008, Kang, Shu et al. 2014), and a decrease in nitric oxide (NO) bioavailability arising from less activity of endothelial nitric oxide synthase (eNOS) (Shu, Park et al. 2009). The following is a more detailed description of these experiments. Results from the following experiments led to the hypothesis that cardiovascular abnormalities in Fabry disease are localized to the endothelium.

Accelerated thrombosis

A study was performed comparing time to occlusion in the carotid artery of $Gla^{-/0}$ and $Gla^{+/0}$ mice (hemizygous and wild type male, respectively), using a common model to study thrombosis, where time to occlusion is measured following rose bengal injection and exposure of the carotid artery to a green laser light (Eitzman, Bodary et al. 2003). The laser causes the local release of reactive oxygen species and subsequent damage to the endothelium. A thrombus forms at the site of endothelial damage, and the time to occlusive thrombus formation is a measure of the thrombotic nature of the system being examined. The results indicated an age-dependent decrease in time to occlusion of the $Gla^{-/0}$ mice that was not corrected upon bone marrow transplantation from $Gla^{+/0}$ mice, indicating the defect is localized to the vascular wall.

Accelerated atherogenesis

A study was performed where both male and female *Gla*-knockout and WT mice were bred on an $ApoE^{-/-}$ background (Bodary, Shen et al. 2005), which is a common model to study atherogenesis (Zhang, Reddick et al. 1992). After mice were fed on a Western chow diet, the aortas from *Gla*-knockout/*ApoE*-knockout mice had significantly greater lesion areas compared to GLA-WT controls bread on $ApoE^{-/-}$ background. In addition, the aortas from GLA-deficient mice had more iNOS and nitrotyrosine staining, which are markers for atherosclerosis. Accumulation of Gb3 in the vessel wall correlated with these markers. The conclusion was that GLA deficiency promotes atherosclerosis.

Impaired vasoreactivity

The vascular abnormalities described in the previous two experiments indicate a change in the arterial wall of mice lacking GLA. Because both defects could be due to

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changes in nitric oxide production, it was important to determine how GLA-deficiency affects the endothelium where NO production occurs. Therefore, vascular aortic rings were isolated from Gla$^{-/0}$ and Gla$^{+/0}$ mice, exposed to phenylephrine to contract them, and then subjected to increasing concentrations of acetylcholine (a vasodilator) (Park, Whitesall et al. 2008). The relaxation response was drastically reduced in rings from Gla$^{-/0}$ mice, indicative of impaired acetylcholine-induced NO production. A subsequent study investigated endothelial dysfunction in the mesenteric arteries from GLA-deficient mice and reported impaired relaxation of vessels to acetylcholine and evidence of eNOS uncoupling (Kang, Shu et al. 2014).

In vitro Fabry disease models

As described previously, GLA deficiency contributes to the accumulation of neutral GSLs in various cell types and organs. Notably, vascular endothelial cells are the most affected cell type (Desnick, Ioannou et al. 2001), which could explain the prevalence of stroke and cardiac abnormalities in patients. The three models of induced vasculopathy described in the previous section indicated a defect localized to the endothelium. To further investigate changes due to GLA deficiency, the Shayman group has utilized *in vitro* models to detect changes in endothelial cells. Results from the following experiments have led to the hypothesis that eNOS uncoupling is the basis for cardiovascular abnormalities in Fabry disease.

   Mouse aortic endothelial cells

Mouse Aortic Endothelial Cells (MAECs) were isolated from aortas of Gla$^{-/0}$ and Gla$^{+/0}$ mice (Shu, Murphy et al. 2005). The activity and expression of eNOS from cells from

Gla$^{-/0}$ mice was decreased (Shu and Shayman 2007, Shu, Park et al. 2009). It was also shown that the composition of caveolae was changed; Gb3 accumulated in the caveolae of MAECs from Gla$^{-/0}$ (Shu and Shayman 2007). Consequently, localization of eNOS to caveolae, one method of eNOS regulation, is disrupted. Decreased eNOS-caveolar association decreases eNOS activity (Garcia-Cardena, Oh et al. 1996). Treatment with the GlcCer synthase inhibitor, D-*threo*-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol, did not restore eNOS activity despite lowering the Gb3 content of GLA-deficient cells. These results are indicative of long-term consequences of GLA-deficiency that cannot be reversed by restoring Gb3 levels to normal levels (that of WT).

The MAEC model provided valuable evidence for how GLA deficiency contributes to changes in eNOS expression and location in endothelial cells. However, the drawback of this model is the difficulty of establishing these primary cell lines and the limited number of passages that can be used. Therefore, a better, permanent endothelial cell line was needed.

GLA-siRNA knockdown in EA.hy926 cells

The EA.hy926 cell line is derived from the fusion of a human umbilical vein cord (HUVEC) cell with an A549 permanent human lung carcinoma cell (Edgell, McDonald et al. 1983). Cells retain properties of endothelial cells, including expression of eNOS, Weibel-Palade bodies, and von Willebrand factor. However, EA.hy926 cells have more chromosomes than do HUVEC and A549 cells, and retain a marker chromosome from A549 cells (Emeis and Edgell 1988, Edgell, Haizlip et al. 1990). These cells express GLA and were chosen to study eNOS uncoupling. GLA expression was silenced with

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siRNA (Shu, Vivekanandan-Giri et al. 2014), and resulted in an accumulation of Gb3 correlating with duration of siRNA treatment (either 3 or 6 days), and a decrease in NOS activity. siRNA targeting β-glucocerebrosidase (GBA) (fig. 1.1), the enzyme deficient in Gaucher disease, did not exhibit a change in NOS activity. Furthermore, a marker for eNOS uncoupling, 3-nitrotyrosine (Heinecke 2002), was found to be increased in GLA-siRNA treated cells. This siRNA model was useful to characterize how even a temporary loss of GLA expression and/or increase in Gb3 content directly impacts eNOS activity. However, to determine long-term changes, a permanent, GLA-deficient cell line was needed. The work described in this dissertation uses EA.hy926 cells with permanent GLA deficiency (induced by CRISPR/Cas9-gene editing) to investigate changes in eNOS function.

Fabry disease models: conclusions

While studies using these models have each contributed to the overall understanding of Fabry disease, there are limitations for each. The *Gla*-knockout mouse was useful for studying the effects of GLA deficiency on intact tissue, but further investigations into changes at the cellular level was limited. The primary cell model using MAECs was useful for bridging the gap between *in vivo* and *in vitro* studies, but was difficult to isolate and maintain in cell culture. The EA.hy926 cell line is often used for studies in endothelial cells because it retains many endothelial cell characteristics and is of human origin (Bouis, Hospers et al. 2001). However, in order to induce a Fabry phenotype, siRNA silencing was used to knockdown GLA expression. This allowed for the discovery of elevated 3-nitrotyrosine as a result of GLA-deficiency, indicating the occurrence of eNOS uncoupling. However, siRNA transfection is limited by the length of

time the treatment is effective for, and by possible off-target effects. Therefore, the work presented in this dissertation uses the newer method of CRISPR/Cas9 gene editing to create cell lines with a permanent *Gla* mutation. To address possible off-target effects, cell lines were generated by targeting two different exons of *Gla*, and growing several single-cell colonies.

The link between GLA deficiency and cardiovascular dysfunction is not clear. The primary hypothesis in the Shayman group is that GLA deficiency leads to endothelial nitric oxide synthase (eNOS) uncoupling resulting in less production of nitric oxide (NO) and increased production of oxidant species.

Nitric oxide, eNOS, and eNOS uncoupling

The reaction catalyzed by endothelial nitric oxide synthase (eNOS) is: L-arginine + O_2 $\rightarrow$ L-citrulline + NO, and is dependent on cofactors, nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH4), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), heme, Ca^{2+}, and calmodulin.

Robert Furchgott discovered the critical role of an intact endothelium for acetylcholine-induced relaxation of aortic rings (Furchgott and Zawadzki 1980). He determined that endothelial cells release a factor, endothelium derived relaxing factor (EDRF), which relaxes vascular smooth muscles cells, resulting in dilation of blood vessels. Later, Louis Ignarro identified EDRF as NO (Ignarro, Buga et al. 1987). Ferid Murad contributed to the field by identifying that many vasodilators act through NO (Pollock, Forstermann et al. 1991). These three individual were awarded the Nobel Prize in

Physiology or Medicine in 1998. NO was the first gas discovered to act as a second messenger (Nobelprize.org 1998).

Abnormal endothelial function is a factor in the development of common vascular diseases, such as stroke and coronary artery disease. An important function of the endothelium is the production of NO by eNOS. NO is a critical regulator of vascular homeostasis. It inhibits platelet aggregation, vascular smooth muscle cell proliferation, and leukocyte adhesion (Ignarro 2002). NO can act within the cell or diffuse locally to signal a variety of physiology responses by generating secondary messengers. For example, NO inhibits contraction of smooth muscle cells and prevents thrombosis by stimulating the release of cGMP. NO also nitrosylates cysteine residues of proteins (S-nitrosylation) (Stamler, Simon et al. 1992); for example, S-nitrosylation of N-ethylmaleimide-sensitive factor (NSF) prevents exocytosis of Weibel Palade bodies (Matsushita, Morrell et al. 2003). Weibel Palade bodies contain the pro-thrombotic factor, von Willebrand factor (Reinders, De Groot et al. 1984). Therefore, the main result of NO in this system is preventing secretion of von Willebrand factor.

There are three members of the NOS family, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), named after the cell type from which they were discovered. eNOS is constitutively expressed in endothelial cells, but expression is regulated in response to various stimuli. For example, eNOS mRNA and protein expression increases when endothelial cells are exposed to shear stress (Nishida, Harrison et al. 1992). eNOS is post-translationally modified. It is *N*-myristoylated (Pollock, Klinghofer et al. 1992, Liu and Sessa 1994) and palmitoylated (Liu, Garcia-Cardena et al. 1995, Robinson, Busconi et al. 1995, Liu, Garcia-Cardena et al. 1996).

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Several protein kinases regulate eNOS activity, including Akt, PKA, PKC, and AMP-activated protein kinase (Chen, Mitchelhill et al. 1999, Fulton, Gratton et al. 1999, Michell, Griffiths et al. 1999, Michell, Chen et al. 2001). Phosphorylation can activate or inhibit eNOS function depending on the residue that is phosphorylated. Other factors, including vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and estrogen also affect eNOS activity (Xia, Aiello et al. 1996, Hisamoto, Ohmichi et al. 2001).

In addition to eNOS mRNA and protein expression and posttranslational modification, the bioavailability of NO is affected by the availability of the substrate, L-arginine, or cofactors, and protein-protein interactions such as caveolin-1 and heat shock protein 90 (Hsp90) (Garcia-Cardena, Fan et al. 1998). An additional factor is the presence of superoxide. NO reacts with superoxide to yield peroxynitrite, which causes further oxidations, including the conversion of BH4 to BH2 (Huang 2009), resulting in eNOS uncoupling.

A common observation with eNOS and vascular disease is that higher eNOS protein expression does not necessarily result in more NO production. eNOS expression is reduced in endothelial cells located over advanced atherosclerotic plaques (Wilcox, Subramanian et al. 1997). However, in diabetes, heart failure, and hypertension, eNOS expression remains normal or is increased (Bouloumie, Bauersachs et al. 1997, Cosentino, Hishikawa et al. 1997, Bauersachs, Bouloumie et al. 1999, Hink, Li et al. 2001). This discrepancy can be attributed to a process known as eNOS uncoupling (fig 1.3).

eNOS becomes uncoupled when electrons from NADPH in the reductase domain flow to molecular oxygen rather than to the oxygenase domain to reduce L-arginine (fig 1.3). This complex forms superoxide instead of NO (Vasquez-Vivar, Kalyanaraman et al. 1998, Xia, Tsai et al. 1998). Superoxide can then react with NO to produce peroxynitrite (ONOO$^-$). ONOO$^-$ is a reactive nitrogen species that can oxidize BH4 to the inactive form, BH2 (Milstien and Katusic 1999). A biomarker for the presence of ONOO$^-$ is 3-nitrotyrosine resulting from protein nitrosylation (Heinecke 2002). A lower ratio of BH4:BH2 and eNOS:BH4 contributes to eNOS uncoupling (Crabtree, Tatham et al. 2009). There are two main pathways of BH4 formation (fig 1.4), which can be pharmacologically manipulated to increase BH4 availability.

eNOS uncoupling is a characteristic of common diseases with a vascular dysfunction component, like hypercholesterolemia (Ohara, Peterson et al. 1993), diabetes (Hink, Li et al. 2001), atherosclerosis (White, Brock et al. 1994), and hypertension (Panza, Garcia et al. 1995). In diabetic mice, the aortic expression of eNOS is increased, but NO availability is reduced. Inhibiting eNOS reduces superoxide levels in vessels from diabetic mice, but increases superoxide levels in healthy mice (Hink, Li et al. 2001). Lower BH4 availability due to a pro-oxidant state has been described in diabetes and hypertension (Landmesser, Dikalov et al. 2003). Supplementation with BH4 improves NOS function in patients with diabetes (Heitzer, Krohn et al. 2000), chronic smokers (Heitzer, Brockhoff et al. 2000, Ueda, Matsuoka et al. 2000), hypertension (Hong, Hsiao et al. 2001), and high cholesterol (Stroes, Kastelein et al. 1997). Decreased BH4 availability has recently been implicated as the basis for eNOS uncoupling in Fabry disease (Shen, Arning et al. 2017). Simply increasing eNOS expression does not

increase NO production because of the requirement of a proper ratio of eNOS:BH4, and

will instead result in more eNOS uncoupling and superoxide production (Bendall, Alp et

al. 2005).

Coupled eNOS   Uncoupled eNOS
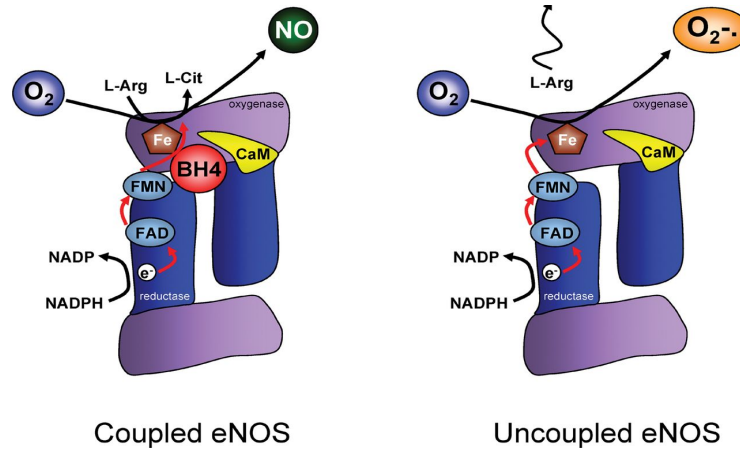
Figure 1.3. Role of BH4 in eNOS uncoupling. *Left*: After calcium activates calmodulin (CaM), and BH4 is available, electrons from the reductase domain of one monomer flow to the oxygenase domain of the other monomer, via electron transfer from NADPH to FAD, FMN, BH4, and the ferrous-dioxygen complex (Fe). Reduction of molecular oxygen (O_2) is coupled to L-arginine (L-Arg) oxidation, resulting in production of L-citrulline (L-Cit) and NO. *Right*: When BH4 is not available, L-Arg and the ferrous-dioxygen complex dissociate. Electrons transfer to O_2, and superoxide ($O_2^{-\cdot}$) is generated from the oxygenase domain.[1]
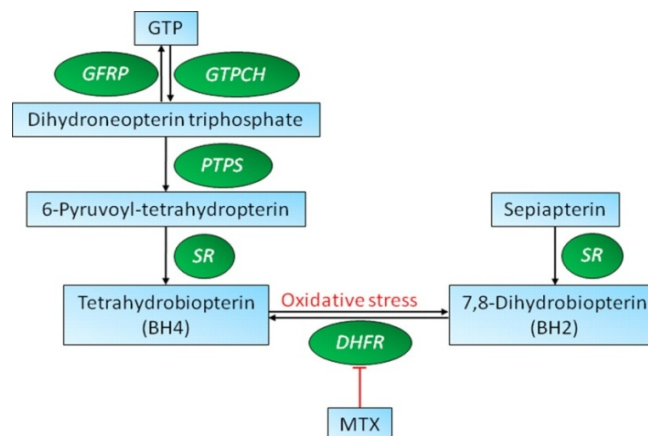
Figure 1.4. The BH4 recycling and *de novo* synthetic pathways. BH4 is synthesized from GTP through a series of reactions involving GTP cyclohydrolase (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), sepiapterin reductase (SR) and dihydrofolate reductase (DHFR). Methotrexate (MTX) inhibits DHFR. Sepiapterin increases BH4 production, and also inhibits GTPCH. GFRP (GTP cyclohydrolase feedback regulatory protein).[2]

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[1] Figure reproduced from: Tim S. Schmidt, and Nicholas *J. Alp Clin. Sci*. 2007;113:47-63

[2] Figure reproduced from: Mark J. Crabtree et al. *J. Biol. Chem*. 2009;284:28128-28136

Overall goal and specific aims

Cardiovascular complications are a major cause of mortality in patients with Fabry disease. The long-term goal of this work is to determine the link between GLA deficiency and vascular dysfunction. The overall objective of this study was to establish an *in vitro* model to investigate potential mechanisms of eNOS uncoupling in the setting of GLA deficiency. The hypothesis was that GLA deficiency leads to a limited availability of a cofactor for eNOS, BH4, and that the reduction of neutral GSLs improves eNOS activity.

Aim 1: Develop CRISPR/Cas9-mediated GLA-deficient EA.hy926 cell model to study eNOS uncoupling

Previously, anti-GLA siRNA treatment of EA.hy926 cells resulted in Gb3 accumulation and eNOS uncoupling. Chapter 2 describes the process of obtaining CRISPR/Cas9 *Gla*-mutated cell lines, with a permanent absence of GLA activity. It was hypothesized that GLA(-) cells would have Gb3 accumulation and decreased NOS activity. It was found that these cells do not accumulate Gb3, but instead accumulate Gb4, but still have less NOS activity. In addition, it was found that the CRISPR/Cas9 and siRNA models have elevated lyso-Gb3. Because decreased NOS activity is observed in both the CRISPR/Cas9 and siRNA models of GLA deficiency, we conclude that elevated level of lyso-Gb3, not Gb3, is a consistent marker for eNOS uncoupling in the setting of GLA deficiency.

Aim 2: Determine if GSL accumulation, BH4 availability, and lyso-Gb3 affect eNOS uncoupling in GLA-deficient cells

Chapter 3 investigates possible mechanisms of eNOS uncoupling in GLA(-) cells, with three main focuses: neutral GSLs, BH4 availability, and lyso-Gb3.

First, it was hypothesized that decreasing GSLs by enzyme replacement or substrate reduction would improve NOS activity in GLA(-) cells. It was found that enzyme replacement with exogenous GLA treatment improved NOS activity, but substrate reduction with eliglustat treatment decreased NOS activity. These contrasting results suggest that it is the absence of functional GLA and not necessarily elevated globo-series GSLs that is the cause of eNOS uncoupling.

Second, it was hypothesized that BH4 availability is limited in GLA(-) cells, and increasing BH4 availability would restore NOS activity. We observed that increasing the availability of the NOS cofactor, BH4, did improve NOS activity in GLA(-) cells. We conclude that GLA deficiency leads to limited BH4 availability, which causes eNOS uncoupling.

Third, it was hypothesized that GLA(-) cells secrete a substance, possibly lyso-Gb3, that inhibits NOS activity. However, we found that GLA(-) cells secrete a substance that increased NOS activity in WT cells. The increase in the observed NOS activity was not necessarily due to increased eNOS expression. We separately demonstrated that lyso-Gb3 inhibits NOS activity. Therefore, we conclude that elevated lyso-Gb3 in the absence of GLA activity causes eNOS uncoupling, and GLA(-) cells increase NOS activity in WT cells partly in an eNOS-independent manner.

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Chapter 2: CRISPR/Cas9-mediated GLA deficient EA.hy926 cells do not accumulate Gb3, but still have decreased NOS activity

Abstract

Patients with Fabry disease, a deficiency of α-galactosidase A (GLA), have an increased risk of vasculopathy compared to the non-Fabry population. Past studies suggest that endothelial nitric oxide synthase (eNOS) dysfunction is the basis for impaired vascular function. In order to better study the mechanism of eNOS dysfunction, permanent GLA-deficient endothelial cell lines were obtained by using CRISPR/Cas9 gene editing to mutate *Gla* and eliminate GLA protein expression in EA.hy926 endothelial cells. Surprisingly, these cells do not accumulate globotriaosylceramide (Gb3), the primary substrate for GLA, but instead have elevated amounts of the downstream glycosphingolipid, globotetraosylceramide (Gb4). In contrast, EA.hy926 cells treated with siRNA against GLA have an accumulation of Gb3, but not Gb4. However, consistent with previous models of GLA deficiency, including the siRNA model, CRISPR/Cas9 GLA-deficient cells had decreased NOS activity. It was found that globotriaosylsphingosine (lyso-Gb3), a deacylated form of Gb3, is elevated in both the siRNA and CRISPR/Cas9 models of GLA deficiency in EA.hy926 cells,

suggesting that level of lyso-Gb3, not Gb3, is a consistent marker for eNOS uncoupling in Fabry disease.

Introduction

Fabry disease is an X-linked lysosomal storage disorder caused by mutations in the *Gla* gene. Patients experience vascular disorders, often resulting in premature death. Fabry disease causes elevated levels of glycosphingolipids (GSLs) with terminal α-1,4-galactose groups due to deficient α-galactosidase A (GLA) activity. GSLs, mainly the globo-series GSLs, accumulate in various cells and tissues, but are especially prominent in endothelial cells (Linhart and Elliott 2007). Both clinical and laboratory studies of Fabry disease indicate the accumulation of globotriaosylceramide (Gb3), the substrate for GLA, in cells. However, in patients, Gb3 is not a good biomarker for individual symptoms (Vedder, Linthorst et al. 2007). The deacylated metabolite of Gb3, globotriaosylsphingosine (lyso-Gb3), better correlates with symptoms, especially in females with the disease (Aerts, Groener et al. 2008). Previous work suggests that the primary cause of endothelial dysfunction in Fabry disease is due to endothelial nitric oxide synthase (eNOS) uncoupling.

One of the greatest challenges of any scientific study is choosing the appropriate model, especially a good *in vitro* model to study molecular mechanisms. Previous *in vitro* studies of Fabry disease in the Shayman group have utilized mouse aortic endothelial cells (MAECs) from *Gla*-knockout mice, and the human endothelial cell line, EA.hy926 with GLA-siRNA knockdown (Shu and Shayman 2007, Shu, Vivekanandan-Giri et al. 2014). Both models have provided insight into how GLA-deficiency affects GSL

accumulation in the cell and affects eNOS function. However, both models have limitations; MAECs are challenging to maintain in culture, and anti-GLA siRNA-treated EA.hy926 cells do not maintain the Fabry phenotype for longer than the duration of the siRNA treatment (3-6 days). Therefore, in this study, cell lines with a permanent absence of GLA protein expression were developed to be able to study eNOS uncoupling in the setting of GLA deficiency. These cells were obtained by using the CRISPR/Cas9 method of gene editing.

