The Role of Acid Sphingomyelinase in Murine Placental Development

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Physiology
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2018

Abstract

Sphingolipids are a diverse class of bioactive signaling lipids that control an array of fundamental cellular processes. Recently, dysregulation of sphingolipid metabolism has been observed in placentas from complicated pregnancies. In the present study, we demonstrate that acid sphingomyelinase (Smpd1), an enzyme involved in sphingolipid metabolism, is critical for normal placental development, and that its deficiency results in fetal/intrauterine growth restriction (IUGR). Smpd1<sup>−/−</sup> placentas display several structural abnormalities, including a reduced labyrinth compartment and increased thickness of the fetal-maternal interhemal membrane. In addition, Smpd1<sup>−/−</sup> placentas exhibit a three-fold decrease in sphingosine-1-phosphate (S1P), a sphingolipid important for promoting angiogenesis. Finally, we observed several hallmarks of defective autophagy and lysosomal impairment in Smpd1<sup>−/−</sup> placentas, which could contribute to the IUGR phenotype. This study proposes Smpd1-deficient mice as a novel model of IUGR, and can aid in the understanding of molecular events which lead to IUGR and placental pathologies in humans.
Acknowledgments

I would first and foremost like to thank my supervisor, Dr. Andrea Jurisicova, for giving me the opportunity to be a part of her laboratory. Thank you for taking the time to personally teach me many important concepts and techniques, and for always being available to talk.

Thank you to my supervisory committee members, Dr. Brian Cox and Dr. Susannah Varmuza for their support and guidance throughout my Master’s.

A big thank you to all of the past and present members of the Jurisicova lab, who have made my graduate experience so enjoyable. I am eternally grateful for all of the guidance provided by Dr. Han Li, who has taught me many of the techniques presented in this thesis, and who always makes time to answer my thousands of questions. Thank you to Rosanne McQuaid, Sally Kim, Rob Babcock, Nicole Zhang and Katherine Szelag for their help, support and friendship inside and outside of the lab.

I would also like to thank my two amazing undergraduate students, Michael Pitino and Jackie Graham, for their commitment and contributions to this project.

Most importantly, I would like to thank my family, who has supported me in every way possible my entire life. Thank you to my parents who have always believed in me and who have sacrificed so much to give me the best opportunities in life. To my sister and brother, thank you for always being there for me. I would not have been able to accomplish nearly as much as I have today without their love and support.
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<tr>
<td>A-SMase</td>
<td>acid sphingomyelinase</td>
</tr>
<tr>
<td>CERs</td>
<td>ceramides</td>
</tr>
<tr>
<td>DHCers</td>
<td>dihydro-ceramides</td>
</tr>
<tr>
<td>Dec</td>
<td>decidua</td>
</tr>
<tr>
<td>EPC</td>
<td>ectoplacental cone</td>
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<tr>
<td>ExE</td>
<td>extraembryonic ectoderm</td>
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<tr>
<td>EVT</td>
<td>extravillous trophoblast</td>
</tr>
<tr>
<td>FC</td>
<td>fetal capillary</td>
</tr>
<tr>
<td>GlyT</td>
<td>glycogen trophoblast</td>
</tr>
<tr>
<td>H&amp;E</td>
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</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>IUGR</td>
<td>intrauterine growth restriction</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>JZ</td>
<td>junctional zone</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
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<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>Lab</td>
<td>labyrinth</td>
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<tr>
<td>LAMP-1</td>
<td>lysosomal associated membrane protein 1</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated protein-1 light chain 3</td>
</tr>
<tr>
<td>MBS</td>
<td>maternal blood space</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PE</td>
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</tr>
<tr>
<td>PL</td>
<td>placental lactogen</td>
</tr>
<tr>
<td>PMSG</td>
<td>pregnant mare serum gonadotropin</td>
</tr>
<tr>
<td>rcf</td>
<td>relative centrifugal force</td>
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<tr>
<td>Sa</td>
<td>sphinganine</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SPH</td>
<td>sphingosine</td>
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<td>sphingosine kinase</td>
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<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>ST</td>
<td>syncytiotrophoblast</td>
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<tr>
<td>SpT</td>
<td>spongiotrophoblast</td>
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<tr>
<td>TE</td>
<td>trophectoderm</td>
</tr>
<tr>
<td>TGC</td>
<td>trophoblast giant cell</td>
</tr>
<tr>
<td>TFEB</td>
<td>transcription factor EB</td>
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<tr>
<td>WT</td>
<td>wildtype</td>
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Chapter 1