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) system is found in bacteria, and is used as a method of protection from foreign DNA (Mojica, Ferrer et al. 1995, Jansen, Embden et al. 2002, Barrangou, Fremaux et al. 2007). The system uses an RNA-guided DNA nuclease to combat foreign nucleic acids. Specific single stranded RNA sequences recognize and bind to complementary sequences in foreign DNA elements, after which the Cas endonuclease cleaves it. The type II CRISPR-Cas system from *Streptococcus pyogenes* consists of a single Cas nuclease, Cas9, two noncoding CRISPR RNAs (crRNAs) – trans-activating (tracrRNA) and precursor (pre-crRNA), and has recently been adapted for use in human cells (Deltcheva, Chylinski et al. 2011, Jinek, Chylinski et al. 2012, Cho, Kim et al. 2013, Cong, Ran et al. 2013, Mali, Yang et al. 2013, Qi, Larson et al. 2013).

In this study, two methods of GLA deficiency are evaluated in EA.hy926 cells. Both models have deficient NO production; however they have different patterns of Gb3 accumulation. siRNA silencing of GLA leads to accumulation of Gb3, as expected. In contrast, CRISPR/Cas9-mediated GLA deficiency does not lead to accumulation of

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Gb3, but instead of Gb4, another globo-series GSL (fig. 1.1). The lipid that is elevated in both models is lyso-Gb3, supporting other studies reporting that lyso-Gb3 is the better marker for Fabry disease.

Materials and Methods

Cell culture

EA.hy926 cells (ATCC, Manassas, VA) were maintained at 37 °C with 95/5 O_2/CO_2, in DMEM/F-12, GlutaMAX (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (source), which will be later referred to as "growth medium."

CRISPR/Cas9 plasmid generation and delivery

In order to disrupt GLA expression in EA.hy926 cells, Single-stranded guide RNA was designed to target Exon 1 or Exon 2 (Table 2.1) using the Optimized Design Tool (crispr.mit.edu). Forward and reverse single-stranded oligonucleotides (Table 1) were annealed to generate a double-stranded oligonucleotide. The oligonucleotide was cloned into the GeneArt CRISPR nuclease vector (Thermo Fisher Scientific, Waltham, MA), which expresses the orange florescence protein (OFP) reporter. The vector was transformed into One Shot TOP10 E. coli cells (Thermo Fisher Scientific, Waltham, MA). Bacteria were grown on LB agar plates with ampicillin and incubated at 37 °C overnight. Clones were selected and grown in LB broth overnight. Plasmids were

purified and sequenced with the U6 forward primer to verify the presence and proper orientation of double-stranded oligonucleotide[3].

EA.hy926 cells were plated at 600,000 cells/well on 6 well dishes. The following day, 2 µg of CRISPR plasmid DNA was transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA), and incubated with DMEM-F12 GlutaMAX™ supplemented with 10% FBS for 24 hours. Cells were FACS sorted on a BD FACSAria™ II flow cytometer (BD Biosciences, San Jose, CA) with BD FACSDiva 8.0 software. OFP positive cells were collected and plated onto 150 mm dishes at low density in normal growth conditions. Cells were allowed to grow for several days, and then cloning cylinders were used to isolate individual colonies. Cells were grown in the same media conditions described for EA.hy926 cells.

Surveyor nuclease assay

Colonies were grown to confluency on 100 mm dishes and harvested for DNA extraction (DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA). The region around the CRISPR-targeted region was PCR amplified with the primers listed in Table 2.2 to detect mutations. PCR products were run on 4% agarose gels to verify that the products were of the expected length. The surveyor nuclease assay (Integrated DNA Technologies) was performed according to the manufacturer's instructions, with slight modification. This nuclease cleaves mismatched DNA (due to mutations), which can be visualized on an agarose gel. Two different reactions were set up per colony. The first reaction used only DNA from that colony; the other reaction used DNA from the colony and DNA from EA.hy926 cells not CRISPR/Cas9 treated. The purpose for carrying out

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[3] CRISPR vector cloning and plasmid purification was performed by Robert Kelly in the Shayman group.

both reactions was to rule out the probability that one colony may have the same *Gla*

mutation on all copies of the gene.

The DNA was denatured and then rehybridized in the thermocycler. $MgCl_2$, surveyor

nuclease, and surveyor enhancer were then added to each reaction. The reaction was

incubated at 42 °C for 60 minutes. Loading dye was added to each tube and the product

was run on a 4% agarose gel with a size ladder. Mutant colonies were identified by

changes in the DNA size.

CRISPR guide RNA	Sequence 5'-3'
Exon 1, set 1	F: GCTAGCTGGCGAATCCCATG (GTTTT)
	R: CATGGGATTCGCCAGCTAGC (CGGTG)
Exon 1, set 2	F: ATTGGCAAGGACGCCTACCA (GTTTT)
	R: TGGTAGGCGTCCTTGCCAAT (CGGTG)
Exon 2	F: GCTAGCTGGCGAATCCCATG (GTTTT)
	R: CATGGGATTCGCCAGCTAGC (CGGTG)

Table 2.1: Forward (F) and reverse (R) nucleotides used to guide Cas9 to *Gla*. Parentheses indicate sequence complementary to the 3' overhang sequence in the CRISPR nuclease vector. Two different sets of gRNAs were used to target exon 1.

PCR Primers	Sequence 5'-3'
Exon 1	F: GCCCCTGAGGTTAATCTTAAAAGCC
	R: AGCTCTCCCTCGGGCTCAACTGTTC
Exon 2	F: GTGAAATCCCAAGGTGCCTAATAAA
	R: TCTAAACAAGCTTCTGTACAGAAGT

Table 2.2: Forward (F) and reverse (R) nucleotides used to amplify the regions of exons 1 and 2 of *Gla* that were targeted by CRISPR/Cas9.

Western blotting

Colonies that were identified as having a mutation were grown to confluency on 100 mm dishes. Cells were harvested with 0.05% trypsin-EDTA and lysed in a buffer consisting of 25 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, 20 mM NaF, 1% Triton X-100, 10% glycerol, and protease inhibitor (P8340, Sigma). Cells were probe sonicated on ice and centrifuged at 10,000 x g for 10 minutes at 4 °C. Supernatants were saved and total cellular proteins determined with bicinchoninic acid (BCA) assay using bovine serum album (BSA) as a standard (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA). Thirty μg of protein were denatured with 2-mercaptoethanol in Laemmli buffer, heated at 95 °C for 10 minutes, and then separated on NuPAGE 4-12% SDS-PAGE (Thermo Fisher Scientific). Proteins were transferred to nitrocellulose membranes, blocked for 1 hour with 5% nonfat dry milk-TBST (tris-buffered saline, tween-20), then incubated with primary antibody against GLA (1:1000 in 5% milk-TBST, EPR5829[4], (ab129173) Abcam, Cambridge MA), eNOS (1:1000 in 5% nonfat dry milk-TBST, M221 (ab76198) Abcam), or GAPDH (1:1000 in 1% BSA-TBST, #MAB374, EMD Millipore, Billerica, MA) overnight at 4 °C. After washing with TBST, membranes were incubated with anti-mouse (for eNOS and GAPDH detection) or anti-rabbit (for GLA detection) secondary antibody at (1:15,000 in 1% BSA-TBST). Membranes were washed with TBST and incubated with enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific). Protein bands were detected with film exposure. The film was scanned and protein bands quantified with ImageJ Software.

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[4] This antibody recognizes a region in the C terminus of GLA.

GLA enzyme activity assay

GLA activity was measured by modification of previously reported methods (Abe, Arend et al. 2000). Cells were grown to confluency in T150 flasks and harvested with 0.05% trypsin-EDTA. Cells were probe sonicated in buffer containing 3 mg/mL sodium taurocholate, 28 mM citric acid, and 44 mM Na_2HPO_4. The lysate was centrifuged for 30 minutes at 20,000 x g at 4 °C and supernatant was saved. Protein concentrations were determined using BCA assay described above. Two µg of protein was assayed in buffer with final concentrations of 5 mM *p*-nitrophenyl α-D-galactopyranoside (PNPαGal, substrate for GLA), 117 mM *N*-acetyl-*D*-galactosamine (NAGA, inhibitor for α-*N*-acetylgalactosaminidase, another lysosomal enzyme that hydrolyzes PNPαGal (Mayes, Scheerer et al. 1981)) 28 mM citric acid, 44 mM Na_2HPO_4, and 5 mg/mL BSA. Reactions were incubated at 37 °C overnight and stopped by adding an equal volume of 200 mM Na_2CO_3. Absorbance was read at 400 nm on a spectrometer (Molecular Devices SpectraMax 250, Sunnyvale, CA).

DNA sequencing

DNA was extracted and PCR amplified as described earlier. PCR products were run on a low melting temperature 2% gel. The DNA bands were viewed under UV light and carefully excised. DNA was extracted from isolated gel fragments using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified DNA fragment was cloned into the 2.1 Topo vector (Thermo Fisher Scientific, Waltham, MA). The vector was transformed into One Shot TOP10 *E. coli* cells (Thermo Fisher Scientific). Bacteria were grown on LB agar plates with ampicillin and incubated at 37 °C overnight. Clones were selected and grown in LB broth with ampicillin overnight. Plasmids were purified (QIAprep Spin

Miniprep Kit, Qiagen) and submitted to the University of Michigan DNA Sequencing Core. Sequences were analyzed using DNAStar software (Madison, WI).

siRNA silencing of GLA in EA.hy926 cells

Transfection of EA.hy926 cells was performed as previously described, but with minor modification (Shu, Vivekanandan-Giri et al. 2014). For cells that were to be harvested after a single transfection, the following protocol was observed: One day before transfection, 0.8×10^6 cells were seeded onto 150 mm cell culture dishes with growth medium. At the time of transfection (Day 0), growth medium was replaced with Opti-MEM-I (Thermo Fisher Scientific, Waltham, MA). Next, anti-GLA siRNA or scrambled-siRNA control (scr-siRNA) was mixed with Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer's protocol. After 8 hours, the medium was replaced with growth medium. Cells were harvested on Day 1 or 3 post-transfection.

For cells that were to be harvested after a double transfection, the following protocol was observed. One day before transfection, 0.7×10^6 cells were seeded onto 150 mm cell culture dishes with growth medium. The same 3-day transfection protocol was followed as described above, but instead of harvesting the cells on Day 3, cells were transfected once more with the same treatment, and later harvested on Day 6.

Lipid extraction

Cells were grown to confluency on 150 mm cell culture dishes and harvested by scraping into 700 µL PBS. The cell suspension was transferred into 16 mm x 100 mm glass tubes, and 1 mL chloroform and 2.4 mL methanol were added. After water bath sonication, protein was precipitated by centrifuging at 2400 x g for 30 minutes. The

supernatant was transferred to a new glass tube and 4.5 mL chloroform and 1.2 mL 0.9% NaCl were added. The sample was centrifuged at 900 x g for 5 minutes. The upper aqueous phase was discarded, and the lower organic phase was washed twice with 2 mL methanol and 0.8 mL 0.9% NaCl. The lower phase was extracted using a 1 mL glass syringe (Hamilton, Reno, NV) and transferred to a new glass tube. Lipids were dried under a stream of nitrogen.

Total phospholipid assay

To measure the lipid content of samples, total phospholipid was determined (Ames 1966). Dried lipids were suspended in 500 μL of chloroform/methanol (2:1, v/v). Thirty μL of sample was transferred to 13 x 100 mm glass tube, and 30 μL of 10% $Mg(NO_3)_2.6H_2O$ in 95% EtOH was added. The mixture was dried under a stream of nitrogen and ashed for 30 seconds over a flame. After cooling to room temperature, 300 μl of 0.5 M HCl was added and incubated at room temperature for 30 minutes. Then, 700 μL of (1:6, v/v) 10% ascorbic acid: 0.42% $(NH_4)_2Mb \cdot 4H_2O$ was added and samples and standards were incubated for 45 minutes at 45 °C. Samples were cooled to room temperature and absorbancy measured at 820 nm. Samples were compared to standards of 0, 5, 10, 20, 30, 40 μL of 1 mM KH_2PO_4, that were prepared simultaneously. Total phospholipid was calculated according to the standard curve. The volume according to 100 nmol of total phospholipid was transferred to a new glass tube and dried under a stream of nitrogen.

Alkaline methanolysis and acid hydrolysis

To dried lipids, 2 mL chloroform and 1 mL 0.21 M NaOH in 100% methanol were added.

Samples were incubated at room temperature for 1 hour. The reaction was terminated

by addition of 0.8 mL 0.25 M HCl and centrifuged at 900 x g for 5 minutes. The upper

aqueous phase was removed and lower organic phase was transferred with a 1 mL

Hamilton syringe to a new 16 mm x 125 mm glass tube. Four mL methanol and 1.6 mL

0.05 M HCl/25 mM $HgCl_2$ were added. The mixture was incubated at 37 °C for 15

minutes. 2 mL of chloroform and 1.6 mL of water was added. Samples were centrifuged

at 900 x g for 5 minutes. The upper aqueous phase was removed and 2 mL methanol

and 1 mL 30 mM EDTA pH 8.0 were added to the lower organic phase. Samples were

centrifuged and the upper phase was removed again. The lower organic phase was

washed twice with 2 mL methanol and 1.6 mL water. The lower layer was transferred

with a 1 mL glass syringe to a new 12 x 100 mm glass tube and dried under a stream of

nitrogen.

Thin layer chromatography – LacCer, Gb3, and Gb4 measurements

One hundred nmol of total phospholipid was applied to a silica high performance TLC

plate (Sigma-Aldrich). The plate was first developed in a solvent system consisting of

chloroform/methanol (98:2, v/v), and air dried. The plate was then developed in a

solvent system consisting of chloroform/methanol/acetic acid/water (61/31/5/3, v/v/v/v)

and air dried. Plates were submerged in 8% (wt/vol) cupric sulfate pentahydrate in

water/methanol/H_3PO_4 (60:32:8, v/v/v), and charred for 10 minutes at 150 °C, or were

sprayed with 1% orcinol in 11% H_2SO_4 and charred at 130 °C for 5 minutes. Plates were

scanned and densinometry measured using ImageJ software. Lipids were quantified by

running GlcCer, LacCer, Gb3, and Gb4 standards on each plate (Matreya LLC, State College, PA).

Exogenous GLA treatment

Cells (1.0×10^6) were seeded on 150 mm cell culture dishes with growth medium. The following day, growth medium was replaced with fresh media, and 3 U of α-galactosidase A (Green coffee bean extract, Sigma), was added. Cells grew for 3 days and were harvested for lipid analysis with TLC.

Eliglustat treatment

Cells (1.0×10^6) were seeded on 150 mm cell culture dishes with DMEM/F12 GlutaMAX supplemented with 5% FBS. The following day, medium was replaced with DMEM/F12 GlutaMAX supplemented with 2% FBS. Cells were treated with either PBS vehicle control, 20 or 200 nM eliglustat. Cells were harvested 3 days later for lipid analysis with TLC.

NOS activity measurement

Endothelial nitric oxide synthase (eNOS) activity in EA.hy926 cell lysates was analyzed by using a NOS activity assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. This assay measures the biochemical conversion of L-arginine to L-citrulline by NOS. Cells were harvested with 0.05% trypsin-EDTA and washed with PBS two times. Cell pellets were lysed in the provided homogenization buffer (final concentration 1 mM EDTA and 1 mM EGTA in 25 mM Tris-HCl (pH 7.4) buffer). After brief sonication, samples were centrifuged at 12,000 x g for 5 minutes, and supernatants transferred to new tubes. Cell lysates (5 μL) were incubated at room

temperature for 3 hours with 1 µCi [3H]L-arginine (PerkinElmer), 100 nM calmodulin, and the provided reaction buffer (final concentration 1 mM NADPH, 600 µM CaCl$_2$, 25 mM Tris-HCl (pH 7.4), 3 µM tetrahydrobiopterin, 1 µM flavin adenine dinucleotide, and 1 µM flavin adenine mononucleotide in 50 µL). The reaction was stopped by adding 400 µL of 5 mM EDTA in 50 mM HEPES (pH 5.5) buffer. The provided resin was added to each sample to remove [3H]L-arginine. Radioactivity due to [3H]L-citrulline was measured as counts per minute (cpm) using a scintillation counter. Cpm were also measured in samples with no cell lysate as a background control. NOS activity of each sample was calculated by subtracting background cpm from the cpm for each sample, and then normalizing to total protein in each 5 µL sample, determined by the BCA protein assay.

Lyso-Gb3 measurement

To measure lyso-Gb3 in EA.hy926-CRISPR/Cas9 clonal cell lines, 0.7 x 10^6 cells were seeded on 100 mm dishes. The following day, media was replaced with fresh growth media. Three days later, media was collected and cells harvested with 0.05% trypsin-EDTA. Cells and media were collected the same way in siRNA-treated EA.hy926 cells with the transfection method described above. Cell pellets were sonicated on ice in 200-250 µL PBS, centrifuged at 12,000 x g, and supernatant collected. BCA protein assay was performed. Lyso-Gb3 was measured with previously published methods[5] (Gold, Mirzaian et al. 2013). Results were normalized to protein concentration.

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[5] Samples were analyzed by the Aerts lab at Academic Medical Center in Amsterdam, Netherlands.

Data were analyzed by using Prism 7.0 software (GraphPad Software Inc, La Jolla, CA).

Significance was determined by two-way analysis of variance (ANOVA) with Dunnett's

or Sidak's multiple comparisons test. Values are shown as mean ± SEM. Differences

between groups were considered statistically significant at a P-value of <0.05.

****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

Results

### *CRISPR/Cas9-mediated gene editing of Gla to obtain GLA-deficient EA.hy926 cell lines*

In order to disrupt GLA expression in EA.hy926 cells, Gla-specific single-stranded guide

RNA (gRNA) was designed to target exon 1 or 2 of *Gla* (table 2.1). EA.hy926 cells were

transfected with a CRISPR/Cas9 vector that contained the exon-specific gRNA and

orange florescence protein (OFP). These cells were FACS sorted (table 2.3), then

grown and analyzed by surveyor mutation analysis for a mutation in the targeted

regions (fig. 2.1). After confirming the presence of mutant cells, single cell colonies were

grown and surveyor mutation analysis was repeated (table 2.3). The first transfection

and FACS sorting using the exon 2-targeted gRNA did not yield GLA(-) clonal cell lines.

Therefore, the procedure was repeated to target exon 2, and this time, GLA(-) clonal

cell lines were obtained. CRISPR/Cas9 transfection with the exon 1, set 1 gRNA did not

yield GLA(-) clonal cell lines. Because GLA(-) clonal cell lines were obtained with the

other gRNA targeting exon 1, transfection with the exon 1, set 1 gRNA was not

repeated. For studies in chapters 2 and 3, clonal cell lines from exon 1, set 2 gRNA,

were used. The DNA sequence of the *Gla*-exon 1 targeted region is shown in figure 2.2. Results from experiments using exon 2 gRNA clonal cell lines are included in the appendix.

Colonies that have a mutation in *Gla* may still express GLA because there is probably more than one copy of the X chromosome in EA.hy926 cells (Edgell, McDonald et al. 1983). Western blot confirmed the absence of GLA protein expression in 4 clonal cell lines from the exon 1 set 2 gRNA group (fig 2.4). Experiments are performed with these clonal cell lines, named 2, 5, 12, and 14. A single base pair deletion was detected in these cells (fig. 2.2). There is a possibility that clones 5, 12, and 14 originated from the same cell due to having the same mutation. One wild type (WT) colony that was negative for a mutation in the surveyor assay, clone number 17, was used as a wild type control in all assays. A western blot of siRNA-GLA knockdown is also shown for comparison (fig. 2.4). Treatment with scrambled siRNA is used as a control for the siRNA transfection procedure.

Clonal cell lines were grown, harvested, and GLA activity was determined by incubated cell lysates with the artificial GLA substrate, PNP-α-Gal, and measuring absorbance at 400 nm for the product, *p*-nitrophenol. The GLA-deficient cells 2, 5, 12, and 14 have only background activity, while the WT control, 17 had significant activity (fig. 2.3).
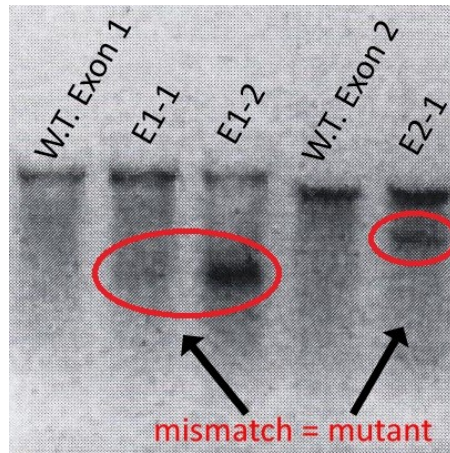
Figure 2.1: Surveyor mutation analysis of CRISPR/Cas9 gRNA targeting exon 1 or 2 of *Gla*.

|	E1-1	E1-2	E2 (first)	E2 (second)
# OFP cells	2106	1160	2028+2414	5732+2668
# Isolated colonies	20	19	28	17
# Surveyor mutants	4	14	0	-
# No GLA expression	0	4	0	4

Table 2.3: Results of CRISPR/Cas9 transfection with gRNAs targeting exons 1 and 2 of Gla. FACS sorted cell count, and number of colonies indicating a mutation in the Surveyor assay, and the number of colonies with no GLA expression on Western blot.

Figure 2.2: Sequence of the region of *Gla* – Exon 1 that was targeted by CRISPR/Cas9. The target sequence is indicated by the red box, which is the sequence complementary to the gRNA used in the CRISPR/Cas9 transfection. CRISPR/Cas9 caused single base pair deletions resulting in frameshift mutations. The deletion affects residue 41 of 429 of the GLA protein.

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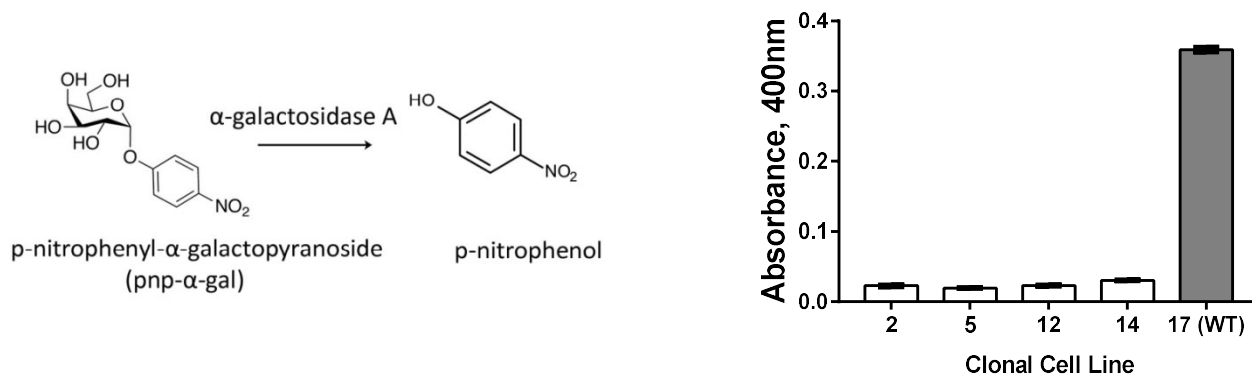
Figure 2.3: GLA Enzyme Assay. *Left*: Reaction scheme of the GLA enzyme activity assay. Pnp-α-gal is an artificial substrate for GLA that is converted to p-nitrophenol. The resulting color change can be measured by reading the absorbance at 400 nm by spectrophotometry. *Right*: Cell lysates from clonal cell lines 2, 5, 12, and 14, exhibit background GLA activity in comparison to 17(WT). n=3
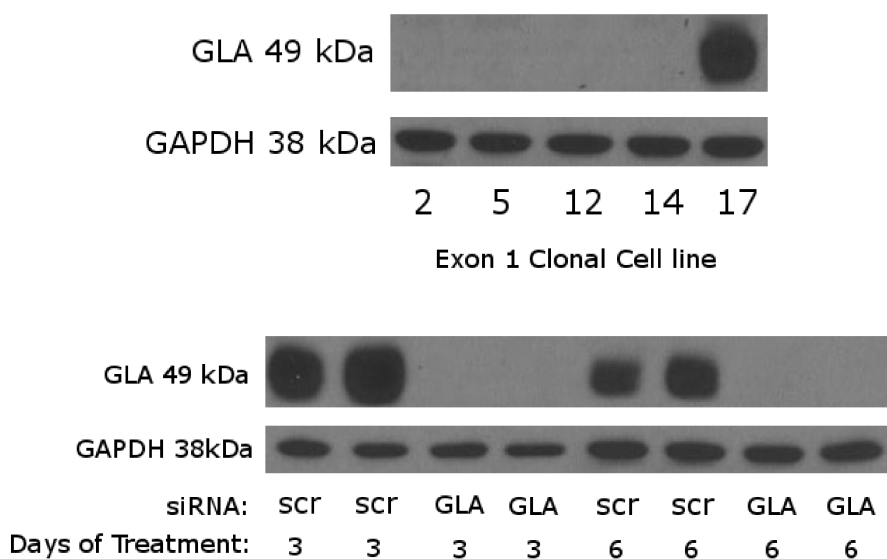
Figure 2.4: CRISPR/Cas9 gene editing or anti-GLA siRNA treatment eliminates GLA expression in EA.hy926 cells. Thirty µg of protein was denatured, separated on a 4-12% gel, transferred to nitrocellulose membranes, and blotted for GLA and GAPDH. *Top:* CRISPR/Ca9-edited clones 2, 5, 12, and 14 do not express GLA. Clone 17 represents the WT control used for all CRISPR/Cas9-model studies. *Bottom*: anti-GLA siRNA treatment for 3 or 6 days eliminates GLA expression. Scrambled (scr) siRNA controls express GLA.

CRISPR/Cas9 GLA-deficient cells have a different lipid profile compared to siRNA-mediated GLA-knockdown cells

Previously published results showed that siRNA silencing of GLA in EA.hy926 cells for 3 or 6 days results in Gb3 accumulation (Shu, Vivekanandan-Giri et al. 2014). The siRNA assay was repeated, and cells harvested for lipid extraction. CRISPR/Cas9 GLA-deficient cell lines were also grown and harvested for lipid extraction. Total phospholipid assay was performed to determine lipid content of samples, and 100 nmol of total phospholipid was subjected to alkaline methanolysis and acid hydrolysis. The amount of globo-series GSLs was determined by TLC with cupric sulfate charring (example shown in fig. 2.7). LacCer and Gb3 were quantified. In agreement with previously reported results (Shu, Vivekanandan-Giri et al. 2014), treating EA.hy926 cells with anti-GLA siRNA for 6 days increased Gb3 content two-fold over treatment with scr-siRNA control (fig 2.5). However, CRISPR/Cas9 GLA(-) cells did not have an increase in Gb3 content compared with WT controls (fig 2.6), and in fact had a slight decrease. Furthermore, while anti-GLA siRNA treatment increased LacCer content, the CRISPR/GLA –deficient cells all have decreased LacCer content. In summary, although both the siRNA silencing and CRISPR/Cas9 methods eliminate GLA expression (fig 2.4) in EA.hy926 cells, the lipid profile was different.
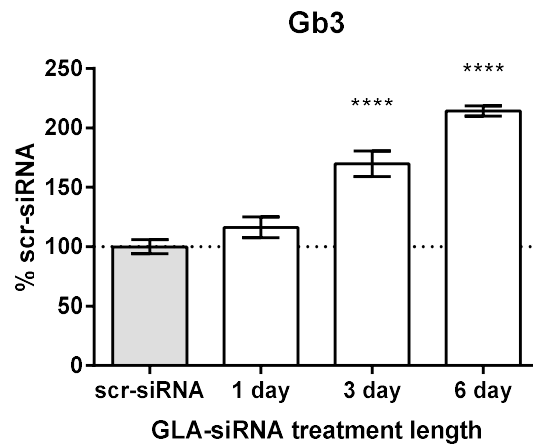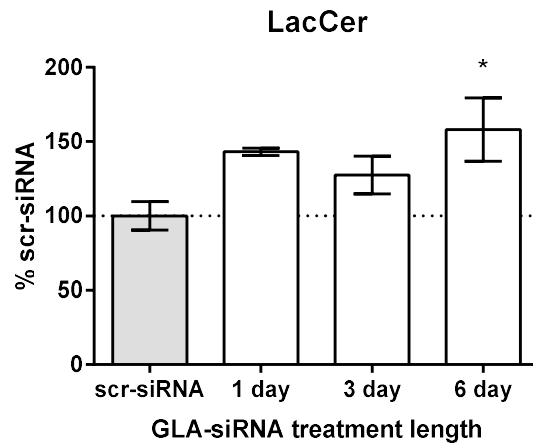
Figure 2.5: siRNA-GLA knockdown increases LacCer and Gb3.
EA.hy926 cells were treated with scrambled siRNA (scr-siRNA) control or anti-GLA siRNA for up to 6 days. Lipids were extracted for TLC analysis. TLC plates were charred with 8% (wt/vol) cupric sulfate pentahydrate in water/methanol/H_3PO_4 (60:32:8, v/v/v). There is a time-dependent accumulation of Gb3. n=3-9 ****p<0.0001, *P<0.05 when compared to scr-siRNA control.
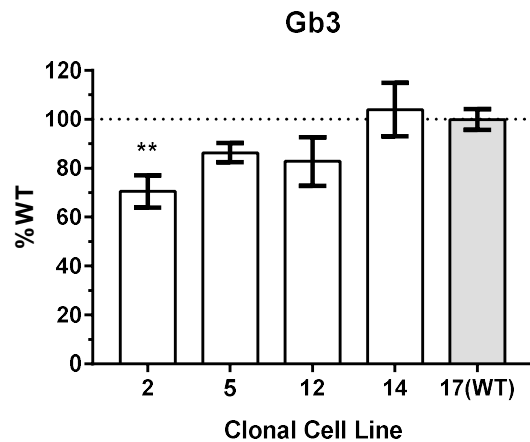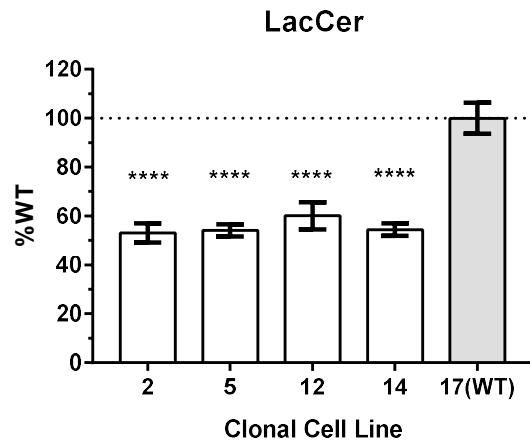
Figure 2.6: CRISPR/Cas9-gene edited GLA(-) cells do not accumulate LacCer or Gb3. GLA(-) clonal cell lines were grown to confluency, and lipids were extracted for TLC analysis. TLC plates were charred with 8% (wt/vol) cupric sulfate pentahydrate in water/methanol/H_3PO_4 (60:32:8, v/v/v). GLA-deficient cells do not have more Gb3 than the WT control. Clone 2 has less Gb3 than WT. All GLA-deficient Clonal cells have less LacCer than WT. n=4-12 ****p<0.0001, **p<0.01 when compared to WT control.

GLA deficient cells accumulate Gb4

In light of these surprising results, we hypothesized that other neutral GSLs may be accumulating in response to deficient GLA activity. In addition, we observed that the band at the bottom of the TLC plate exhibited noticeable differences between WT and GLA(-) samples. Specifically, the GLA(-) samples had a band with a higher Rf value that was very faint or absent in samples from clone 17 (WT) cells (fig. 2.7, top). Based on this mobility, we hypothesized that this band may represent the downstream globo series GSL, globotetraosylceramide (Gb4). Because of similar properties to sphingomyelin, with respect to polarity, Gb4 cannot be clearly separated on the TLC plate in order to quantify it. Therefore, instead of charring the TLC plate with cupric sulfate solution, the TLC plate was stained with 1% orcinol (w/v) solution in 11% H_2SO_4 (v/v) to detect only carbohydrate moieties. This staining solution was used for the remaining studies to measure levels of neutral GSLs in cell lysates. Representative TLC plates with both staining methods are shown in figure 2.7.

Cell lysates from GLA-deficient cells have a 2.5 to 4 fold higher amount of Gb4 content over the WT cell line (fig. 2.9). siRNA-GLA knockdown also increases Gb4, but not to the same extent (fig. 2.8).
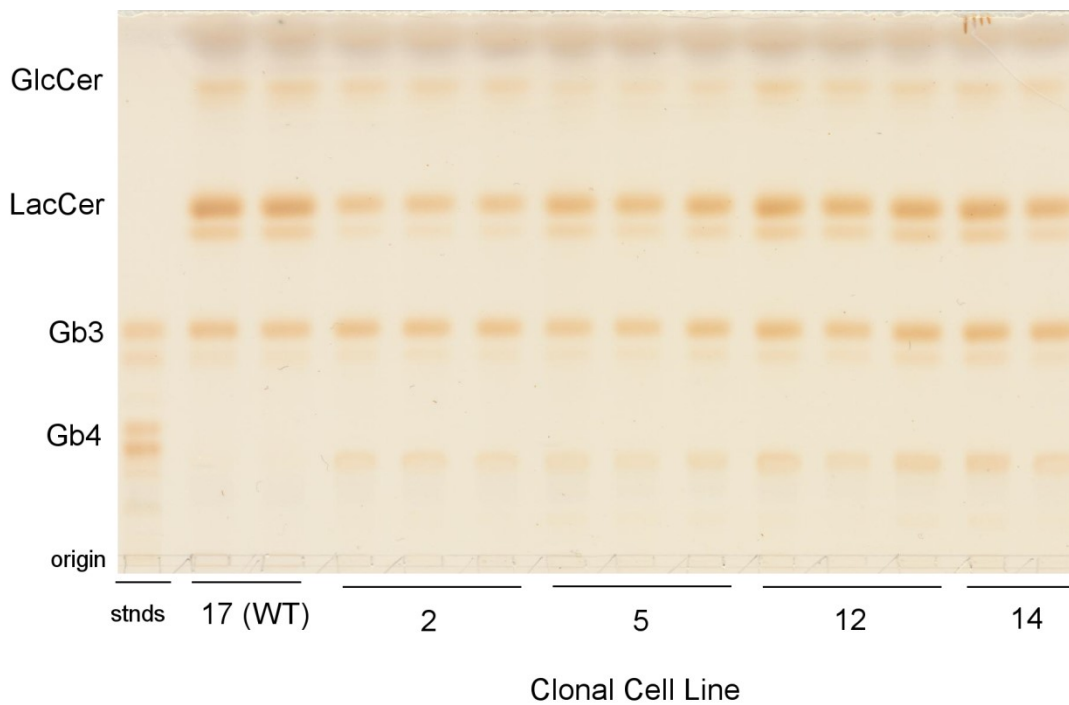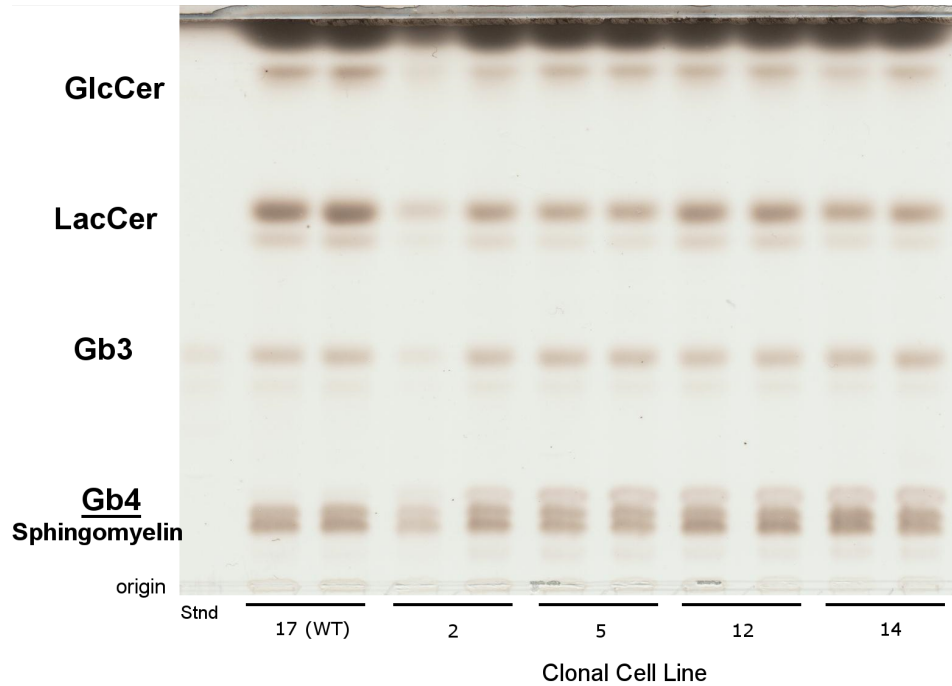
Figure 2.7: Neutral GSLs are analyzed by TLC. *Top*: A representative TLC plate charred with 8% (wt/vol) cupric sulfate pentahydrate in water/methanol/H$_3$PO$_4$ (60:32:8, v/v/v) is shown. GLA-deficient cells exhibit changes in lipids near the bottom of the plate, which is most likely to be Gb4. *Bottom*: A representative TLC plate charred with 1% (wt/vol) orcinol in 11% (v/v) H$_2$SO$_4$ is shown. GLA-deficient cells have more Gb4 content than the WT control.
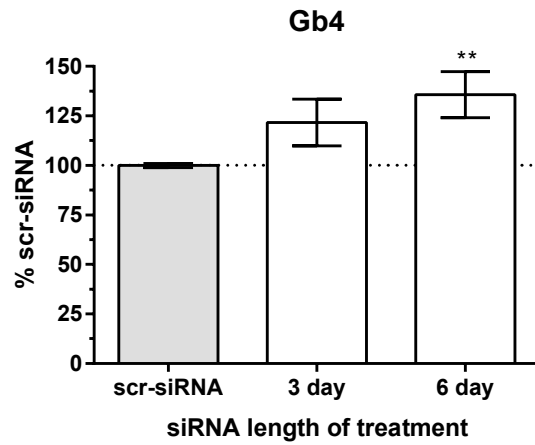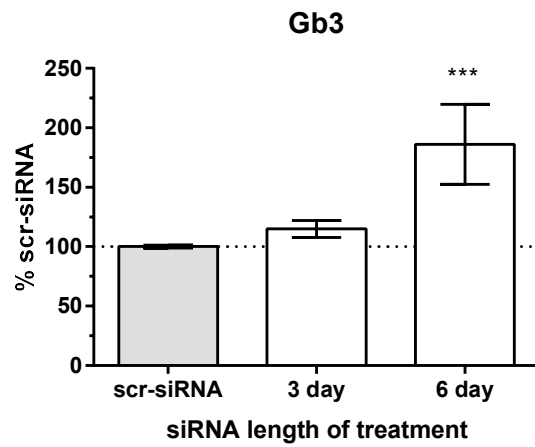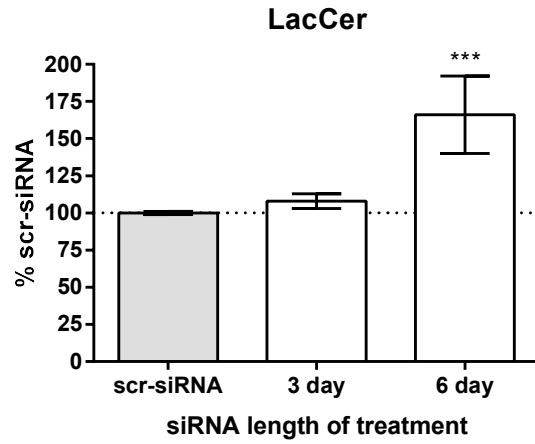
Figure 2.8: GLA knockdown causes lipid accumulation. EA.hy926 cells were treated with scrambled siRNA (scr-siRNA) control or anti-GLA siRNA for up to 6 days. Lipids were extracted for TLC analysis. TLC plates were charred with 1% (wt/vol) orcinol in 11% (v/v) H_2SO_4. There is a time-dependent accumulation of LacCer, Gb3, and Gb4. n=10 ***p<0.001, **p<0.01, when compared to scr-siRNA control (100%, dotted line).
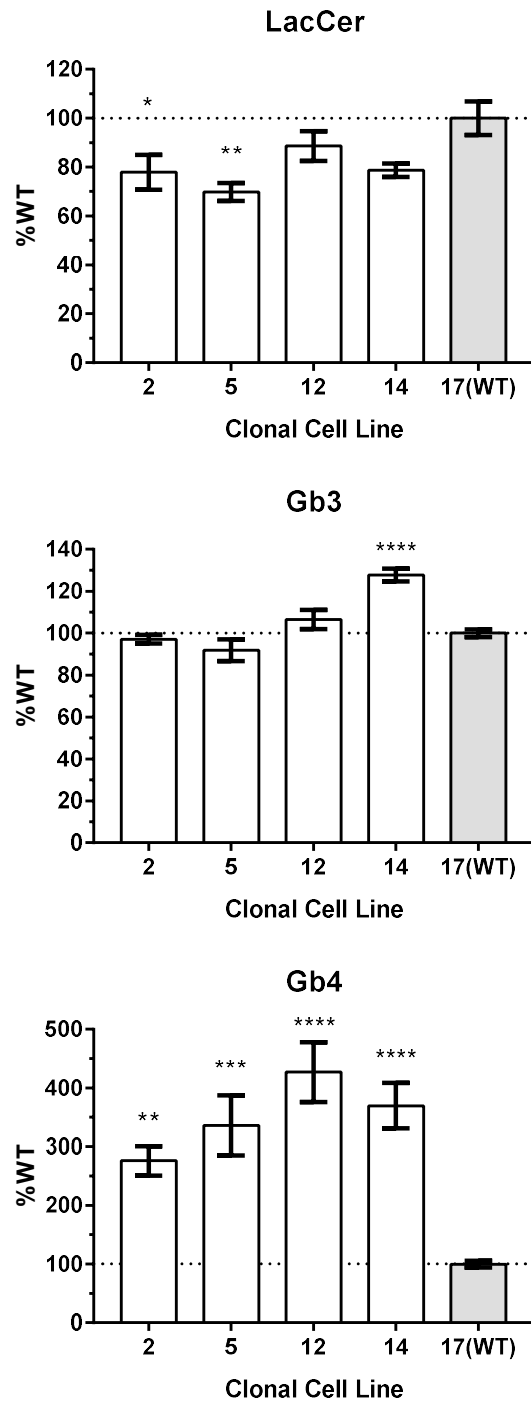
Figure 2.9: GLA(-) cells (CRISPR/Cas9 gene edited) accumulate Gb4
CRISPR/Cas9 clonal cell lines were grown to confluency, and lipids were extracted for TLC analysis. TLC plates were charred with 1% (wt/vol) orcinol in 11% (v/v) H_2SO_4 for detection of carbohydrate groups. All GLA-deficient clonal cells have more Gb4 than WT (100%, dotted line). n=10 ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Exogenous GLA and eliglustat treatments decrease neutral GSLs