1 Introduction

1.1 The Placenta

The placenta is a transient, yet essential organ that connects the fetus to the mother during pregnancy. It acts as the primary interface that facilitates the exchange of nutrients, gases and wastes between the mother and baby. In addition to its role in mediating fetal-maternal exchange, the placenta also provides immune protection to the fetus, and produces various hormones and angiogenic factors that are crucial for regulating maternal adaptations to pregnancy (Georgiades, Fergyson-Smith, and Burton 2002). Any event that impairs the development of the placenta can compromise the growth and survival of the fetus. Indeed, placental dysfunction has been linked to several life-threatening pregnancy disorders, including fetal growth restriction and maternal preeclampsia. While placental abnormalities have been observed in these disorders, the mechanisms by which they occur are still poorly understood. This lack of knowledge is partially attributed to the fact that experimental manipulation of the human placenta is particularly challenging and often hampered by practical and ethical restrictions. To circumvent this, scientists have relied on the use of animal models to study placental development and the etiology of placental disease. The mouse is a particularly useful model due to its similarities to the human placenta, relatively short lifespan, and ease of genetic and physiological manipulation.

1.1.1 The mouse placenta as a model of human placentation

Although distinct, considerable similarities exist between the human and mouse placenta, including several analogous cell types (Georgiades, Fergyson-Smith, and Burton 2002). The mature mouse placenta consists of three distinct anatomical regions: the outer decidua basalis, the middle junctional zone and the inner labyrinth. Likewise, the human placenta contains three regions which are structurally analogous to that of the mouse: the decidua basalis, the basal plate, and the chorionic villi, also referred to as the fetal placenta. In both species, the decidua basalis is the maternal compartment of the placenta which is composed of maternal uterine cells that have undergone decidualization. Maternal blood enters the placenta via the maternal spiral arteries
(SAs) within the decidua, which coalesce to deliver blood to the fetal unit of the placenta. Bordering the decidua is an avascular region termed the junctional zone (JZ) in mice, or basal plate in humans, which forms the “junction” between the maternal compartment and the fetal placenta/labyrinth. Although its function is not fully understood, the JZ is believed to provide structural support to the surrounding vasculature of the placenta, and is an important source of pregnancy related hormones. Finally, the labyrinth layer of the mouse placenta, which is closest to the fetus, is the primary site of fetal-maternal exchange. It consists of fetal capillaries (FC) and maternal blood spaces (MBS) or ‘sinuses’ which are separated by fetal-derived trophoblast cells that facilitate oxygen, nutrient and waste exchange between fetal and maternal blood. The function of the mouse labyrinth is completely analogous to that of the human chorionic villi (Georgiades, Fergyson-Smith, and Burton 2002).

In addition to structural similarities, the human and mouse placenta have also been shown to share several genetic and molecular properties. A recent microarray analysis revealed highly conserved gene expression patterns between human and mouse placentas (Soncin et al. 2018). Overall, there was a closer fit between the patterns observed when the inter-species comparison was restricted to human placentas up to gestational week 16 (thus, excluding full-term placentae), suggesting that murine placental development parallels the first half of human placentation (Soncin et al. 2018). The human placenta has also been shown to express several genes which are known to be required for proper development of the mouse placenta (Rossant and Cross 2001). Importantly, over 80% of genes which are known to be involved in abnormal development or dysfunction of the mouse placenta, are co-expressed in human (Cox et al. 2009). Together, the structural, transcriptional, molecular and functional analogies between human and mouse placentas warrant the use of the mouse as a valid model for the study of human placentation.
1.2 Murine placentation