Previously, it was shown that two different pharmacological methods, treatment with recombinant GLA (enzyme replacement) or an inhibitor of glucosylceramide synthase (substrate deprivation), can lower Gb3 content (Shu, Murphy et al. 2005, Shu and Shayman 2007). Although Gb3 is not increased in the CRISPR/Cas9 GLA-deficient cells, it remained to be determined whether Gb4 content is affected by these treatments. Cells were treated with exogenous GLA (3 U) or eliglustat (20 or 200 nM) in cell culture media for 3 days. Lipids were extracted and analyzed by TLC and GSLs were visualized by charring with orcinol-sulfate solution. Both treatment methods decreased levels of LacCer and Gb3 in all cells. Gb4 was only lowered in GLA-deficient cells. GLA treatment resulted in comparable levels of LacCer and Gb3 in GLA-deficient and WT cells, but not Gb4 (fig. 2.10). In contrast, 20 nM eliglustat treatment did not affect Gb3 levels compared to the vehicle control for each cell line, but decreased LacCer and Gb4. The 200 nM treatment more severely reduced Gb4 content in GLA-deficient cells (fig. 2.11).
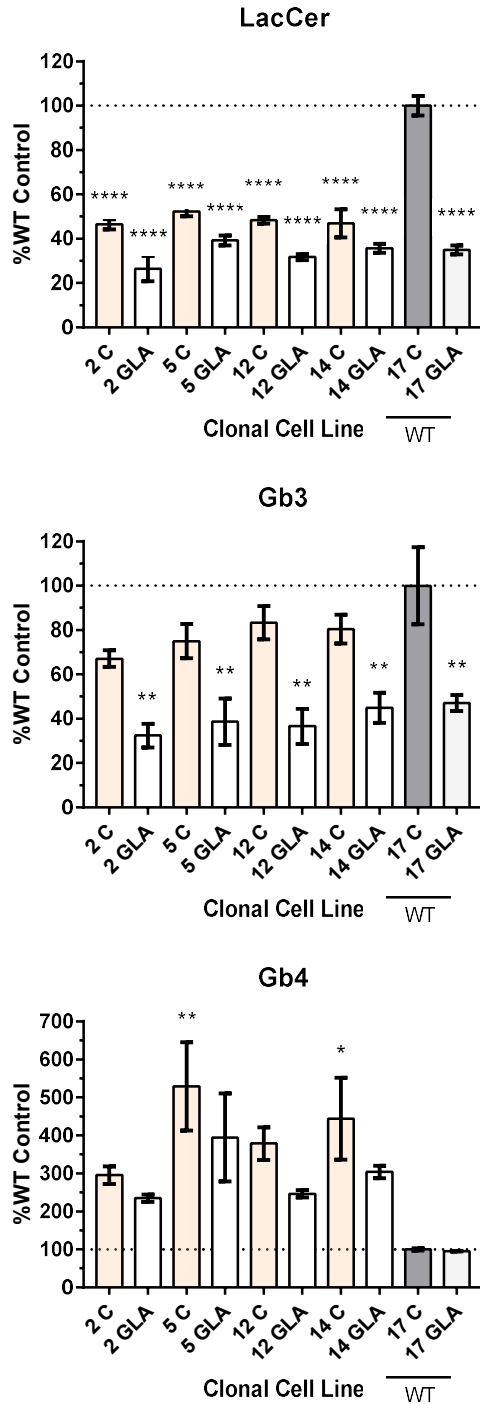
Figure 2.10: Treatment with GLA decreases globo-series GSLs.
CRISPR/Cas9 clonal cell lines were treated with exogenous α-galactosidase A
(empty bars) or vehicle (shaded bars) for 3 days and lipids were extracted for
TLC analysis. TLC plates were charred with 1% (wt/vol) orcinol in 11% (v/v)
H_2SO_4 for detection of carbohydrate groups. Dotted line represents 100% of WT
control treatment. Values presented as % of WT control (dark gray bars).
Significance is indicated in comparison to 17(WT) control. n=2 ****P<0.0001,
**P<0.01, *P<0.05.
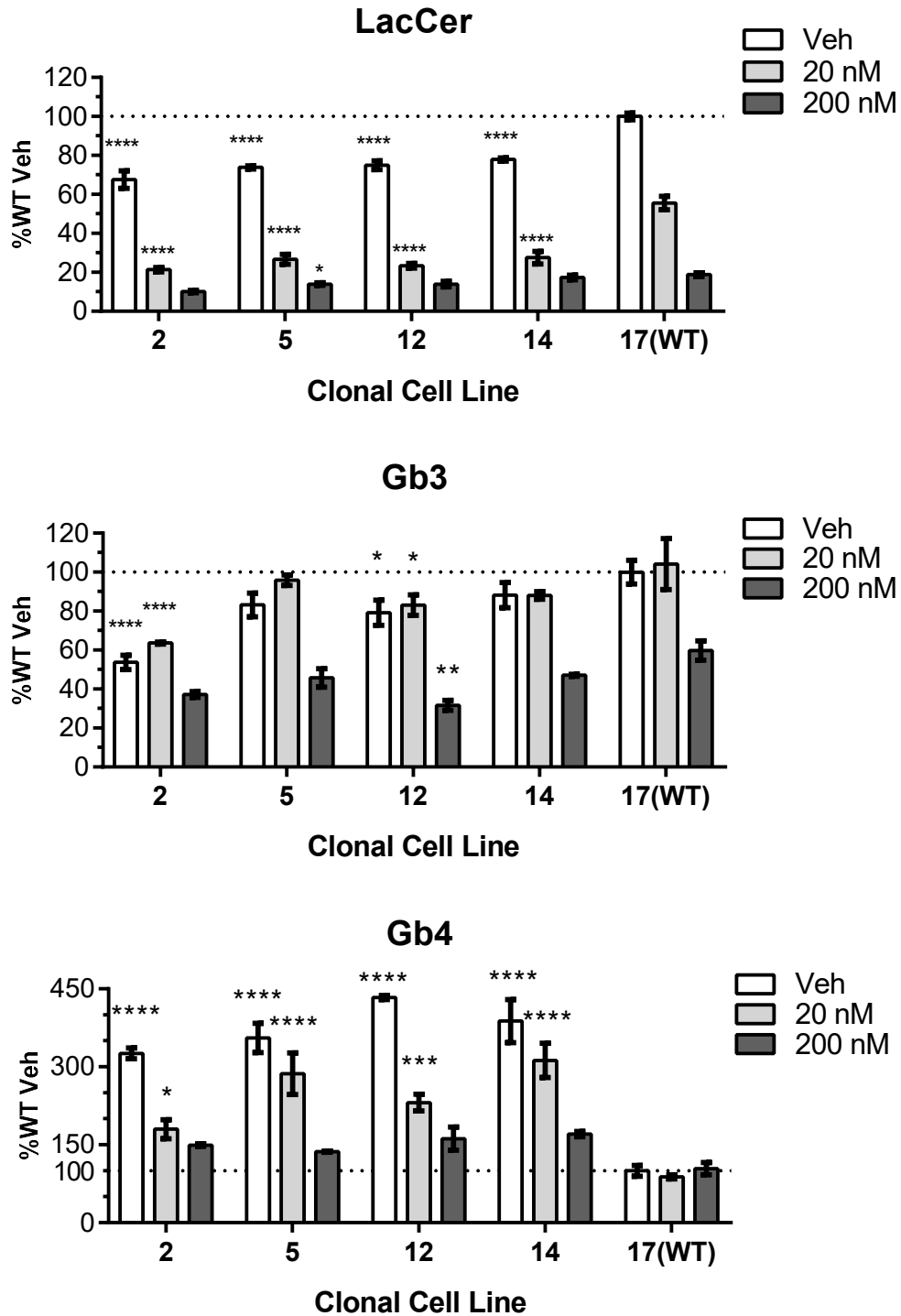
Figure 2.11: Treatment with eliglustat decreases globo series GSLs.
LacCer, Gb3, and Gb4 content of cells treated with vehicle, 20 nM, or 200 nM eliglustat for 3 days. Values presented as %WT - vehicle (empty bars). Dotted line represents 100% of WT control treatment. Significance is indicated in comparison to 17(WT) of each treatment. n=3 ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Specificity of exogenous GLA for Gb3 catabolism

To confirm the specificity of GLA for Gb3, extracted lipids (100 nmol of total lipid phosphate) were incubated with GLA overnight at 37 °C. The reaction was stopped, and samples were washed to obtain neutral GSLs and developed by TLC. The most prominent changes are seen with Gb3 content. A representative TLC plate is shown (fig. 2.12) to exhibit how the reaction mixture contaminates the samples with substances that run alongside LacCer, but do not interfere with Gb3 measurement. GLA digest specifically decreases Gb3 content in lipid samples (fig. 2.13). These results also confirm the identity of the lipid that labeled as "Gb3" on the TLC plate.

Figure 2.12: GLA specifically degrades Gb3
Cells were grown to confluency and lipids extracted. 100 nmol aliquots of lipids were incubated overnight with either α-galactosidase A (GLA) or vehicle control (V), and developed on a TLC plate, then charred with 1% (wt/vol) orcinol in 11% (v/v) H_2SO_4. Reagents in the reaction mixture do not interfere with quantification of Gb3.
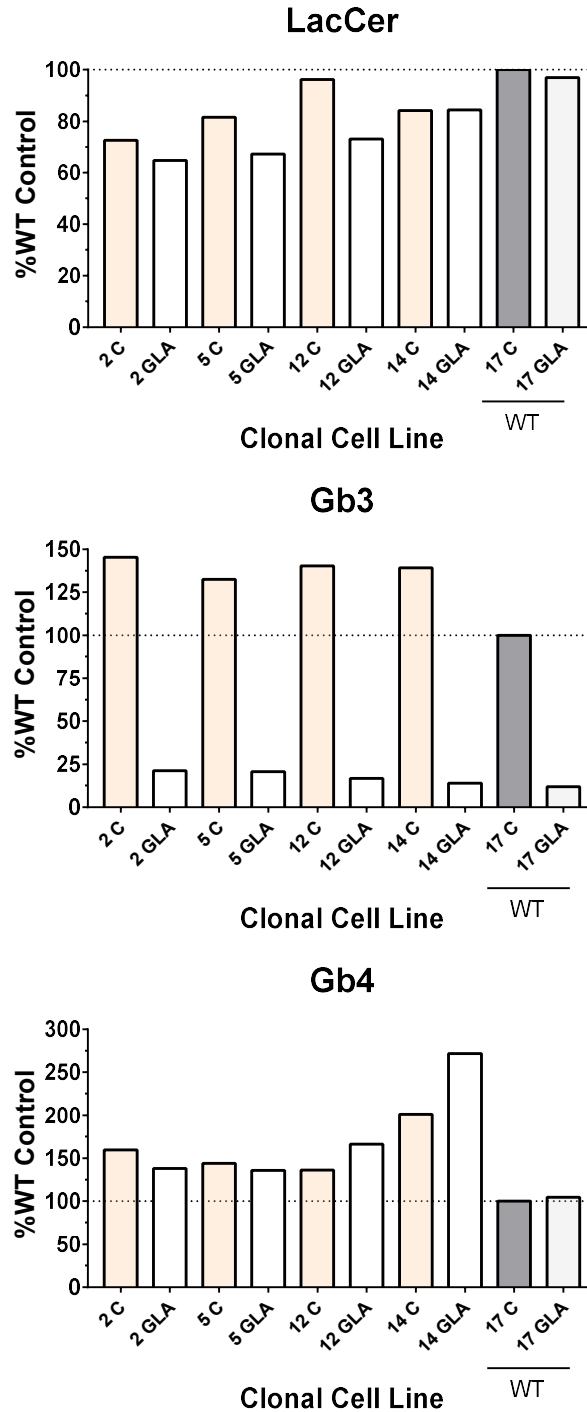
Figure 2.13: Incubation of extracted lipids with GLA decreases Gb3 content. Cells were grown to confluency and lipids extracted. 100 nmol aliquots of lipids were incubated overnight with either α-galactosidase A (GLA, empty bars) or vehicle control (shaded bars), and developed on a TLC plate, then charred with 1% (wt/vol) orcinol in 11% (v/v) H_2SO_4. GLA treatment specifically decreases Gb3 content in lipid samples. Dotted line indicates 100% clone 17(WT) control (gray bar). n=1

Elevated lyso-Gb3 in GLA-siRNA knockdown and CRISPR/Cas9 GLA(-) cells

EA.hy926 cells were treated with siRNA against GLA or with scr-siRNA control for 3 or 6 days, or CRISPR/Cas9 *Gla*-edited cells were grown to confluency on 100 mm dishes. All cells were harvested and sonicated in PBS, and lyso-Gb3 was measured with UPLC-MS[6] (Gold, Mirzaian et al. 2013). Cell lysates from CRISPR/Cas9 GLA(-) clonal cell lines had a 3 to 4-fold increase of lyso-Gb3 compared to WT control (fig. 2.14). Similarly, 6-day treatment of anti-GLA siRNA in EA.hy926 cells caused a significant increase of lyso-Gb3 compared to scr-siRNA control and 3-day anti-GLA siRNA treated cells. In contrast with LacCer, Gb3, and Gb4 measurements between the siRNA and CRISPR/Cas9 models of GLA-deficiency, lyso-Gb3 is a marker that is common between the two models. This corroborates clinical studies reporting that lyso-Gb3 is a more reliable marker for Fabry disease than is Gb3 (Vedder, Linthorst et al. 2007, Smid, van der Tol et al. 2015).

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[6] UPLC-MS was performed by the Aerts group at Academic Medical Centre, University of Amsterdam, Netherlands.
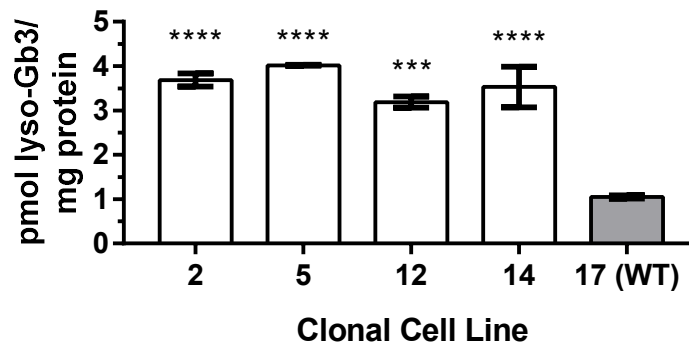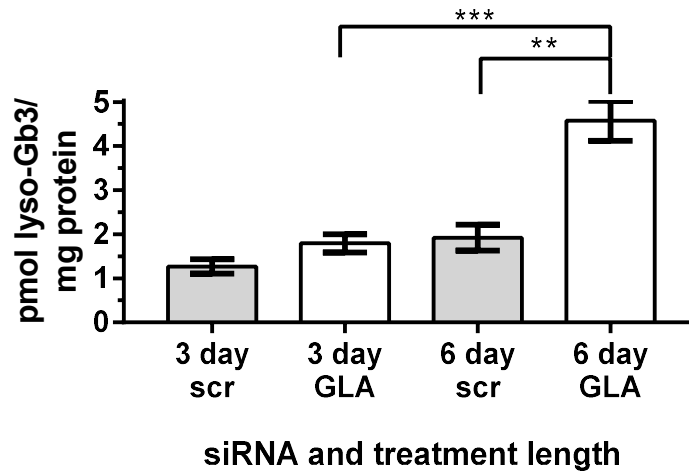
Figure 2.14: Elevated lyso-Gb3 in GLA deficient cell lysates.
Lyso-Gb3 is elevated in 6-day anti-GLA siRNA treated cells compared to 3-day anti-GLA siRNA treatment and 6-day scrambled-siRNA (scr) treatment. Lyso-Gb3 is elevated in CRISPR/Cas9 GLA-deficient cells compared to clone 17 (WT). n=3. ****$P<0.0001$, ***$P<0.001$, **$P<0.01$.

Decreased NO production and eNOS expression in GLA deficient cells

The CRISPR/Cas9-EA.hy926 GLA deficient cell model is the only *in vitro* model that we are aware of that has been reported to not have a consistent increase in Gb3 content. However, consistent with previous models of GLA deficiency, GLA-deficient cells had decreased NO production (fig. 2.15). Three of the four GLA(-) cell lines had 40-50% of NOS activity compared to WT cells. One GLA(-) cell line, clone 2, had a 14% reduction

of NOS activity compared to WT. To verify [^{3}H]L-citrulline that was measured in the

NOS activity assay originated from NOS activity, reactions were repeated with the

addition of a competitive NOS inhibitor, N^G-nitro-L-arginine (L-NNA) to the NOS reaction

mixture. There was no detectable [^{3}H]L-citrulline above background with L-NNA (data

not shown).

A possible reason for decreased NOS activity is reduced eNOS expression. A Western

blot was performed to detect eNOS expression in WT and GLA deficient cells (fig. 2.16).

Although there is a trend of decreased eNOS expression in GLA-deficient cells that

correlates with decreased NOS activity, the change was not statistically significant

(p=0.08 for clone 12 compared with 17(WT)).
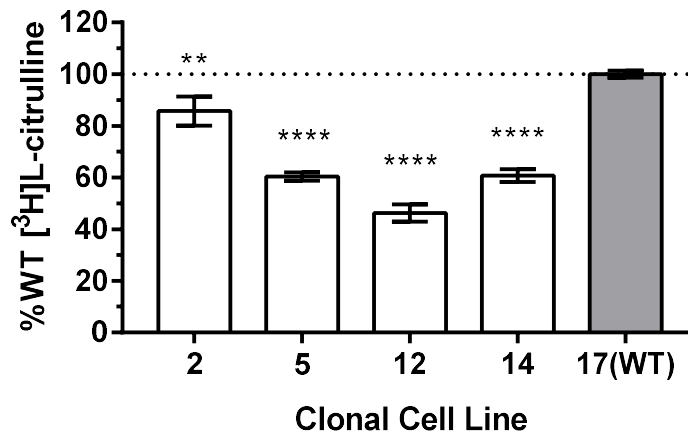
Figure 2.15: GLA(-) cells have less NOS activity. NOS activity was measured
in cell lysates from clonal cell lines. NO production was significantly lower in
GLA(-) cell lines, 5, 12, and 14, when compared to the WT control 17. n=18
**P<0.01, ****P<0.0001 compared to 17(WT).
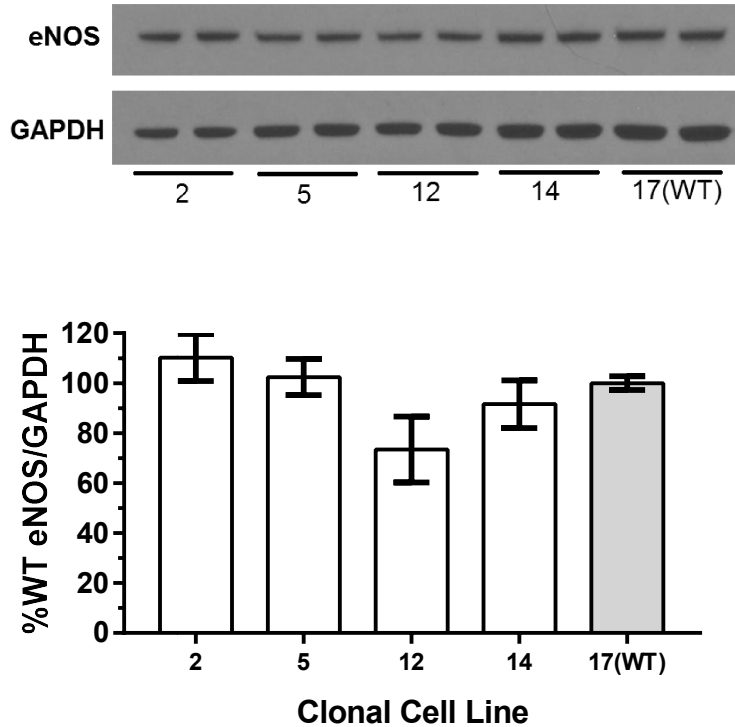
Figure 2.16. GLA(-) cells have less eNOS protein expression.
Thirty µg of protein was separated on 4-12% SDS-PAGE, transferred to nitrocellulose membranes, and blotted for eNOS and GAPDH. *Top:* representative Western blot from CRISPR/Cas9 clonal cell lines. Clone 17 is the WT control. *Bottom:* Quantification of Western blots. GLA-deficiency causes a decrease in eNOS expression in clone 12, but is not statistically significant (p=0.08). Clone 2, n=3; clones 5, 12, and 14, n=4; clone 17(WT), n=6.

Discussion

In the United States, the only therapeutic to treat the underlying cause of Fabry disease

is enzyme replacement therapy (ERT) with recombinant GLA (Eng, Banikazemi et al.

2001, Eng, Guffon et al. 2001). Based on clinical trials, ERT is the standard of care as it

has been shown to clear Gb3 from kidney cells (Thurberg, Rennke et al. 2002) and

reduce renal, cardiac, and CNS events (Banikazemi, Bultas et al. 2007). However ERT

has limitations in improving some clinical manifestations of Fabry disease, including

cardiovascular outcomes (Hopkin, Cabrera et al. 2016). The following are a few reasons why the benefit of ERT may be limited. First, infusions are administered on a weekly or bi-weekly basis, which may be insufficient for Fabry patients to have normal, consistent, GLA activity (Schiffmann, Askari et al. 2007). Second, patients may develop an immune reaction to the infused enzyme, which can decrease the efficacy (Linthorst, Hollak et al. 2004, Rombach, Aerts et al. 2012). Third, when ERT is started after there has been organ damage, ERT does not halt disease progression (Cabrera, Politei et al. 2017).

Considering the high cost of ERT ($200,000 per year for life), there is a need for a good biomarker of Fabry disease. A biomarker would be useful for predicting who may benefit from ERT or if ERT is effective for a particular Fabry patient. Although Gb3 is the primary substrate of GLA, no strict correlation exists between plasma Gb3 levels and clinical manifestations of Fabry disease. In hemizygotes, Gb3 accumulation begins *in utero*, before clinical symptoms, whereas heterozygotes may have normal Gb3 levels with or without clinical symptoms. In addition, Gb3 is not a good biomarker for monitoring the efficacy of ERT unless Gb3 is elevated prior to initiation of treatment (Young, Mills et al. 2005, Vedder, Strijland et al. 2006, Vedder, Linthorst et al. 2007).

Lyso-Gb3, a deacylated form of Gb3, has emerged as a biomarker for Fabry disease (Aerts, Groener et al. 2008). Lyso-Gb3 is elevated in plasma from Fabry patients and in GLA-deficient mice. The level of lyso-Gb3 in plasma decreases after initiation of ERT. Furthermore, lyso-Gb3 is useful for monitoring Fabry disease in females. Heterozygous females have normal circulating levels of GLA and Gb3, but may still experience symptoms of the disease. Symptomatic heterozygous females have elevated plasma lyso-Gb3, while lyso-Gb3 cannot be detected in asymptomatic heterozygous females

(Aerts, Groener et al. 2008, Rombach, Dekker et al. 2010, Togawa, Kodama et al. 2010, Smid, van der Tol et al. 2015).

Lyso-lipids found to be elevated in other lipid storage diseases, for example, Krabbe disease (galactocerebroside), and Gaucher disease (glucosylsphingosine), have been shown to have toxic effects (Tanaka and Webster 1993, Schueler, Kolter et al. 2003). Lyso-Gb3 has also been shown to be involved in the pathogenesis of Fabry disease. Lyso-Gb3, but not Gb3 or lactosylsphingosine, promotes smooth muscle cell proliferation, which may contribute to the increased intima-media thickening seen in patients (Aerts, Groener et al. 2008). Lyso-Gb3 induces podocyte injury, which is an early feature of Fabry nephropathy (Sanchez-Nino, Sanz et al. 2011).

In this study, we have shown that contrary to other *in vitro* models of Fabry disease, CRISPR/Cas9 – induced GLA(-) cells do not accumulate Gb3 (figs. 2.6 and 2.9), the primary substrate for GLA. However, there is consistency with other models and data from Fabry patients regarding elevated lyso-Gb3 (Aerts, Groener et al. 2008, Gold, Mirzaian et al. 2013, Smid, van der Tol et al. 2015). GLA(-) EA.hy926 cells, either CRISPR/Cas9 or siRNA – mediated, exhibit an increase of lyso-Gb3 (fig. 2.14). Experiments in chapter 3 will address the direct effect of lyso-Gb3 on eNOS uncoupling.

We chose the CRISPR/Cas9 method of gene editing to obtain permanent cell lines that are GLA deficient. Previously, the Shayman group used primary cells isolated from mouse aortas of *Gla* knockout mice (mouse aortic endothelial cells, MAECs), which were useful for characterizing lipid changes and effectiveness of GlcCer synthase inhibition for decreasing accumulating Gb3 (Shu, Murphy et al. 2005). However, this model was not sustainable for studying long-term consequences of treating cells with

different agents since these cells could only be maintained in cell culture for a limited time (3 to 6 days). The anti-GLA siRNA EA.hy926 cell model was useful for making initial observations of how GLA-deficiency affects NOS activity, even when GLA was absent for only a few days, and for identifying 3-nitrotyrosine (a marker of eNOS uncoupling (Heinecke 2002)) as a biomarker for GLA deficiency (Shu, Vivekanandan-Giri et al. 2014). However, similar to MAECs, this *in vitro* model is not sustainable because a single siRNA treatment lasts for only 3 days. There can also be off-target effects of the siRNA.

One advantage of the CRISPR/Cas9 GLA(-) cell model is that they can be continually passaged and retain the GLA(-) characteristic with no additional treatment. To account for off-target effects of the initial CRISPR/Cas9 transfection, multiple single cell colonies were isolated, grown, and analyzed. In this study, lyso-Gb3 is the most consistent marker of GLA deficiency. While there was variability of measurement of Gb3, Gb4, and NOS activity (figs. 2.6, 2.9, and 2.15), the lyso-Gb3 levels were consistently elevated in all four GLA(-) clonal cell lines (fig. 2.14). GLA(-) clonal cell lines isolated from treatment with a CRISPR/Cas9 vector targeting exon 2 of *Gla* also have variability in the lipid measurements (figs. A.3, A.4) and NOS activity (fig A.5).

As described above, ERT with recombinant GLA is currently the only approved treatment for Fabry disease in the United States. Substrate deprivation is an alternative approach to reducing accumulation of globo-series GSLs; using a small molecule inhibitor of glucosylceramide (GlcCer) synthase prevents the *de novo* synthesis of downstream GSLs, including LacCer, Gb3, and Gb4 (Abe, Arend et al. 2000, Abe, Gregory et al. 2000). Experiments performed in an *in vitro* model of Fabry disease using

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recombinant GLA and GlcCer synthase inhibitors have indicated that both pharmacological methods can reduce the accumulation of Gb3 in GLA-deficient cells (Shu, Murphy et al. 2005). Similarly, in our study, treating GLA(-) EA.hy926 cells with exogenous GLA or the GlcCer inhibitor, eliglustat, decreases the accumulation of globo-series GSLs (figs 2.10 and 2.11).

A hypothesis for the vasculopathy of Fabry disease is that GLA deficiency causes changes to the plasma membrane. A previous study of mouse aortic endothelial cells reported that although GLA is a lysosomal enzyme, the primary substrate, Gb3, accumulates in the lysosome and in the plasma membrane (Shu and Shayman 2007). In the present study, accumulation of GSLs was not examined in the caveolar membrane, however it is possible that Gb3 (or Gb4) is accumulating at the plasma membrane but total cell Gb3 is unchanged. The plasma membrane is the site of eNOS activity. eNOS uncoupling is characterized by the production of superoxide instead of nitric oxide (NO), and occurs when electrons from NADPH in the reductase domain of eNOS flow to molecular oxygen rather than to the oxygenase domain to reduce L-arginine to L-citrulline with the NO byproduct (fig. 1.3). The reduction of molecular oxygen forms superoxide (Vasquez-Vivar, Kalyanaraman et al. 1998, Xia, Tsai et al. 1998), which reacts with NO to produce peroxynitrite ($ONOO^-$). $ONOO^-$ is a reactive nitrogen species that can oxidize the eNOS cofactor, tetrahydrobiopterin (BH4) to the inactive form, dihydrobiopterin (BH2) (Milstien and Katusic 1999).

A biomarker for the presence of $ONOO^-$, 3-nitrotyrosine, is derived from protein nitrosylation (Heinecke 2002). A previous study of Fabry disease showed that 3-nitrotyrosine is elevated in the siRNA-EA.hy926 GLA(-) and *Gla*-knockout mouse

models, as well as in plasma from Fabry patients (Shu, Vivekanandan-Giri et al. 2014). In the current study, NOS activity was shown to be decreased in GLA(-) cells. There is a decrease in eNOS protein expression, but the difference did not reach statistical significance (fig. 2.15). Although 3-nitrotyrosine was not measured in this study, to determine if GLA(-) cells had increased oxidative stress, total glutathione was measured, and found to be decreased compared to WT, indicating the cells had increased oxidative stress (fig A.1). A recent study (Shen, Arning et al. 2017) observed a decrease of BH4 in the heart and kidney of Fabry mice, and an inverse correlation between Gb3 and BH4 levels in tissues. Glutathione levels in Fabry mouse tissues were also found to be decreased compared to WT. BH4 availability in our CRISPR/Cas9 GLA(-) cells will be addressed in chapter 3.

eNOS uncoupling is a characteristic of common vascular diseases that arise from a variety of genetic or environmental factors, like hypercholesterolemia (Ohara, Peterson et al. 1993), diabetes (Hink, Li et al. 2001), atherosclerosis (White, Brock et al. 1994), and hypertension (Panza, Garcia et al. 1995). Assessing the cause of eNOS uncoupling in a rare, monogenic disorder provides a window of opportunity to uncover novel mechanisms of eNOS regulation that would not be possible in a disease model with multiple variables.

In summary, GLA-deficient EA.hy926 cells were obtained using CRISPR/Cas9 gene editing. Although these cells do not accumulate the primary substrate of GLA, Gb3, they exhibit decreased NOS activity, and have elevated lyso-Gb3. Lyso-Gb3 is better than Gb3 for correlating GLA deficiency with eNOS uncoupling. The CRISPR/Cas9 GLA(-) cells can be used to further assess how GLA deficiency leads to eNOS uncoupling.

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Chapter 3: Mechanisms of eNOS uncoupling in GLA-deficient cells

Abstract

Fabry disease arises from mutations in *Gla,* resulting in deficiency of the lysosomal hydrolase, α-galactosidase A (GLA). A prominent feature of Fabry disease is vascular dysfunction. One of the effects of GLA deficiency is endothelial nitric oxide synthase (eNOS) uncoupling, where eNOS produces superoxide instead of the important messenger, nitric oxide (NO). In the previous chapter, CRISPR/Cas9 gene editing was used to eliminate GLA expression in EA.hy926 endothelial cells as a model to investigate the link between GLA deficiency and eNOS uncoupling in Fabry disease. Experiments performed in this chapter used two different methods of reducing neutral glycosphingolipid content to indicate that GLA function, and not necessarily elevated GSLs, leads to eNOS uncoupling in Fabry disease. It is also reported that eNOS activity was improved by increasing the availability of tetrahydrobiopterin (BH4), an important cofactor of eNOS. Furthermore, globotriaosylsphingosine (lyso-Gb3), which is elevated in GLA-deficient cells, inhibited NOS activity in cell lysates.

Introduction

Fabry disease is caused by deficiency of the lysosomal hydrolase, α-galactosidase A (GLA). The primary substrate for GLA is the globo-series glycosphingolipid (GSL),

globotriaosylceramide (Gb3). Loss of GLA function results in accumulation of neutral GSLs in various cells and tissues (Brady, Gal et al. 1967). This is a rare, X-linked, disease, but is often not diagnosed until later in life due to symptoms being attributed to other causes (Eng, Fletcher et al. 2007).

Complications of the disease include kidney failure, neuropathy, and cardiovascular disease. Cardiovascular events, like myocardial infarction and stroke, are often the cause of premature death in Fabry patients. The only approved therapy for Fabry disease is enzyme replacement therapy (ERT). ERT has been shown to clear Gb3 from various cells and tissues. However, unless ERT is initiated early in life, before any major cardiovascular event, it does not prevent the occurrence of cardiovascular events (Hopkin, Cabrera et al. 2016).

An alternative approach to reducing accumulation of globo-series GSLs is substrate deprivation, where a small molecule is used to inhibit GlcCer, which prevents the *de novo* synthesis of the downstream GSLs, including Gb3. Eliglustat tartrate (Cerdelga®, Genzyme) has been approved for clinical use to treat Gaucher disease, another lysosomal storage disease. However, proof-of-concept studies for these inhibitors were performed in the mouse model for Fabry disease (Abe, Arend et al. 2000, Abe, Gregory et al. 2000). Experiments performed in an *in vitro* model of Fabry disease using recombinant GLA and GlcCer synthase inhibitors have indicated that both pharmacological methods can reduce the accumulation of Gb3 in GLA-deficient cells (Shu, Murphy et al. 2005).

Several experimental models of Fabry disease have suggested that endothelial nitric oxide synthase (eNOS) dysfunction is the basis for vasculopathy that occurs in many

patients with Fabry disease (Eitzman, Bodary et al. 2003, Bodary, Shen et al. 2005, Park, Whitesall et al. 2008, Shu, Park et al. 2009, Shu, Vivekanandan-Giri et al. 2014). eNOS uncoupling occurs when there is improper flow of electrons from the reductase domain of eNOS, to the oxidase domain. Normally, NADPH-derived electrons are transferred from the reductase to the oxygenase domain to oxidize L-arginine to NO and L-citrulline. In the case of eNOS uncoupling, however, the electrons are not transferred to L-arginine and are instead transferred to O_2, forming O_2^- instead of NO (Vasquez-Vivar, Kalyanaraman et al. 1998, Xia, Tsai et al. 1998). O_2^- can then react with NO to produce a reactive nitrogen species, peroxynitrite ($ONOO^-$). $ONOO^-$ can then rapidly oxidize tetrahydrobiopterin (BH4) to the inactive form, dihydrobiopterin (BH2), which causes further uncoupling (Milstien and Katusic 1999). A biomarker for $ONOO^-$ is 3-nitrotyrosine, which results from protein nitrosylation (Heinecke 2002).

eNOS dysfunction has been extensively studied in more common diseases that have a cardiovascular dysfunction component. In diabetic mice, aortic expression of eNOS is increased, but NO availability is reduced. Inhibiting eNOS reduces superoxide levels in vessels from diabetic mice, but in healthy mice, increases superoxide levels (Hink, Li et al. 2001). The major cause of eNOS uncoupling in diseases with cardiovascular risk factors is BH4 deficiency due to increased oxidant stress. Therefore, methods to prevent eNOS uncoupling often target the pathways that regenerate BH4 (Li and Forstermann 2014).

BH4 was discovered to be important for the NOS reaction by Stuehr and colleagues (Kwon, Nathan et al. 1989), and Tayeh and Marletta (Tayeh and Marletta 1989). BH4 is a key intermediate in the electron transfer between the reductase and oxygenase

domains of eNOS. BH4 was first found to stabilize the neuronal NOS (nNOS) homodimer (Klatt, Schmidt et al. 1995), and later the eNOS homodimer (Rodriguez-Crespo, Gerber et al. 1996).

BH4 is generated by two pathways (schematic fig 1.4). The *de novo* pathway converts GTP to BH4 via two intermediates. The rate-limiting step is GTP cyclohydrolase I (GTPCH), which catalyzes the first step in this pathway. GTPCH is regulated at the transcriptional and post-transcriptional levels. Sepiapterin inhibits GTPCH (Gross and Levi 1992) and is a substrate for the salvage pathway.

The salvage pathway regenerates BH4 from the oxidized form, BH2. Sepiapterin reductase produces BH2 from sepiapterin. Then dihydrofolate reductase (DHFR) reduces BH2 to BH4 (Nichol, Lee et al. 1983). An increase of superoxide in endothelial cells causes a down-regulation of DHFR expression and BH4 levels, resulting in uncoupling of eNOS, which further generates superoxide. Increasing DHFR expression restores eNOS coupling (Chalupsky and Cai 2005). Methotrexate inhibits DHFR activity

BH4 availability is reduced when it is oxidized, which happens in the presence of peroxynitrite. Peroxynitrite is formed by the reaction of superoxide and NO. Low levels of peroxynitrite can oxidize physiologically relevant levels of BH4 (Milstien and Katusic 1999); impaired vasorelaxation in aortic rings from *apoE$^{-/-}$* mice can be overcome by treatment with the peroxynitrite scavenger, uric acid (Laursen, Somers et al. 2001).

In the following study, decreased NOS activity in GLA-deficient cells is improved by treating cells with exogenous GLA or with sepiapterin. In contrast, NOS activity is

reduced when treating with eliglustat or methotrexate. Lyso-Gb3 is also found to inhibit NOS activity.

Materials and Methods

Cell culture

EA.hy926 cells (ATCC, Manassas, VA) were maintained at 37 °C with 95/5 O_2/CO_2, in DMEM/F-12, GlutaMAX (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (source), which will be later referred to as "growth medium." CRISPR/Cas9 gene editing was used to create 4 clonal cell lines (2, 5, 12, 14), originating from single cell colonies, and that do not express GLA (described in detail in Chapter 2). One clone (17) does not have a mutation in *Gla*, expresses GLA, and is used as the wild type (WT) control in these studies.

NOS activity

A commercially available NOS assay kit (Cayman Chemical, Ann Arbor, MI) was used according to the manufacturer's instructions. This assay measures the biochemical conversion of L-arginine to L-citrulline by NOS. Harvested cell pellets were briefly sonicated on ice, centrifuged at 12,000 x g for 5 minutes, and supernatants transferred to new tubes. Cell lysates were incubated at room temperature for 3 hours with 1 µCi [^{3}H]L-arginine (PerkinElmer), 100 nM calmodulin, and the provided reaction buffer (final concentration 1 mM NADPH, 600 µM $CaCl_2$, 25 mM Tris-HCl (pH 7.4), 3 µM tetrahydrobiopterin, 1 µM flavin adenine dinucleotide, and 1 µM flavin adenine mononucleotide). The reaction was stopped by adding 5 mM EDTA in 50 mM HEPES

(pH 5.5) buffer. The provided resin was added to each sample to remove [^{3}H]L-arginine. Radioactivity due to [^{3}H]L-citrulline was measured as counts per minute (cpm) using a scintillation counter. Cpm was also measured in samples with no cell lysate as a background control. NOS activity of each sample was calculated by subtracting background cpm from the cpm for each sample, and then normalizing to total protein in each 5 µL sample, determined by the BCA protein assay. For assays with lyso-Gb3, Gb3, or glucosylsphingosine (Matreya), the reaction was carried out with the indicated lipid. 5 µL of cell lysate was incubated with the lipid on ice for 1 hour before adding the NOS assay reaction mixture, or the lipid was added to the reaction mixture and then added to the cell lysate.

Exogenous GLA treatment

Cells (1 x 10^6) were seeded on 150 mm cell culture dishes with growth medium. The following day, growth medium was replaced with fresh media, and 3 U of α-galactosidase A (Green coffee bean extract, Sigma), was added. Cells grew for 3 days and were harvested for eNOS activity measurement or lipid analysis with TLC.

Eliglustat treatment

Cells (1 x 10^6) were seeded on 150 mm cell culture dishes with DMEM/F12 GlutaMAX supplemented with 5% FBS. The following day, medium was replaced with DMEM/F12 GlutaMAX supplemented with 2% FBS. Cells were treated with either PBS vehicle control, 20 or 200 nM eliglustat. Cells were harvested 3 days later for NOS activity measurement.

Methotrexate and sepiapterin treatment

Cells were seeded on 100 mm cell culture dishes (1 x 10^6). The following day, growth medium was replaced with fresh growth medium, and cells were treated with either methotrexate (Cayman Chemical, Ann Arbor, MI), or sepiapterin (Sigma). After 18 hours, cells were harvested for NOS activity measurement as described above.

Conditioned media assay

On Day 1, clonal cell lines 12 (GLA-deficient) and 17 (GLA-WT) were seeded on T75 flasks (1 x 10^6 cells), "donor cells," with growth media. On day 2, the media was replaced with fresh growth media. In a separate set of flasks with no cells, growth media was added as a control ("cell-free media"). On Day 4, 1 x 10^6 cells were seeded on T75 flasks, "recipient cells," with growth media. On Day 5, media was removed from "donor cells," centrifuged to remove debris, and used to replace the media on "recipient cells." Donor cells were harvested with 0.05% trypsin-EDTA, washed with PBS, and frozen at -80 °C. On Day 8, "recipient cells" were harvested the same way. eNOS activity was assayed in lysates from donor and recipient cells as described earlier. Lysates were also used for Western blot analysis of eNOS expression.

Western blotting

Cells were harvested with 0.05% trypsin-EDTA and lysed in a buffer consisting of 25 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, 20 mM NaF, 1% Triton X-100, 10% glycerol, and protease inhibitor (P8340, Sigma). Cells were probe sonicated on ice and centrifuged at 10,000 x g for 10 minutes at 4 °C. Supernatants were saved and total cellular proteins determined with bicinchoninic acid (BCA) assay using bovine serum

album (BSA) as a standard (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA). Thirty μg of protein were denatured with 2-mercaptoethanol in Laemmli buffer, heated at 95 °C for 10 minutes, and then separated on NuPAGE 4-12% SDS-PAGE (Thermo Fisher Scientific). Proteins were transferred to nitrocellulose membranes, blocked for 1 hour with 5% nonfat dry milk-TBST (tris-buffered saline, tween-20), then incubated with primary antibody against eNOS (1:1000 in 5% nonfat dry milk-TBST, M221 (ab76198) Abcam, Cambridge MA), or GAPDH (1:1000 in 1% BSA-TBST, #MAB374, EMD Millipore, Billerica, MA) overnight at 4 °C. After washing with TBST, membranes were incubated with anti-mouse (for eNOS and GAPDH detection) or anti-rabbit (for GLA detection) secondary antibody at (1:15,000 in 1% BSA-TBST). Membranes were washed with TBST and incubated with enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific). Protein bands were detected with film exposure. The film was scanned and protein bands quantified with ImageJ Software.

Statistical analysis

Data were analyzed by using Prism 7.0 software (GraphPad Software Inc, La Jolla, CA). Significance was determined by two-way analysis of variance (ANOVA) with Dunnett's or Sidak's multiple comparisons test. Values are shown as mean ± SEM. Differences between groups were considered statistically significant at a P-value of <0.05. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.
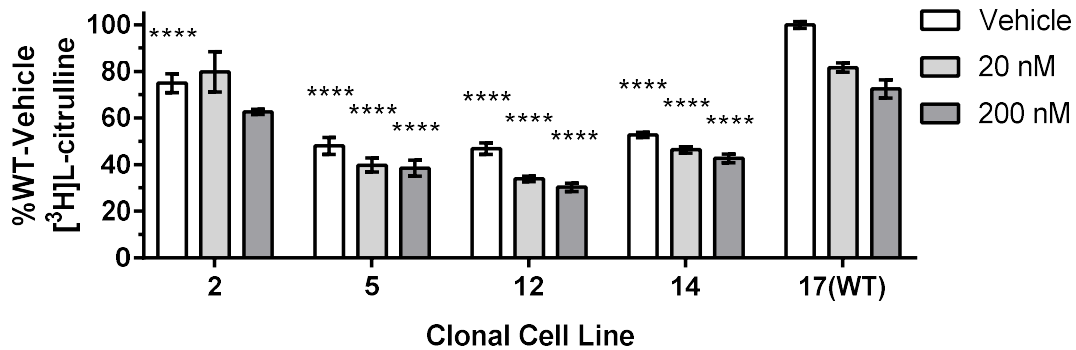
Results

Two methods of reducing neutral GSLs have different effects on NOS activity

Previously, it was shown that two different methods, exogenous GLA treatment or

substrate deprivation with eliglustat treatment, can lower neutral GSL content in GLA(-)

cells (chapter 2). To determine if diminished NOS activity is due to the absence of

functional GLA or accumulation of neutral GSLs, these two pharmacological methods

were used. Cells were treated with either exogenous GLA or eliglustat (20 or 200 nM)

for 3 days, and then harvested for NOS activity measurements. GLA enzyme treatment

increased NOS activity in all cells (fig. 3.1), indicating a direct correlation between

presence of the functional GLA enzyme and functioning NOS. In contrast, treating with

eliglustat decreased NOS activity in a dose-dependent manner (fig 3.2).

Clonal Cell Line and Treatment
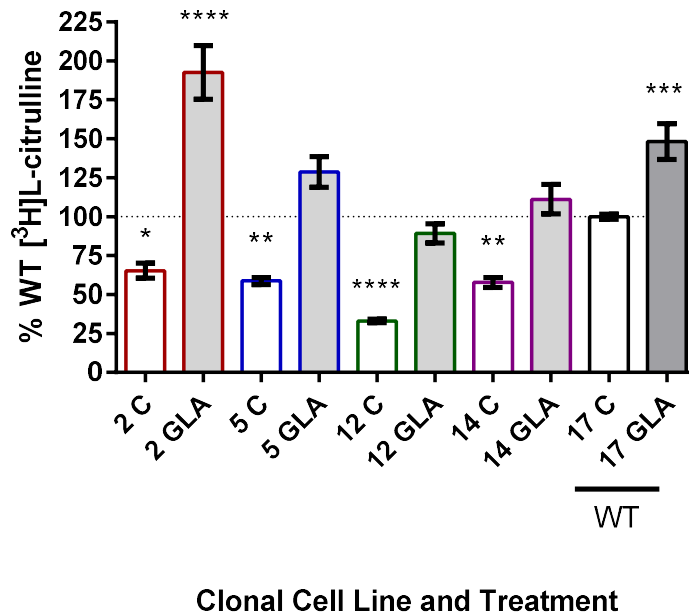
Figure 3.1: Exogenous GLA improves NOS Activity
GLA(-) cell lines (2, 5, 12, 14) and the WT cell line (17) were treated with
exogenous GLA (shaded bars), resulting in increased NO production. Dotted line
indicates WT control (C) normalized to 100%. n=8, ****P<0.0001, ***P<0.001,
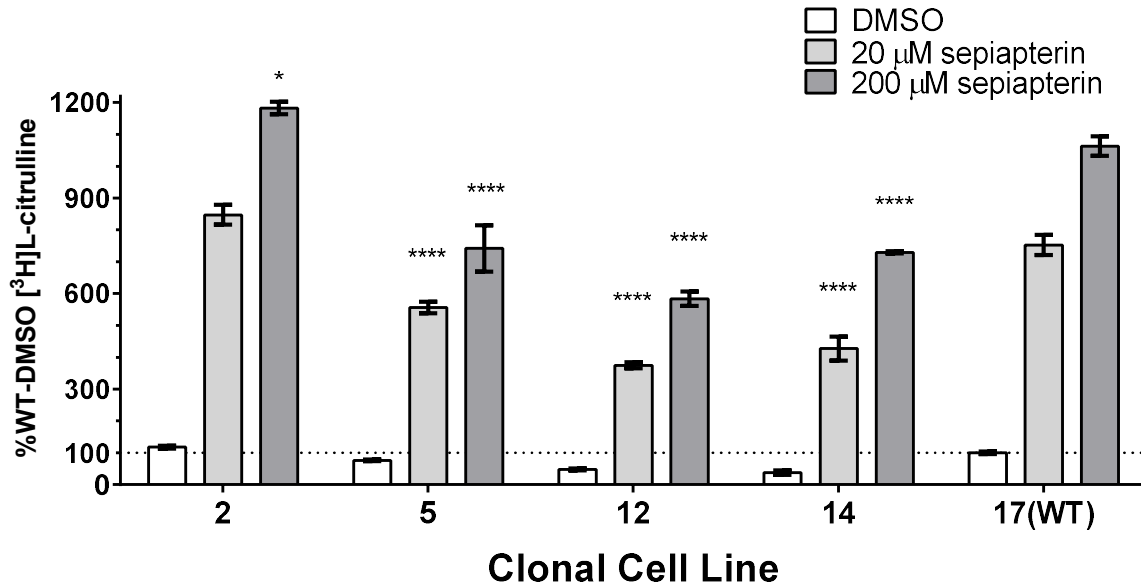**P<0.01, *P<0.05 compared to 17(WT) control

**Figure 3.2: Eliglustat treatment reduces NOS activity.**
GLA(-) cell lines (2, 5, 12, 14) and the WT cell line (17) were treated with
eliglustat (20 or 200 nM) for 3 days, resulting in decreased NO production.
Results are reported as %17(WT)-vehicle. n=5 ****P<0.0001 compared to
17(WT) each treatment.

Sepiapterin and methotrexate treatments affect NOS Activity

A previous study using the anti-GLA siRNA EA.hy926 cell model reported an increase in

3-nitrotyrosine (Shu, Vivekanandan-Giri et al. 2014), which is a marker indicating the

presence of reactive nitrogen species and eNOS uncoupling (Heinecke 2002). To

determine if BH4 supplementation would reverse eNOS uncoupling in GLA(-) cells, cells

were treated with DMSO vehicle control, or sepiapterin (20 or 200 µM). As expected,

sepiapterin treatment increased NO production in all clonal cell lines (fig. 3.3). However,

sepiapterin treatment did not restore NO production to the same level as WT for each

treatment. In contrast, treating with the DHFR inhibitor, methotrexate, there was a

decrease in NOS activity (fig. 3.4). The increase in NOS activity seen with sepiapterin

was confirmed to be biopterin-dependent by treating cells with both sepiapterin and

methotrexate (data not shown). In the presence of methotrexate, sepiapterin decreases

BH4 availability by inhibiting GTP cyclohydrolase I (GTPCH), the first step in *de novo*

BH4 synthesis (fig 1.4) (Gross and Levi 1992).