The early mouse blastocysts (d3.5) contains three specified lineages—the inner cell mass (ICM), primitive endoderm (PE), and trophectoderm (TE). The TE gives rise to the trophoblast lineage, a specialized epithelial cell type that makes up the majority of the fetal portion of the placenta. It contains two populations of TE cells based on its proximity to the ICM. Polar TE cells are in direct contact with the ICM and mural TE cells surround the blastocoel. The post-implantation development of the polar and mural TEs is highly regulated by signals originating from the ICM, the details of which are out of scope for this thesis (reviewed in Simmons and Cross 2005). Following implantation (~d4.5), mural TE cells stop dividing and instead begin to endoreduplicate their DNA, forming large, polyploid trophoblast giant cells (TGCs) in a process termed primary differentiation. Primary TGCs are highly invasive cells that facilitate implantation by secreting various proteases, including cathepsin and metalloproteinases, which help to digest the surrounding maternal tissue (Screen et al. 2008). In contrast, polar TE cells overlying the ICM continue to proliferate and give rise to nearly all of the trophoblast lineages that make up the mature placenta. After implantation, polar TE cells form the diploid extra-embryonic ectoderm (ExE) and ectoplacental cone (EPC), which together contain progenitors for the labyrinth trophoblast, spongiotrophoblast (SpT), glycogen trophoblast (GlyT) and secondary TGCs.

1.2.1 Secondary TGCs – hormonal factories and leaders of invasion

After implantation, a subpopulation of EPC progenitors differentiate into secondary TGCs which, similar to primary TGCs, contain large, polyploid nuclei and have highly invasive properties (Screen et al. 2008; Varanou et al. 2006). Secondary TGCs play a number of important roles throughout placental development and pregnancy, including facilitating placental invasion, maternal vasculature remodeling, and the secretion of important pregnancy related hormones. Four different types of secondary TGCs are recognized, which are classified based on their location and function within the placenta: sinusoidal (S-TGC), parietal (P-TGC), maternal canal-associated (C-TGC) and spiral artery-associated (SA-TGC) (Hu and Cross 2010).

P-TGCs surround the transient parietal yolk sac of the early embryo (d6.5), which acts as the initial site of fetal-maternal exchange (Hu and Cross 2010). As development proceeds, they continue to invade into the maternal decidua, eventually forming a layer of cells within the
mature placenta that separates the decidua from the fetal compartment (Figure 1). P-TGCs have been shown to express specific placental lactogen (PL) hormones, including Prl3d, which distinguishes them from other secondary TGC subtypes (Simmons et al. 2008). PLs are a family of prolactin-like hormones that are exclusively expressed in the placenta, and which regulate several key maternal adaptations to pregnancy, including mammary gland development and insulin secretion (Hu and Cross 2010). The spatial distribution and highly invasive nature of P-TGCs makes them functionally analogous to the early extravillous cytotrophoblast (EVTs) that invade into the decidua basalis of the human placenta (Georgiades, Fergyson-Smith, and Burton 2002; Rossant and Cross 2001).

Another family of highly invasive secondary TGCs within the mouse placenta are the SA-TGCs. Around mid-gestation, SA-TGCs invade beyond the P-TGC layer and digest the smooth muscle surrounding the maternal SAs, resulting in trophoblast-lined or hemochorial blood spaces (Adamson et al. 2002). Hemochorial remodeling of maternal SAs also occurs in the human placenta, and is mediated by endovascular EVTs (Pijnenborg et al. 1981). This process is believed to promote blood flow to the growing fetus, as decreased remodeling has been shown to result in reduced placental perfusion (Verlohren et al. 2010). In addition to SA-TGCs, both C-TGCs and S-TGCs are also in direct contact with maternal blood. C-TGCs line the large central canals that bring maternal blood from SAs to the labyrinth, and S-TGCs are found within the labyrinth, surrounding the maternal blood sinuses (Figure 1).

1.2.2 Junctional zone – structural and hormonal support layer

Progenitors within the EPC also give rise to two distinct trophoblast cell types that form the junctional zone (JZ) of the mature placenta— the spongiotrophoblast (SpT) and glycogen trophoblast (GlyT). While these cells both exist within the JZ, they can be easily differentiated based on their morphology and spatial distribution. SpT cells form a dense layer of ovoid, mononuclear cells between the outer P-TGCs and the labyrinth (Figure 1). They express certain placental lactogens such as Prl3a1, Pr4a1 and Prl8a9, as well as other hormones that are essential for the maintenance of pregnancy (Georgiades, Fergyson-Smith, and Burton 2002). GlyT cells on the other hand, are only detected within the JZ beginning at ~d12.5. They are also believed to arise from the EPC, and have gene expression patterns that closely resemble SpT cells (Bouillot et al. 2006; Coan et al. 2006). However, GlyT cells are morphologically different, with small condensed nuclei and glycogen filled granules occupying the cytoplasm. Moreover,
unlike SpT cells, they have migratory properties. After ~d13.5, GlyT cells begin to migrate from the JZ and into the interstitial space of the decidua (Figure 1). This invasion is accompanied by a drastic increase in cell number that continues until ~d15.5 (Coan et al. 2006). While their ultimate function in the placenta is still not well understood, it has been postulated that they may act as a nutritional reserve for the surrounding placental vasculature. They have also been shown to express several placental lactogens, including Prl7c1, Prl4a1 and Prl8a9, and may have additional hormonal roles in the maintenance of pregnancy (Simmons et al. 2008).