Figure 3.3: Sepiapterin treatment increases NOS activity. Cells were treated with sepiapterin (20 or 200 μM) for 18 hours and then harvested for NOS activity measurements. Dotted line indicates WT control normalized to 100%. n=4 ****P<0.0001, *P<0.05 compared to 17 (WT) each treatment.
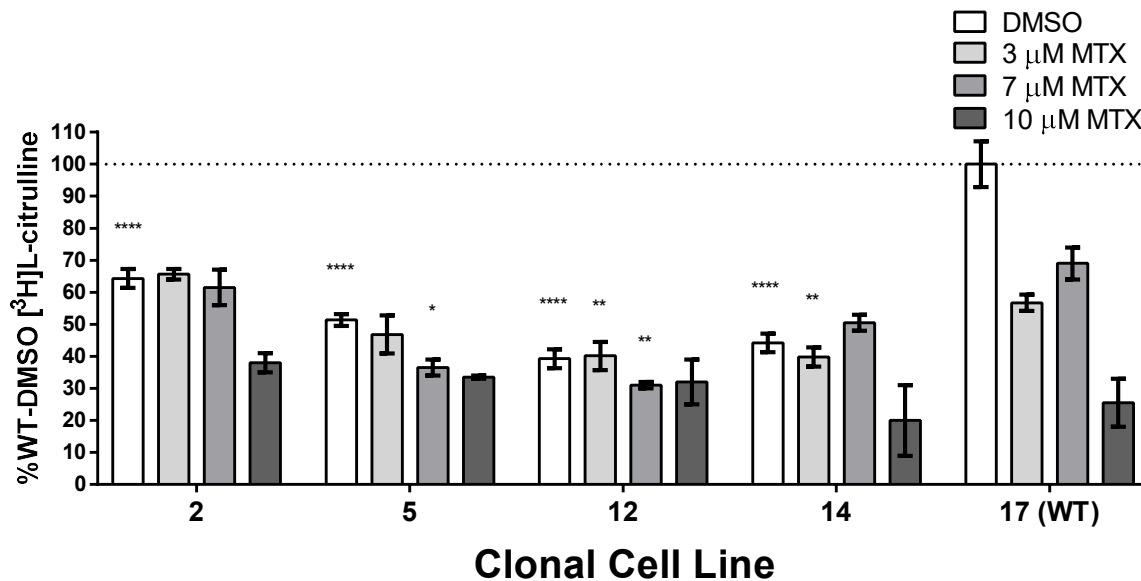
Figure 3.4: Methotrexate treatment decreases NOS activity.
Cells were treated with methotrexate (MTX, 3, 7, or 10 μM) for 18 hours and then harvested for NOS activity measurements. Dotted line indicates WT-DMSO normalized to 100%. n=6 for DMSO, 3, and 7 μM; n=2 for 10 μM,. ****P<0.0001, **P<0.01, *P<0.05 compared to 17 (WT) each treatment.

*Conditioned media from GLA(-) cells increases NOS activity in WT cells*

To determine if GLA(-) cells release a substance into the media that could affect NOS

activity, a conditioned media experiment was performed. Media was incubated with the

GLA(-) clone 12 for 3 days, and then transferred to WT (clone 17) cells for 3 days. The

opposite was also performed. There was ~80% increase in NOS activity in WT cells

treated with media from GLA(-) cells (fig. 3.5). To explore a possible mechanism for this

increase, eNOS protein expression was measured by Western blot (fig. 3.6). Incubating

WT cells with media from GLA(-) media caused ~20% increase in eNOS expression

compared to treating with cell-free media, which correlates with the trend of increased

NO production in this condition. In addition, GLA(-) cells incubated with GLA(-) media

from separately plated cells showed about a 2-fold increase of eNOS protein

expression.
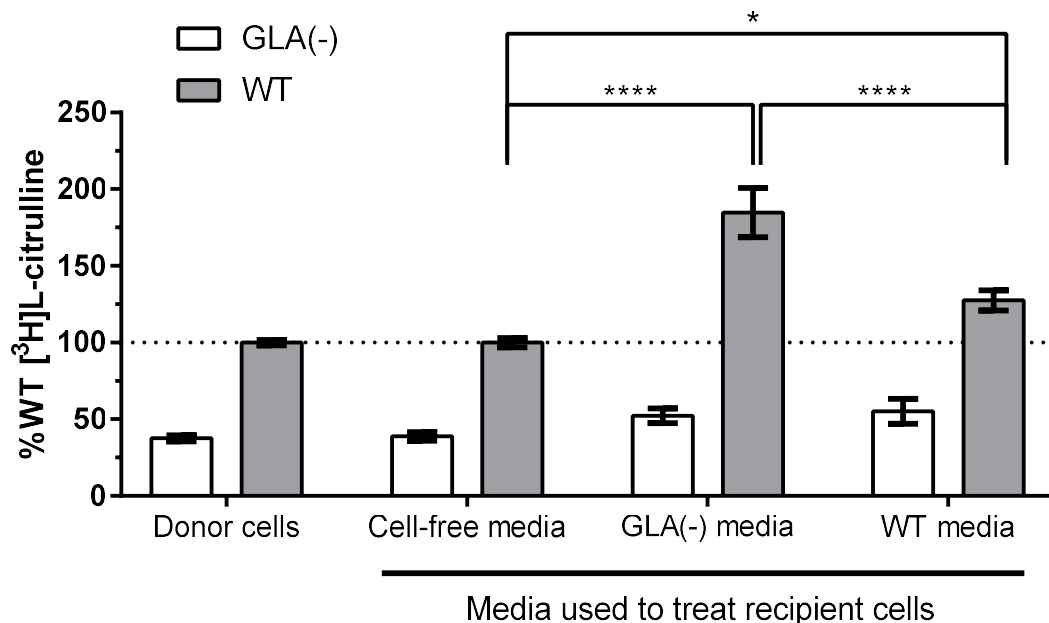
Figure 3.5: GLA(-) media increases NOS activity in WT cells.
Cells were treated with media that was incubated with WT or GLA(-) cells, or no cells as a control (cell-free media). There is a significant increase in [^{3}H]L-citrulline production in WT cells that were incubated with media from GLA(-) cells. n=6. ****P<0.0001, *P<0.05 compared to cell-free media or WT media as indicated.
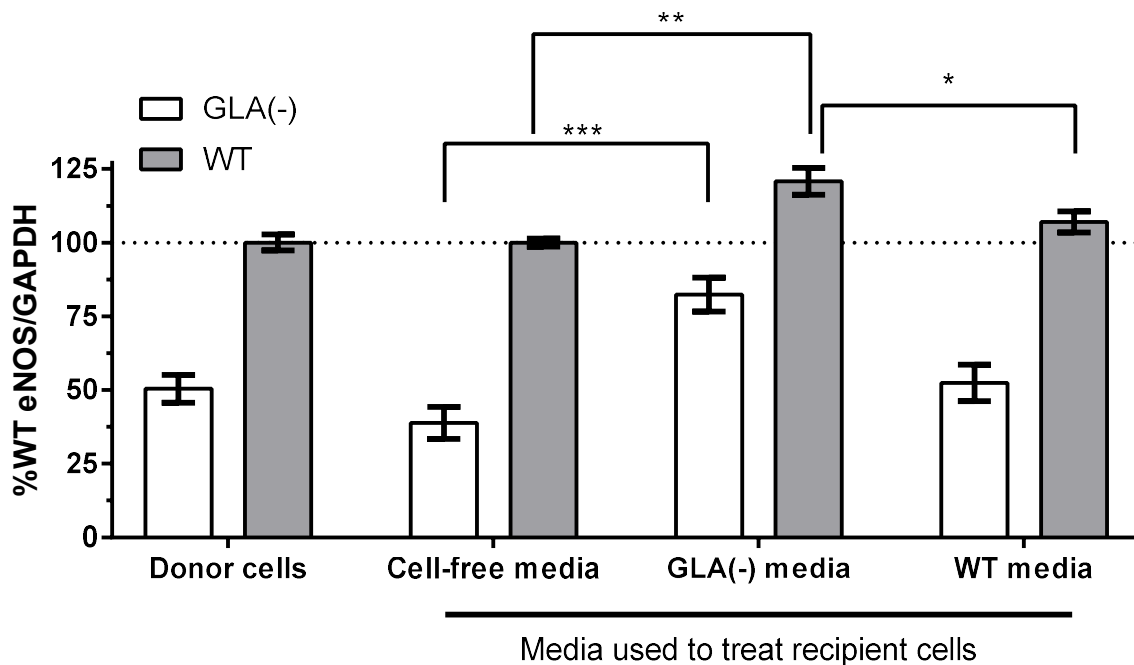
Figure 3.6: GLA(-) media increases eNOS protein expression in WT cells.
Cells were treated with media that was incubated with WT or GLA(-) cells, or no cells as a control (cell-free media). There is a significant increase in eNOS expression in WT cells and GLA(-) cells that were incubated with media from GLA(-) cells. eNOS expression is also increased GLA(-) incubated with media from GLA(-) cells. n=2-6. ***$P<0.001$, **$P<0.01$, *$P<0.05$ when compared to cell-free media or WT media as indicated.

Lyso-Gb3 inhibits NOS activity

Previously, it was determined that GLA(-) EA.hy926 cells have elevated globotriaosylsphingosine (lyso-Gb3) compared to WT cells (chapter 2). It has also been shown that GLA(-) cells have decreased NOS activity. To determine if there is a correlation between presence of lyso-Gb3 and NOS activity, lyso-Gb3 (250 or 500 nM) was added to cell culture media for 3 days, and NOS activity was measured in cell lysates. The concentrations used are within the range reported to increase smooth muscle cell proliferation (Aerts, Groener et al. 2008). However, there was no change in

NOS activity compared to cells treated with DMSO or glucosylsphingosine (GlcSph) (data not shown).

To directly assess the effect of lyso-Gb3 on eNOS, lyso-Gb3, GlcSph, or Gb3 were added to the NOS reaction mixture for a final concentration of 10-60 µM lyso-Gb3 in the reaction. NOS activity is severely inhibited by lyso-Gb3, and to a lesser extent, GlcSph (fig. 3.7). Gb3 in the reaction mixture only slightly affects NOS activity. There is an increase in activity at the 50 pmol/µg protein concentration for both lyso-Gb3 and Gb3. The experiment was repeated with GLA(-) clonal cell line 12 (fig. 3.8).

To determine if the effect seen is due to a direct interaction of the lipid and protein, cell lysates were incubated with lyso-Gb3 or GlcSph on ice for 1 hour before adding the other reagents into the NOS reaction mixture. Lyso-Gb3 and GlcSph inhibit the reaction in this condition (fig. 3.9). In GLA(-) lysates, the effect is not as drastic, presumably due to the low activity with DMSO-control (~50% WT-DMSO-control).
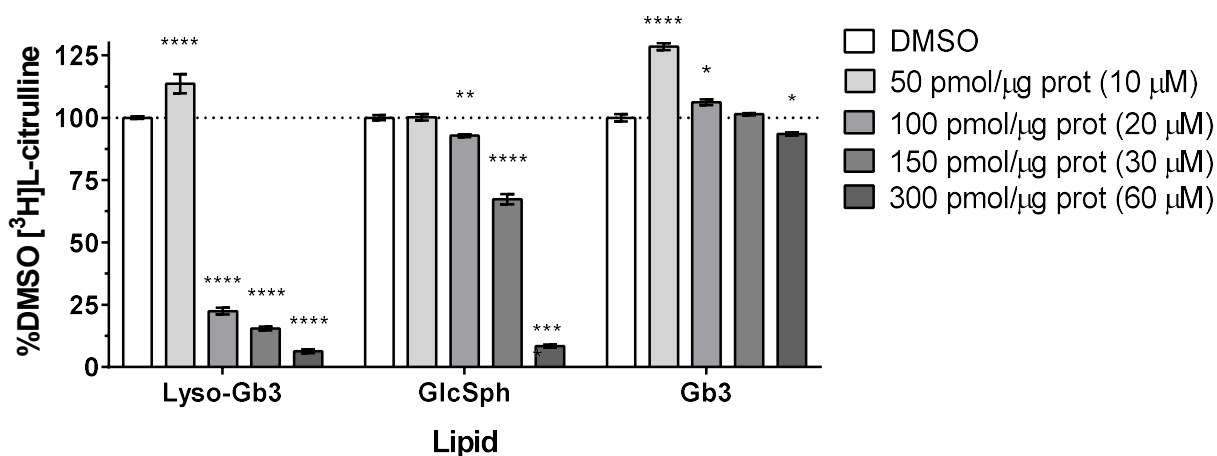
Figure 3.7: Lipids in NOS reaction inhibit activity.
The NOS reaction was performed with different amounts of Lyso-Gb3, GlcSph, and Gb3 in the reaction mixture. Each reaction contained 10 µg of protein from clone 17 (WT) lysates. Results are reported as %DMSO. n=3 ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$ compared to DMSO.
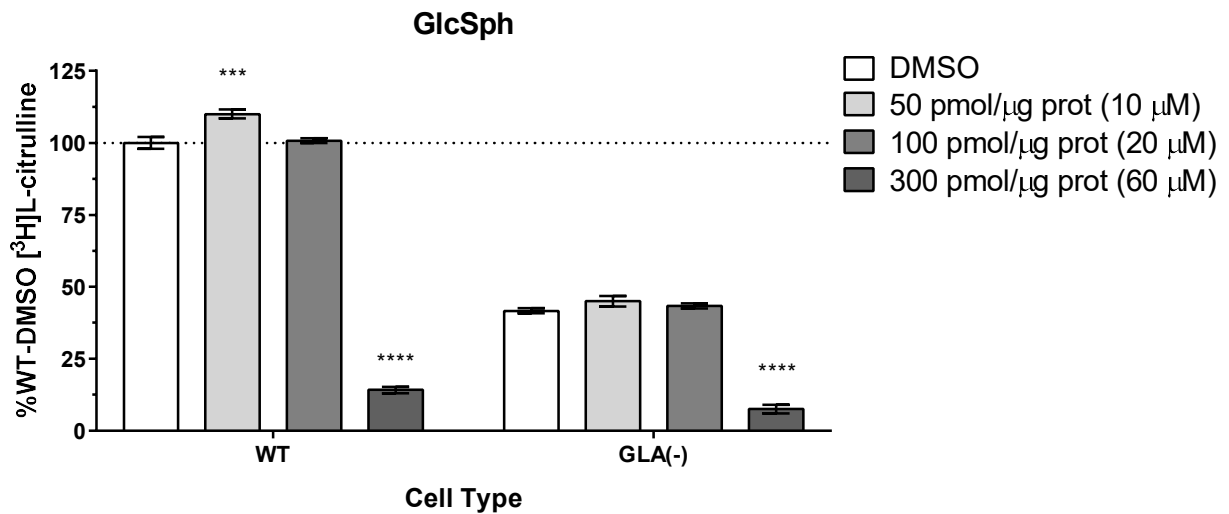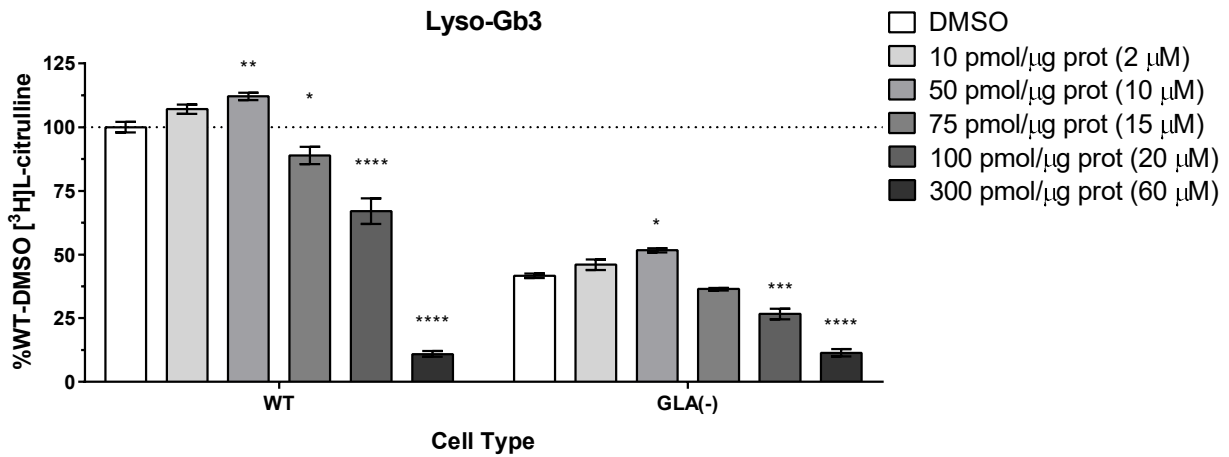
Figure 3.8: Lyso-Gb3 inhibits NOS activity in a dose-dependent manner.
The NOS reaction was performed with different amounts of Lyso-Gb3 in the
reaction mixture. Each reaction contained 10 μg of protein from WT or GLA (-)
lysates. Results are reported as %WT-DMSO (dotted line).n=3. ****P<0.0001,
***P<0.001, **P<0.01, *P<0.05 compared to DMSO each cell type.
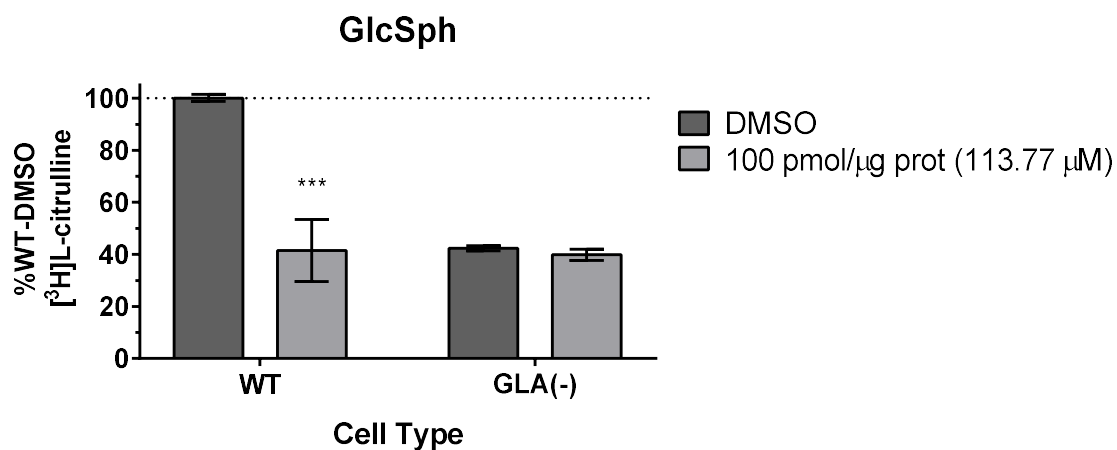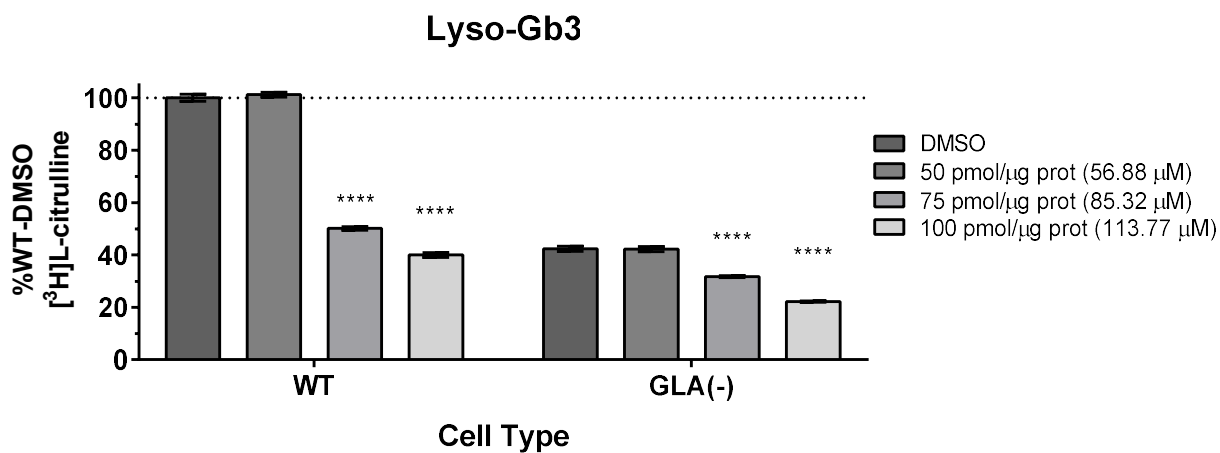
Figure 3.9: Preincubation of cell lysates with lyso-lipids inhibits NOS activity.
10 µg of protein from WT and GLA (-) cells were incubated with different amounts of lyso-Gb3 or GlcSph for 1 hour before beginning the NOS reaction. Results are reported as %WT-DMSO (dotted line). n=3. ****P<0.0001, ***P<0.001 compared to DMSO each cell type

Presence of inhibitor in lysates from GLA-deficient cells

To determine the possibility that lyso-Gb3 or another substance in GLA(-) cell lysates inhibits NOS activity, the NOS assay was performed with 10 µg protein per reaction, made up of combinations of cell lysates from WT and GLA(-) cells in various proportions. The expected results are calculated based on the results of 10 µg of WT or GLA (-) – only samples. There is a trend of experimental values being lower than expected values, but only one combination reached statistical significance (8 µg GLA (-) and 2 µg WT protein, p=0.0132).
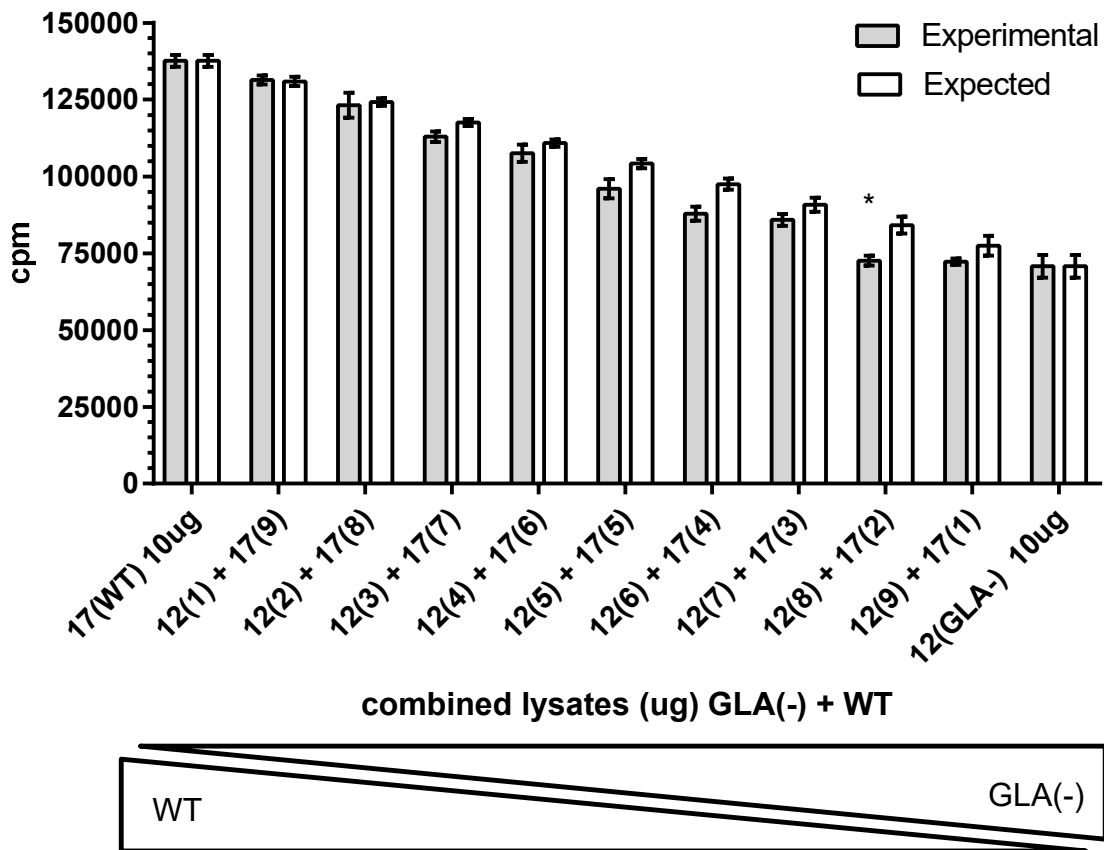
Figure 3.10: GLA (-) cell lysates decrease activity of WT cell lysates.
NOS reactions were setup with a total of 10 µg protein lysate in each reaction, containing different proportions of WT and GLA (-) lysates. Expected values are calculated from experimental results of 10 µg homogenous samples. n=3.
*P<0.05 between experimental and expected values.