1.2.3 Labyrinth – fetal-maternal exchange unit

As the embryo continues to grow, so does its metabolic demand for oxygen and nutrients. The formation of the placental labyrinth allows for a higher capacity of fetal-maternal exchange, in order to meet the metabolic needs of the rapidly growing fetus. Labyrinth formation is initiated by the merging of the chorionic epithelium (derived from the ExE) to the mesodermal structure called the allantois—a process termed chorioallantoic fusion. The allantois forms folds in the chorion that become fetal blood vessels. At the same time, the trophoblast from the chorion undergoes extensive villous branching to create an intertwined network of trophoblast and open sinuses that are filled with maternal blood. During branching, the chorionic trophoblast also begins to differentiate to form specific trophoblast cells of the labyrinth. In the mouse labyrinth, two layers of multinucleated syncytiotrophoblast (ST) cells line the fetal and maternal blood spaces. Syncytiotrophoblast layer I (ST-I) lines the maternal blood spaces, while syncytiotrophoblast layer II (ST-II) is in direct contact with fetal endothelial cells. This ST interface is commonly referred to as the interhemal membrane, and is the main regulator of substrate exchange between fetal and maternal blood (Figure 1). ST cells express various transporters that facilitate nutrient exchange, such as glucose (GLUT), monocarboxylate lactate (MCT), and amino acid transporters (Nagai et al. 2010). As differentiation proceeds, ST cells fuse, bringing fetal capillaries and maternal blood spaces into close proximity. In both the human and mouse placenta, fusion is mediated by the transmembrane proteins Syncytin (Sha et al. 2000). Syncytin 1 and Syncytin 2 in the human, and the homologous Syncytin a and b in mouse, are placenta specific envelope genes of retroviral origin that have been shown to induce cell-to-cell fusion in vitro. Deletion of Syncytin a in the mouse results in mid-gestational embryonic lethality due to improper fusion of the syncytial layers, and decreased vascularization of the labyrinth (Dupressoir et al. 2009).
The labyrinth also contains a population of mononuclear sinusoidal trophoblast giant cells (S-TGC) that reside adjacent to the maternal blood spaces (MBS). While their role in the labyrinth is still not fully understood, they are believed to facilitate nutrient exchange between fetal and maternal blood. S-TGCs can be distinguished from other TGCs by their unique expression of placental specific cathepsins, such as Cathepsin Q (CtsQ) and Cathepsin 6 (Cts6) (Huh 2000; Sol-Church, Frenck, and Mason 2000). Genetic ablation of S-TGCs results in fetal IUGR and embryonic death by d18.5, highlighting the importance of these cells in placental function (Outhwaite, McGuire, and Simmons 2015).

**Figure 1. The mature murine placenta at gestational day 15.5 (d15.5).**
The mature murine placenta consists of the maternal decidua (Dec) and three fetal regions: a layer of parietal trophoblast giant cells (P-TGC), the junctional zone which contains spongiotrophoblast (SpT) cells and glycogen trophoblast (GlyT) cells, and the labyrinth (Lab). The Lab contains maternal blood spaces/sinuses (MBS) and fetal capillaries (FC), separated by two layers of syncytiotrophoblast cells (STI and II) and a discontinuous layer of mononuclear sinusoidal trophoblast giant cells (S-TGC). This is commonly referred to as the interhemal membrane (black arrow). By d15.5, spiral artery associated TGCs (SA-TGC) and GlyT cells have invaded into the Dec. C-TGC=canal associated TGC. Figure adapted from Hu and Cross 2010.
1.3 Placental disorders