Discussion

Patients with Fabry disease have an increased incidence of vasculopathy compared to the non-Fabry population (Sims, Politei et al. 2009). GLA deficiency causes accumulation of globo series glycosphingolipids (GSLs), most notably in vascular endothelial cells (Desnick, Ioannou et al. 2001). Past studies indicate that defects of vascular dysfunction can be localized to the endothelium, and endothelial nitric oxide synthase (eNOS) uncoupling may be the basis for impaired vascular function (Shu, Park et al. 2009, Shu, Vivekanandan-Giri et al. 2014). To further investigate biochemical changes due to GLA deficiency, this study used GLA(-) EA.hy926 cells to address whether eNOS uncoupling is due to accumulation of GSLs, limited availability of the eNOS cofactor, tetrahydrobiopterin (BH4), or elevated globotriaosylsphingosine (lyso-Gb3).

The primary treatment method for Fabry disease is enzyme replacement therapy (ERT) with recombinant GLA. Substrate deprivation is an alternative approach to reducing accumulation of globo-series GSLs, where a small molecule inhibitor of glucosylceramide (GlcCer) synthase prevents the *de novo* synthesis of downstream GSLs, including lactosylceramide (LacCer), globotriaosylceramide (Gb3), and globotetraosylceramide (Gb4) (Abe, Arend et al. 2000, Abe, Gregory et al. 2000). A previous *in vitro* experiment performed with mouse aortic endothelial cells (MAECs) from *Gla*-knockout mice reported that treating cells with recombinant GLA or a GlcCer synthase inhibitor reduced the accumulation of Gb3 (Shu, Murphy et al. 2005). Similarly, treating CRISPR/Cas9 GLA(-) EA.hy926 cells with exogenous GLA or the GlcCer

inhibitor, eliglustat, decreased the accumulation of globo-series GSLs (figs 2.10 and 2.11).

Although both exogenous GLA and eliglustat reduce neutral GSLs, they have different mechanisms of action. GLA decreases the level of its primary substrate, Gb3, and decreases of LacCer and Gb4 levels are secondary (chapter 2). On the other hand, eliglustat, a GlcCer synthase inhibitor, prevents the synthesis of GlcCer, and subsequently of LacCer, Gb3, and Gb4. We report here that eliglustat treatment decreases NOS activity, even in WT cells (fig.3.2). These results are in agreement with a previous study where treatment of MAECs from *Gla*-deficient mice with the GlcCer synthase inhibitor, D-*threo*-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol, did not improve NOS activity, although Gb3 was cleared from cells (Shu, Park et al. 2009). In contrast, treating EA.hy926 cells with exogenous GLA increased NOS activity, in both GLA(-) and WT cells (fig. 3.1). The opposite effects of two methods of reducing GSLs on NOS activity suggests that restoring GLA activity is more important than decreasing the level of GSLs for restoring eNOS function.

Several experimental models of Fabry disease have suggested that eNOS dysfunction is the basis for vasculopathy that occurs in many patients with Fabry disease (Eitzman, Bodary et al. 2003, Bodary, Shen et al. 2005, Park, Whitesall et al. 2008, Shu, Park et al. 2009, Shu, Vivekanandan-Giri et al. 2014). eNOS uncoupling occurs when there is improper flow of electrons from the reductase domain of eNOS, to the oxidase domain. Normally, NADPH-derived electrons are used to convert L-arginine and molecular oxygen to NO and L-citrulline. In the case of eNOS uncoupling, however, the electrons are not transferred to L-arginine and are instead transferred to O_2, forming superoxide

instead of NO (Vasquez-Vivar, Kalyanaraman et al. 1998, Xia, Tsai et al. 1998). Superoxide then reacts with NO to produce a reactive nitrogen species, peroxynitrite (ONOO⁻). ONOO⁻ rapidly oxidizes BH4 to the inactive form, dihydrobiopterin (BH2) (Milstien and Katusic 1999). BH4 is a cofactor of eNOS that keeps eNOS in the active, homodimer form (Rodriguez-Crespo, Gerber et al. 1996). A decrease in BH4 availability causes further uncoupling.

Lower BH4 availability due to a pro-oxidant state has been described in the more common diseases, diabetes and hypertension (Landmesser, Dikalov et al. 2003). Supplementation with BH4 improves NOS function in patients with diabetes (Heitzer, Krohn et al. 2000), chronic smokers (Heitzer, Brockhoff et al. 2000, Ueda, Matsuoka et al. 2000), hypertension (Hong, Hsiao et al. 2001), and hypercholesterolemia (Stroes, Kastelein et al. 1997). In this study, we have reported that GLA(-) cells also have lower BH4 availability, and BH4 supplementation improves NOS function. BH4 levels can be pharmacologically modulated with sepiapterin and methotrexate. Sepiapterin is the substrate for the first step in the salvage pathway that regenerates BH4. Methotrexate is an inhibitor of DHFR, thus inhibiting regeneration of BH4 from BH2 (fig. 1.4). These reagents were used in this study to show that GLA(-) cell have limited BH4 availability, and increasing BH4 availability improves NOS activity. Treating cells with sepiapterin significantly increases NOS activity in GLA(-) cells (fig. 3.3). On the other hand, treating with methotrexate decreases NOS activity in GLA(-) cells (fig. 3.4) to a level that is not significantly different than WT with the same methotrexate treatment.

A recent study (Shen, Arning et al. 2017) reported that BH4 was decreased in the heart and kidney of Fabry mice, and that Gb3 levels were inversely correlated with BH4 levels

in tissues. The study also reported decreased glutathione levels in Fabry mouse tissues. These results correlate with the present study. Increasing BH4 availability in GLA(-) cells increased NOS activity, and GLA(-) cells have less glutathione availability (fig. A.1). The same study also reported that restoring GLA activity in the Fabry mice for 6 months did not improve BH4 availability, but treating mice with a GlcCer synthase inhibitor (GZ161) for 6 months did (Shen, Arning et al. 2017). These results conflict with results from our study as eliglustat treatment decreased NOS activity. Although we did not measure BH4 levels, if BH4 levels were improved with eliglustat, then we would have expected to observe an increase in NOS activity.

Previous results indicated that lyso-Gb3 is elevated in cell lysates from GLA(-) cells (fig. 2.14). We now have shown that lyso-Gb3 can inhibit NOS activity (figs. 3.7, 3.8, 3.9). A discrepancy between the two studies is the higher concentration of lyso-Gb3 in the NOS reaction needed to elicit a response. A possible explanation is that it is not known where in the cell lyso-Gb3 is concentrated. eNOS is active when it is localized to caveolae in the plasma membrane (Shaul, Smart et al. 1996). Caveolae are rich in GSLs. Lyso-GSLs may tend to accumulate in acidic compartments (Ferraz, Marques et al. 2016). It is unclear if they are found in the plasma membrane where eNOS is active. It remains to be determined if the concentration of lyso-Gb3 in the NOS inhibition experiment is physiologically relevant. Also, other lipids, such as LacCer or Gb4, should be tested in this system to better design a possible a structure-activity relationship between lipids and NOS activity. GlcSph, another lyso lipid, also inhibits NOS, but at a higher concentration than lyso-Gb3. No change in NOS activity was observed when adding

lyso-Gb3 or GlcSph to the cell culture. It is possible that the reagents are not stable in the cell culture media, or that they are not getting into cells.

Treatment that improves endothelial function would be useful for managing life-threatening complications of Fabry disease. In this study, mechanisms are uncovered to improve NOS activity in the setting of GLA deficiency. NOS activity is increased upon treating cells with GLA, but not with eliglustat (figs. 3.1 and 3.2). This indicates that GLA activity, not lower GSL levels, is critical for maintaining proper NOS activity. Another finding of this study was that increasing BH4 availability improves NOS activity in the setting of GLA deficiency (fig. 3.3). GLA deficiency may be causing a decrease in BH4 that is corrected upon supplementation with the precursor of BH4, sepiapterin. It remains to be seen whether BH4 levels correlate with 3-nitrotyrosine, a marker of eNOS uncoupling in Fabry disease (Shu, Vivekanandan-Giri et al. 2014).

We observed was that GLA(-) cells release a substance into cell culture media that increases NOS activity in WT cells (fig. 3.5). This was surprising because it was expected that GLA(-) cells secrete a substance, possibly lyso-Gb3, that would decrease NOS activity. The increase of NOS activity is partially due to increased eNOS expression in these samples. However, further investigations assessing the expression of another NOS isoform, inducible NOS (iNOS), need to be performed to explain the increase of NOS activity.

Finally, lyso-Gb3, which is found to be elevated in Fabry disease, inhibits NOS activity (figs. 3.7, 3.8, and 3.9). This is further evidence that lyso-Gb3 is not just a biomarker of Fabry disease, but is involved in the pathogenesis of the disease. Published reports indicate that lyso-Gb3 promotes smooth muscle cell proliferation, which may contribute

to the increased intima-media thickening seen in patients (Aerts, Groener et al. 2008),

and mediates podocyte injury in Fabry nephropathy (Sanchez-Nino, Sanz et al. 2011).

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Chapter 4: Discussion

Summary

In this thesis I have reported potential mechanisms of endothelial nitric oxide synthase (eNOS) uncoupling in Fabry disease. In chapter 2, I detail the process of using CRISPR/Cas9 gene editing to obtain multiple single-cell colonies that have a frameshift mutation in *Gla* and do not express α-galactosidase A (GLA, referred to as GLA(-) cells). These cells have less NOS activity compared to the wild type (WT) GLA control. Globotriaosylsphingosine (lyso-Gb3) was found to be the lipid that is consistently elevated in both siRNA and CRISPR/Cas9 models of GLA deficiency. The purpose of using CRISPR/Cas9 to obtain GLA(-) cells was to be able to investigate the effects of GLA deficiency in an endothelial cell model of Fabry disease that could be continually passaged without further treatment, and maintain the Fabry phenotype of elevated glycosphingolipids (GSLs) and decreased NOS activity. In chapter 3, I report mechanisms of eNOS uncoupling in GLA(-) cells: restoration of GLA activity, and not necessary reduction of GSLs, is important for proper NOS activity; GLA(-) cells have limited tetrahydrobiopterin (BH4) availability, and BH4 supplementation improves NOS activity in GLA(-) cells; and lyso-Gb3 may be inhibiting NOS activity directly. Furthermore, I report that GLA(-) cells release a substance into cell culture media that

increases NOS activity in WT cells, but the increase in activity is not completely due to increased eNOS expression.

GLA deficiency affects neutral GSL levels

The EA.hy926 cell line was chosen for this study because previous work had shown that siRNA-mediated GLA knockdown caused an accumulation of globotriaosylceramide (Gb3) (Shu, Vivekanandan-Giri et al. 2014), which is what is seen in Fabry patients and other models of Fabry disease (Sweeley and Klionsky 1963, Ohshima, Murray et al. 1997).

Surprisingly, GLA(-) EA.hy926 cells, induced by CRISPR/Cas9 gene mutation, have a different lipid profile compared to siRNA-induced GLA(-) EA.hy926 cells (figs 2.5, 2.6, 2.7, 2.8). Notably, the CRISPR/Cas9 GLA(-) cells do not accumulate Gb3, the primary substrate for GLA. This finding was explained by observing that the CRISPR/Cas9 GLA(-) cells have a significant accumulation of globotetraosylceramide (Gb4), whereas 3-6 day anti-GLA siRNA treatment caused only a moderate increase of Gb4.

Because cell lines were expanded from a single cell, it was not possible to measure lipid content until each single cell colony had millions of cells. Therefore, it is possible that the CRISPR/Cas9 GLA(-) cells had an increase in Gb3 during the first few days after the initial *Gla* disruption post-CRISPR/Cas9 transfection, but after several passages, Gb4 became the prominent GSL.

Despite differences of Gb3 and Gb4 accumulation, both the CRISPR/Cas9 and siRNA models of GLA deficiency have decreased NOS activity (fig. 2.15 and (Shu, Vivekanandan-Giri et al. 2014)) and elevated lyso-Gb3 (fig. 2.14). Our study supports

data indicating that lyso-Gb3, not Gb3, is a biomarker for Fabry disease (Smid, van der Tol et al. 2015). Lyso-Gb3 level in human plasma correlates with GLA deficiency, and is more consistently increased in heterozygous females than is Gb3. Furthermore, lyso-Gb3 levels go down post-enzyme replacement therapy (ERT) (van Breemen, Rombach et al. 2011).

GSLs and caveolae

Caveolae are structures on the plasma membrane that were first described in the 1950s (Palade 1953, Yamada 1955), and are found in many mammalian cells, such as adipocytes, muscle cells, and endothelial cells (Parton and Simons 2007). They were later discovered to be involved in the endocytosis of large molecules from the extracellular space, and important for many signaling processes (Lisanti, Tang et al. 1995, Pelkmans and Zerial 2005). Caveolae are rich in GSLs and cholesterol compared to the surrounding plasma membrane (Ortegren, Karlsson et al. 2004). GSLs in the plasma membrane have been shown to regulate signaling molecules, like receptor tyrosine kinases (Bremer, Schlessinger et al. 1986), phospholipase C (Shu, Lee et al. 2002) and Src kinases (Shu and Shayman 2003). Prior to a study in the Shayman group indicating that Gb3 accumulates in caveolae (Shu, Lee et al. 2002), the idea that sphingolipids can accumulate outside of the lysosome in lysosomal storage diseases was not well studied. A later study found that high molecular weight oligomers of caveolin-1 are reduced in mouse aortic endothelial cells (MAECs) isolated from *Gla*-knockout mice (Shu, Park et al. 2009).

The caveolin-1 protein is mainly in the oligomeric form, and is essential for caveolae formation (Drab, Verkade et al. 2001). Caveolin-1 acts as a scaffold for signaling molecules in caveolae, like eNOS which, upon palmitoylation, gets trafficked to the plasma membrane by caveolae, (Garcia-Cardena, Oh et al. 1996). Under conditions of reduced tetrahydrobiopterin (BH4) availability, caveolin-1 associates with eNOS to inhibit eNOS and prevent eNOS-derived superoxide formation (Karuppiah, Druhan et al. 2011). In chapter 3, I reported that decreased NOS activity in GLA(-) cells is likely due to decreased BH4 availability. These results are supported by a recent report that Fabry mice have decreased BH4 in the heart, and plasma BH4 is decreased in both Fabry mice and Fabry patients (Shen, Arning et al. 2017). Considering the association of BH4, eNOS, and caveolin-1, it is likely that in the setting of GLA-deficiency, BH4 deficiency causes eNOS uncoupling and superoxide production that is not attenuated by caveolin-1 because of the disruption of caveolae in the setting of GLA-deficiency.

Future directions

Which lipids directly or indirectly inhibit NOS activity?

In this study, I showed that lyso-Gb3 dose-dependently inhibits eNOS activity (fig. 3.7, 3.8, and 3.9). GlcSph, another lyso-lipid, also inhibits NOS activity, but at higher concentrations. Gb3 only slightly, if at all, seemed to affect NOS activity. To build on this preliminary work, the effect of additional GSLs and lyso-lipids on NOS activity should be evaluated. Other lipids to test include ceramide, lactosylceramide (LacCer), globotetraosylceramide (Gb4), and lactosylsphingosine. A structure-activity relationship would be useful to determine how lyso-Gb3 interacts with eNOS.

Creating a cell library of deficiencies of globo-series GSL catabolic and anabolic enzymes using CRISPR/Cas9

To determine which GSLs contribute to eNOS uncoupling, it would be useful to develop groups of cells that lack expression of genes along the catabolic and anabolic pathways of neutral GSLs. Some metabolic genes in the globo-series GSL pathway are shown in figure 4.1. It is possible that just like in the case of *Gla* and Gb3, a specific enzyme knockout may not lead to accumulation of its substrate. However, it is expected that knockouts of each of these genes would results in a different lipid profile that can be correlated with NOS activity. The results would indicate if a specific lipid, or group of lipids, is causing eNOS uncoupling. Furthermore, eNOS uncoupling could be correlated with either a specific loss of GLA activity, or another GSL metabolic enzyme. In a previous study from the Shayman group (Shu, Vivekanandan-Giri et al. 2014), siRNA was used to silence GLA or GBA in EA.hy926 cells. Only anti-GLA siRNA treatment resulted in decreased NOS activity and increased 3-nitrotyrosine production (marker for eNOS uncoupling).
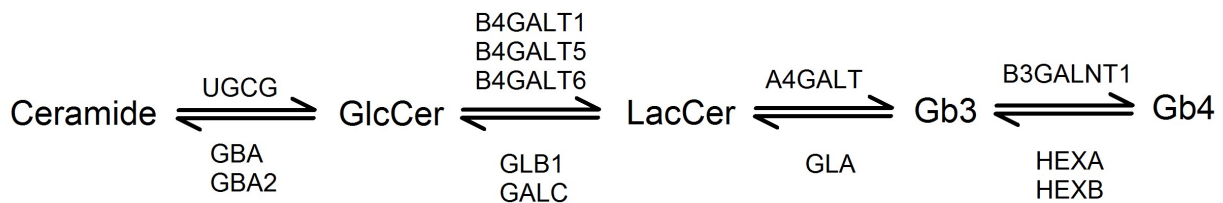
Figure 4.1: Globo-series GSL catabolic and anabolic genes. Genes that synthesize and degrade GSLs are listed. Fabry disease arises from mutations in GLA. Gaucher disease, another lysosomal storage disease, arises from mutations in GBA. GlcCer: glucosylceramide; LacCer: lactosylceramide; Gb3: globotriaosylceramide; Gb4: globotetraosylceramide (Shayman and Larsen 2014).

Why is Gb4, not Gb3, elevated in GLA-deficient EA.hy926 cells?

A surprising finding in the CRISPR/Cas9 GLA(-) cells was the accumulation of Gb4, instead of Gb3, which is the primary substrate of GLA. One way to address why Gb4 is elevated in CRISPR/Cas9 GLA(-) cells is to determine if expression of Gb4 synthase (B3GALNT1) is increased. I performed a pilot study where I measured mRNA expression of B3GALNT1 in GLA(-) EA.hy926 cells (qRT-PCR methods described in appendix). The results show an increase in expression of B3GALNT1 in GLA(-) clonal cell lines, 5, 12, and 14 (fig. 4.2). Future studies should investigate changes in mRNA expression of other GSL metabolic genes (fig. 4.1). There is a possibility that the Gb4 catabolic enzyme, hexosaminidase (*HEXA* and *HEXB*), is downregulated in EA.hy926 cells, which would explain the higher level of Gb4 in the absence of GLA-mediated Gb3 catabolism.
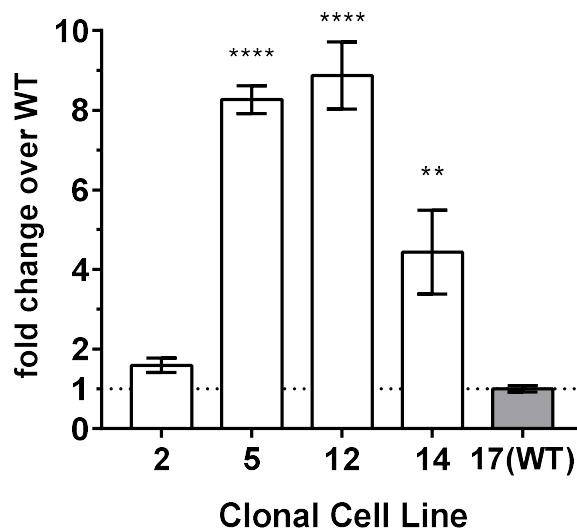
Figure 4.2: GLA(-) cells have increased B3GALNT1 mRNA expression. Cells were grown to confluency, harvested, and mRNA was extracted. After reverse transcriptase reaction, cDNA was used for real-time qRT-PCR measurement for expression of *B3GalNT1*, which encodes the enzyme, Gb4 synthase. Expression levels were normalized to GAPDH and WT control and presented as fold change. Clonal cell lines 5, 12, and 14 had a significant increase in mRNA expression for β3GalNT1. This may explain the elevated level of Gb4 in lipid extracts from these cell lines. n=6, ****P<0.0001, **<0.01 compared to clone 17(WT) control. For method, please refer to the appendix.

How do GLA and eliglustat treatments affect BH4 levels?

GLA and eliglustat treatment have opposite effects on NOS activity (figs. 3.1 and 3.2), although they both reduce neutral GSL levels (figs. 2.10 and 2.11). To explain one of the mechanisms of this observation, it would be useful to determine how GLA and eliglustat affect BH4 levels. GTP cyclohydrolase 1 (GTPCH1) catalyzes the first step of *de novo* BH4 synthesis, and is the rate limiting step (fig 1.4). To determine the reason for deficient BH4, GTPCH1 expression should be determined in GLA(-) cells. A recent study reported that GTPCH1 protein expression was downregulated in Fabry mouse heart, and treatment with a GlcCer synthase inhibitor (GZ161) increased GTPCH1 protein expression (Shen, Arning et al. 2017). Considering these results and the results from our own experiments, if treatment with the GlcCer synthase inhibitor eliglustat increased GTPCH1 protein expression in EA.hy926 cells, then we would have observed an increase in NOS activity. However, in our study, eliglustat treatment decreased NOS activity. Determining changes in GTPCH1 expression could help explain the discrepancy between the two studies.

Other NOS isoforms in EA.hy926 cells

The work in this thesis focused primarily on the endothelial isoform of NOS, eNOS. Another isoform that is pertinent to vasculopathy in Fabry disease is the inducible NOS (iNOS) isoform. iNOS is not constitutively expressed like the endothelial and neuronal NOS isoforms, but expression is induced by inflammatory cytokines, and its activity is Ca^{2+}-independent (Aktan 2004). While eNOS has a protective role in maintaining a functioning vascular endothelium and preventing the development of atherosclerosis, increased iNOS expression is found in many inflammatory diseases, including

atherosclerosis (Luoma, Stralin et al. 1998). In a study evaluating the development of atherosclerosis in Fabry mice, it was found that *Gla*-deficient mice bred on an *ApoE*(-) background had increased iNOS immunostaining in atherosclerotic plaques (Bodary, Shen et al. 2005). Because the EA.hy926 cells I am working with are derived from endothelial cells (human umbilical vein cord cells), it is presumed that the major NOS isoform expressed is eNOS. However, a few studies report that EA.hy926 cells do in fact express iNOS under inflammatory conditions (Toma, Stancu et al. 2011, Lee, Wang et al. 2016).

In chapter 3, I reported that WT cells incubated with cell culture media that was previously incubated with GLA(-) cells for 3 days increased NOS activity by ~80%, but only increased eNOS protein expression by ~25%. Follow up studies to the conditioned media experiment should include Western blot analysis of iNOS protein expression. If iNOS expression is increased in WT cells treated with GLA(-) media, it could explain the large increase of NOS activity, while there is only a small increase of eNOS expression.

Furthermore, iNOS expression should be evaluated in all CRISPR/Cas9 clonal cells lines. If iNOS protein expression is increased in GLA(-) clone 2, it would help explain why NOS activity in clone 2 cell lysates is consistently higher than the other GLA(-) clones (fig. 2.15), but has similar lyso-Gb3 and total glutathione levels to the other GLA(-) clones (figs. 2.14 and A.1).

LAESI-MS method of identifying and quantifying GSLs

High performance thin layer chromatography (HPTLC) is the method I used to measure neutral GSLs in chapter 2. The basis for TLC is separation of molecules based on polarity. It is a robust and relatively inexpensive method to rapidly analyze lipid content

(Skipski 1975, van Echten-Deckert 2000). Known standards are developed alongside samples, but the exact identity of a species cannot be confirmed by TLC alone. Many groups have developed methods to scrape the silica of the lipid of interest, elute the lipid, and analyze the lipid by mass spectrometry (MS) (Meisen, Peter-Katalinic et al. 2004). MS gives detailed information about the structure of the lipid, especially the carbon length of the ceramide moiety of the GSL. However, this indirect method of combing TLC and MS is time consuming and can lead to loss of material.

A new method that can directly analyze lipids on a TLC place is laser ablation with electrospray ionization (LAESI) (Nemes and Vertes 2007). Samples are applied to a TLC plate, developed in the appropriate solvent systems, and lipids are visualized with the nondestructive agent, primuline (Skipski 1975). The spot of interest is wetted (eg with water) and a laser is directed to the location. Analytes are transferred into a mass spectrometer through desorption/ionization. I have done some pilot studies where I analyzed Gb3 and Gb4 in EA.hy926 cells with this method[7]. This method confirmed the identity of Gb3 and Gb4 in samples based on mass (data not shown). More assays need to be done in order to identify differences in ceramide species among the clonal cell lines and if GLA deficiency causes changes in GSLs with a specific carbon length

Conclusions

Studying a rare disease arising from a single genetic defect provides a window of opportunity to gain understanding of normal physiology. Considering the vast number of questions left to answer in biology, there is always a need to develop better models to

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[7] LAESI-MS performed in collaboration with Ashootosh Tripathi in the Sherman group.

study diseases. The intention of the work presented in this dissertation was to do just that – develop an endothelial cell line with a permanent absence of GLA protein expression to better understand eNOS uncoupling in Fabry disease. Previous studies had reported Gb3 accumulation as a major contributor to the pathology of Fabry disease, but the CRISPR/Cas9 model of GLA-deficiency I developed and analyzed indicated that eNOS uncoupling still occurs even when Gb3 is not elevated. I also report that eNOS uncoupling in the setting of GLA deficiency may be due to decreased availability of the eNOS cofactor, BH4, and by the presence of lyso-Gb3.

The only approved therapeutic for Fabry disease in the United States, enzyme replace therapy, was approved for use less than 20 years ago. Although this treatment has improved the lives of patients, it does not completely reverse disease progression, and there remains a need for better therapeutics. Understanding the mechanism behind one of the complications of the disease, endothelial dysfunction, is a step towards improving the treatment for Fabry disease.

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Appendix

Quantitative real-time polymerase chain reaction (qRT-PCR) method for measuring Gb4 Synthase expression

Total cellular RNA was isolated from 3 x 10^6 cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The setup for the reverse transcriptase (StepOne Real-Time) reaction was: 10 µg RNA, 10x buffer (20 µL), 25x dNTP (8 µL), 10x random primers (20µL), reverse transcriptase (10 µL), RNase inhibitor (10 µL), and water, in 200 µL total volume. The reaction was carried out as follows: 25°C 10 min, 37°C 120min, 85 °C 5 sec.

The primers and RT-PCR program were based on those reported for β1,3-*N*-acetylgalactosaminyltransferase (B3GALNT1) and GAPDH (Schweppe, Bielaszewska et al. 2008). Primers (Table A.1) were purchased from ThermoFisher Scientific. Each reaction had 2 µL cDNA, 12.5 µL SYBRGreen (Qiagen), 1.5 µL of each forward and reverse primer (0.3 µM). The RT-PCR cycle was performed on an Applied Biosystems StepOne™ Real-Time PCR instrument (ThermoFisher Scientific), and results quantified with the StepOne™ Software. The PCR cycle was: 94 °C 3 min 30 sec, 30 cycles: 94 °C 15 sec, 63 °C 30 sec, 72 °C 1 min; 72 °C 10 min. The 125 bp product for B3GALNT1,

and 94 bp product for GAPDH verified by electrophoresis on ethidium bromide stained

4% agarose gels.

Gene	Sequence 5'-3'
B3GALNT1 NM_003781.3	F: ATGGCCTCGGCTCTCTGGACT
	R: TTGTAGTGGGGAAGGCTGAGGT
GAPDH	F: CACTAGGCGCTCACTGTTCT
	R: GACCAAATCCGTTGACTCCG

Table A.1: qRT-PCR primer sequences. Genes and NCBI Reference sequence identification; Forward (F), and reverse (R) nucleotides used to quantify gene expression in qRT-PCR. B3GALNT1: B1,3-*N*-acetylgalactosaminyltransferase (β1,3-Gal-NAcT, EC 2.4.1.79; GenBank accession no. Y15062).

GLA-deficient cells have less glutathione availability

Total glutathione (GSH) was measured in cells using the Glutathione Assay Kit

(Cayman Chemical) according to manufacturer's instructions. Cells were harvested and

lysed in RIPA buffer. GSH is significantly lower in GLA(-) cells (fig A.1). GSH is the most

abundant endogenous antioxidant. These results are in agreement with data reporting

increased oxidant production in EA.hy926 cells treated with anti-GLA siRNA (Shu,

Vivekanandan-Giri et al. 2014), and a recent study reporting decreased total GSH in

Fabry mice (Shen, Arning et al. 2017).
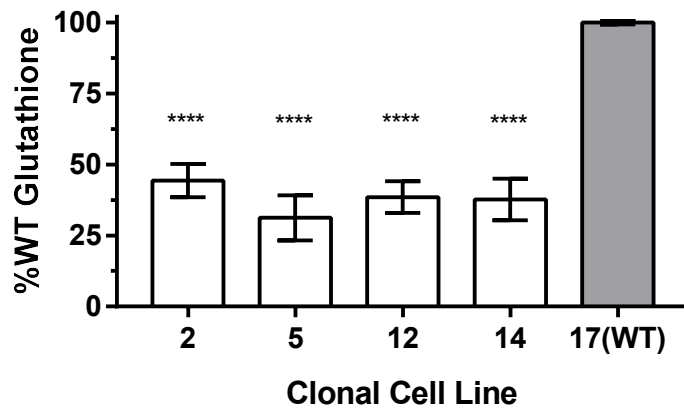
Figure A.1: GLA-deficient cells have less total glutathione.
Total glutathione (GSH) was measured in cells. GLA(-) clonal cell lines, 2, 5, 12, and 14 have significantly lowered GSH availability compared to 17(WT). n=8 ****p<.0001 compared to 17(WT).

**Experiments performed with CRISPR/Cas9 – Exon 2 targeted GLA-deficient cells**
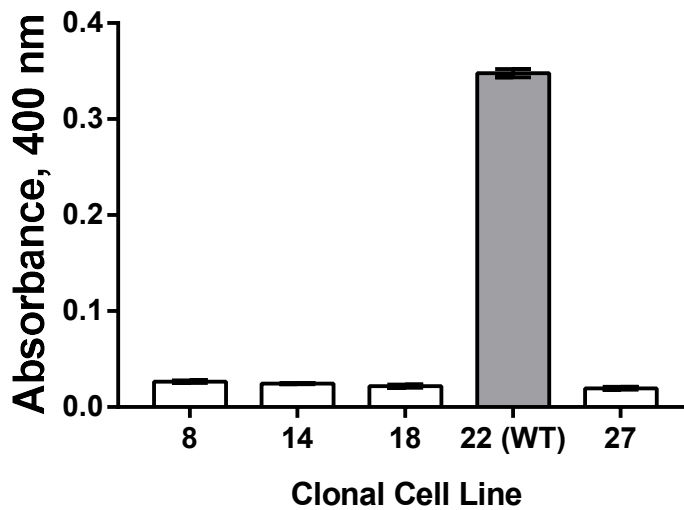
Figure A.2: GLA Activity in CRISPR/Cas9-Exon 2 GLA(-) cell lines. GLA activity was measured with methods described in chapter 2. GLA(-) clonal cell lines 8, 14, 18, and 27 have negligible GLA activity compared to the 22(WT) control clonal cell line. n=6. Results correlate with CRISPR/Cas9 Gla-Exon 1 edited cells (fig. 2.1).

LacCer

Gb3
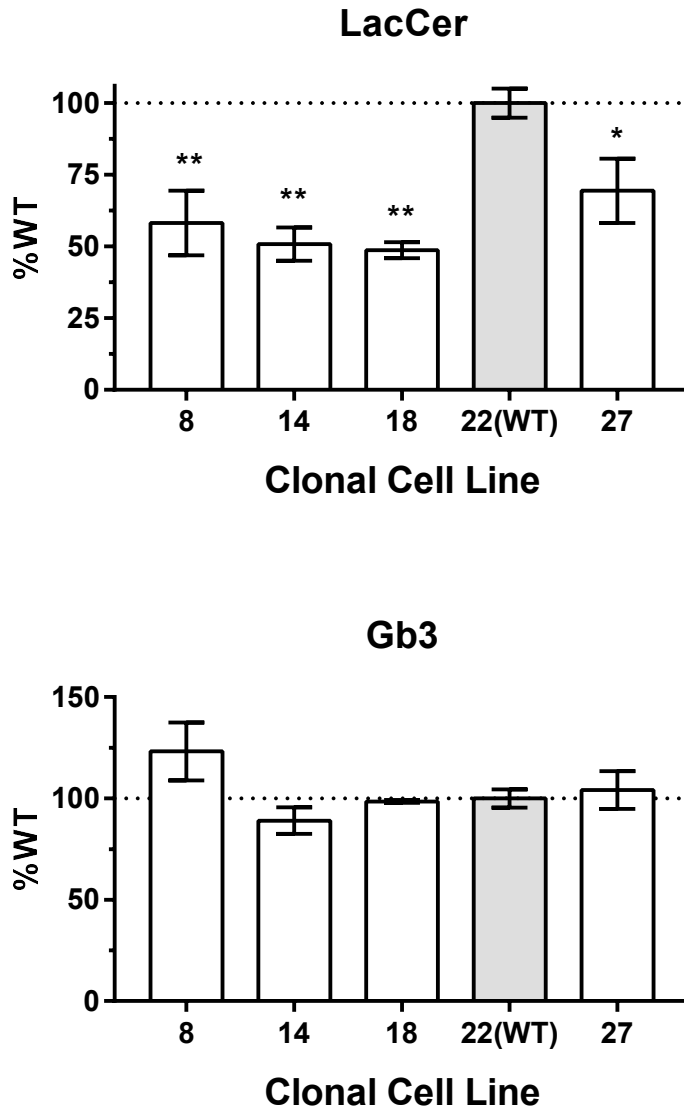
Figure A.3: CRISPR/Cas9-Exon 2 GLA(-) cell lines have no change in Gb3 accumulation. Neutral GSLs were isolated and analyzed by TLC with cupric sulfate charring as described in chapter 2. There is no significant change in Gb3 accumulation in GLA(-) cells compared to the WT control (22). LacCer is diminished in GLA(-) cells. These results correlate with that of CRISPR/Cas9 Gla-exon 1 edited clonal cell lines (fig 2.6). n=3-6. **P<0.01, *P<0.05 compared to 22(WT).
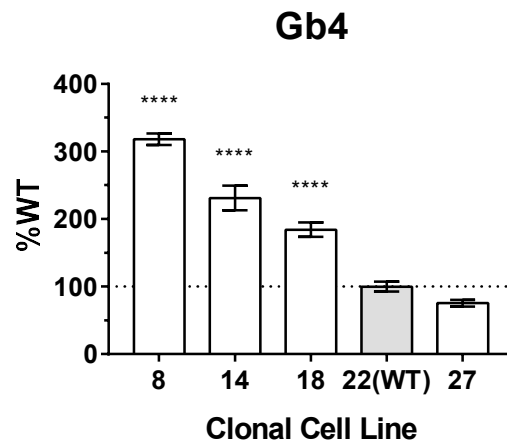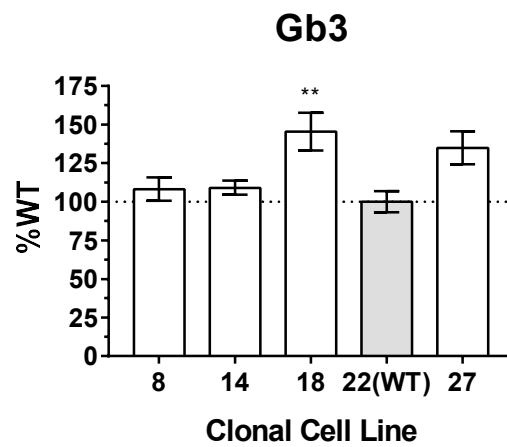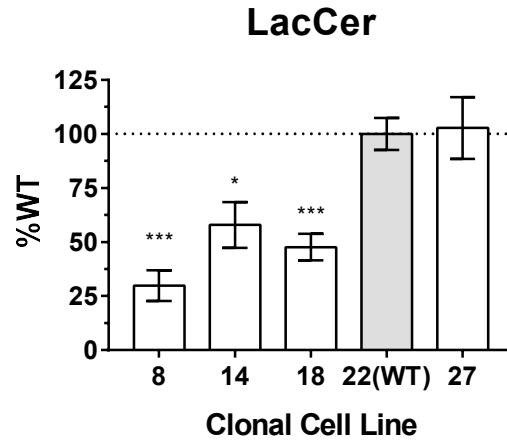
Figure A.4: CRISPR/Cas9-Exon 2 GLA(-) cell lines accumulate Gb4. Neutral GSLs were isolated and analyzed by TLC with orcinol-sulfate charring as described in chapter 2. GLA(-) clonal cell lines, 8, 14, and 18 have significantly increased amounts of Gb4 compared to 22(WT). GLA(-) clone 27 has no significant change in Gb4 or LacCer, but has slightly elevated Gb3 compared to 22(WT) (p=0.0590). Results are comparable to CRISPR/Cas9 Gla-Exon 1 edited clonal cell lines (fig. 2.7). n=3-12****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 when compared to 22(WT).
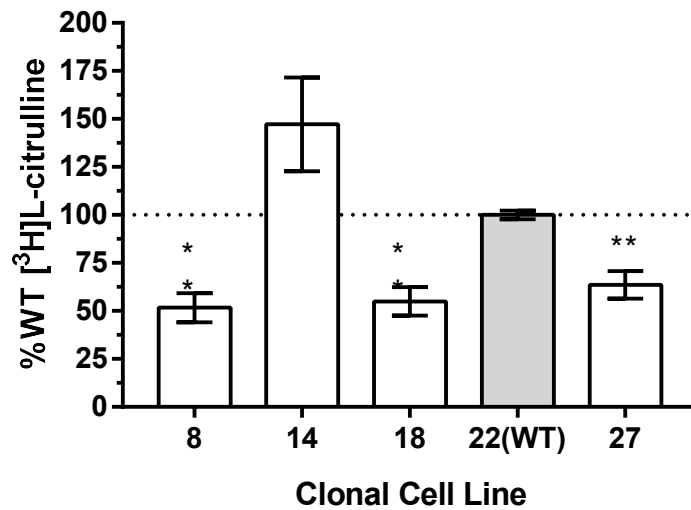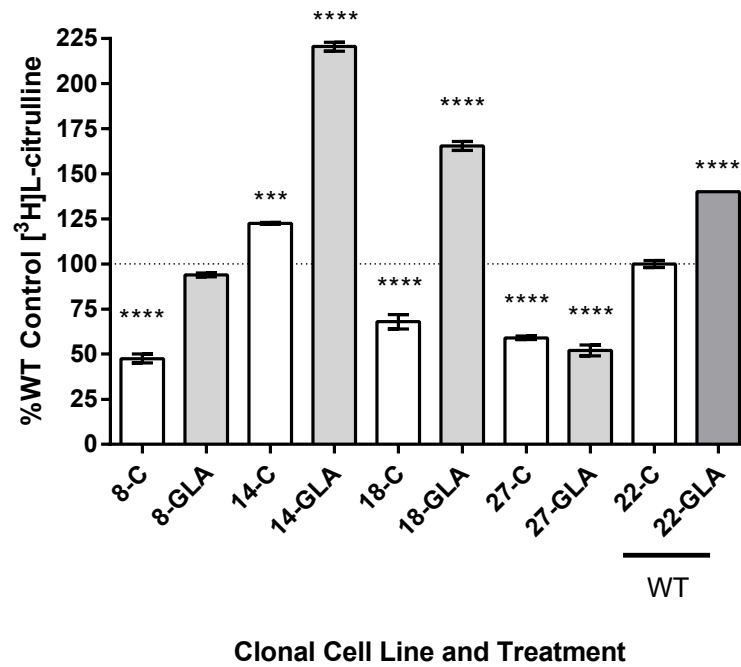
Figure A.5: CRISPR/Cas9-Exon 2 GLA(-) cell lines have decreased NOS activity. NOS activity was measured as described in chapter 2. GLA(-) clonal cell lines, 8, 18, and 27 have decreased NOS activity compared to 22(WT). GLA(-) clonal cell line 14 has elevated NOS activity, but is not statistically significant. These results correlate with CRISPR/Cas9 Gla-exon 1 edited cells (fig 2.15). n=8. **P<0.01 when compared to 22(WT).
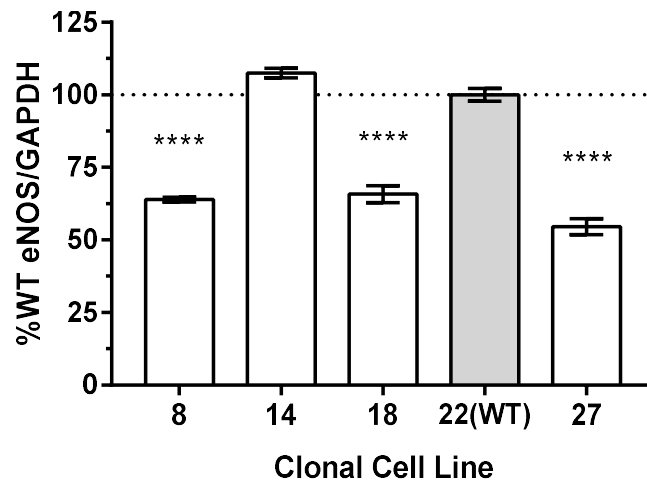

Figure A.6: CRISPR/Cas9-Exon 2 GLA (-) cell lines have decreased eNOS expression. Western blot was performed as described in chapter 2. GLA(-) clonal cell lines, 8, 18, and 27 have decreased eNOS expression compared to 22(WT). GLA(-) clonal cell line 14 has no change of eNOS expression. These results correlate with CRISPR/Cas9 Gla-exon 1 edited cells (fig 2.16). n=4. ***P<0.001 when compared to 22(WT).

Figure A.7: Exogenous GLA treatment improves NOS activity in CRISPR/Cas9-Exon 2 GLA(-) cell lines. Exogenous GLA treatment and NOS assay were performed as described in chapter 3. These results correlate with CRISPR/Cas9 Gla-exon 1 edited cells (fig 3.1). n=2. ***P<0.001, ****P<0.0001 compared to 22(WT)-control.

Correlation of µg protein in NOS assay to cpm measurement
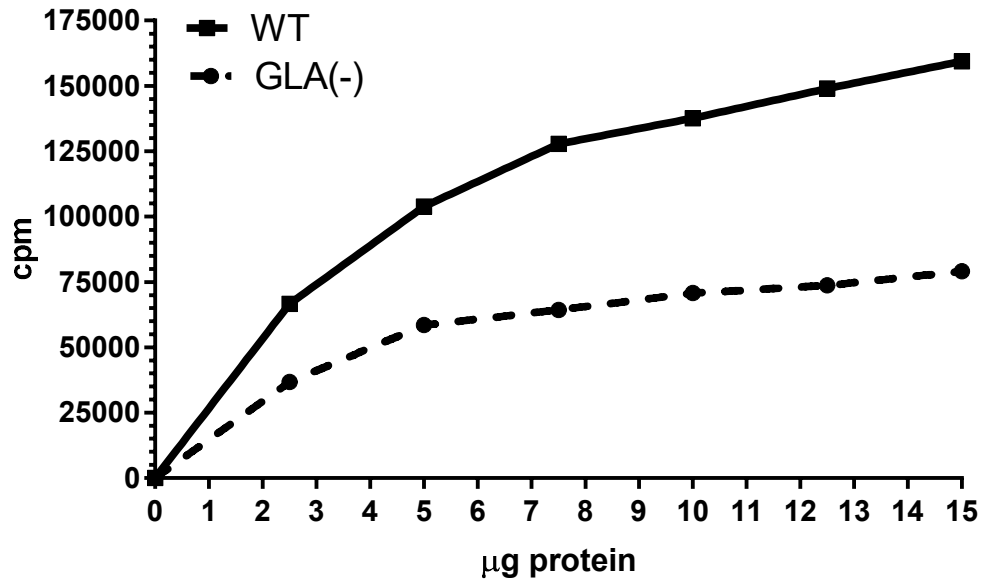
Figure A.8: NOS reaction is dependent on µg protein in reaction. NOS reaction was performed as described in chapter 2 and 3, but with different amounts of lysates from WT or GLA(-) cells. NOS assays described in chapters 2 and 3 were performed with 5-10 µg protein. The cell lysates in this figure originate from CRISPR/Cas9 Gla-Exon 1 edited clonal cell line 17(WT) and 12(GLA-). n=3

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