Placental abnormalities have been associated with two of the most common and severe disorders of human pregnancy—maternal preeclampsia and fetal growth restriction. Preeclampsia (PE) is a complex, heterogenous disorder of pregnancy that is defined by the onset of maternal hypertension and proteinuria in the latter half of gestation (Proia and Hla 2015). It affects up to 5% of all pregnancies and is one of the leading causes of fetal and maternal mortality and morbidity (Vigil-De Gracia 2009). Although the cause of PE remains elusive, placental dysfunction is assumed to play a central role since removal of the placenta in most cases results in termination of the disease. PE placentas exhibit several pathological features, including poor trophoblast invasion and a lack of maternal spiral artery remodeling (Lyall, Robson, and Bulmer 2013). This is believed to result in diminished uteroplacental circulation, which creates a hypoxic environment in the placenta leading to increased trophoblast cell death (Badalà, Nouri-mahdavi, and Raoof 2008; Crocker et al. 2003; N Soleymanlou et al. 2005; Nima Soleymanlou et al. 2007). In addition to elevated cell death, increased proliferation of the villous trophoblast has also been observed PE placentas. This increased proliferation and death (i.e. trophoblast turnover) leads to the formation of syncytial “knots”, which are clusters of dying syncytiotrophoblast cells that protrude from the surface of the terminal villi. Syncytial knots are shed into the maternal circulation and are believed to trigger an inflammatory response in the mother, leading to the canonical symptoms of PE.

Another common pregnancy disorder that exhibits placental involvement and can occur in severe cases of PE, is intrauterine growth restriction (IUGR). IUGR is a condition in which the fetus fails to reach its genetically determined growth potential. It is estimated to affect approximately 8% of all pregnancies worldwide and is the cause of 10% of fetal mortalities and stillbirths (Mandruzzato et al. 2008). In addition to endangering the life of the fetus, comprised in utero growth can contribute to several long-term health complications including obesity, type-II diabetes, cardiovascular disease and cognitive disorders (Bamfo and Odibo 2011; Chan et al. 2010; Geva 2006). IUGR is most commonly due to placental insufficiency, a condition in which the placenta is unable to provide an adequate supply of oxygen and nutrients to the developing fetus (American College of and Gynecologists 2013). A combination of reduced vascularization, as well as poor trophoblast development and function has been observed in placentas from IUGR pregnancies. In these placentas, extra-villous cytotrophoblast cells (EVTs) exhibit abnormally
shallow invasion into the maternal decidua and surrounding vasculature. In some cases, increased thickness and abnormal morphology of the syncytiotrophoblast layer (ST) is observed, presumably impairing the exchange of oxygen and nutrients between the mother and fetus (Krebs et al. 1996; Macara et al. 1996). These placentas also exhibit heightened levels of trophoblast cell death, mainly among the villous trophoblast (Scifres and Nelson 2009).

It is clear that normal development and function of the placenta requires a delicate balance of trophoblast proliferation, differentiation and death. Imbalances in these processes are hallmarks of PE and IUGR. Unfortunately, the molecular pathways leading to increased trophoblast death, turnover and aberrant function are still poorly understood. Recent work has implicated sphingolipids, a unique class of bioactive signaling lipids, as key morphogens and second messengers that regulate a variety of fundamental cellular processes including proliferation, differentiation, death and survival. Interestingly, dysregulation of these lipids has been observed in placentas from IUGR and PE pregnancies, indicating that they may play an important role in the etiology of placental disease (Chauvin et al. 2015; Melland-Smith et al. 2015; Mizugishi et al. 2007).
1.4 Sphingolipids

Sphingolipids are a distinct class of lipids that are essential components of all eukaryotic cell membranes. They are composed of a common sphingosine (SPH) backbone which consists of a polar amine head group attached to a single, long-chain hydrophobic tail (2-amino-4-octadecene-1,3diol) (Figure 2). Various sphingolipid metabolizing enzymes can modify this backbone and give rise to a variety of unique sphingolipids, each with its own specific cellular function. For instance, the addition of a fatty acid residue to the amine headgroup of SPH yields a ceramide (CER), while the addition of a phosphate group will form sphingosine-1-phosphate (S1P). In the plasma membrane, sphingolipids can interact with cholesterol to form sphingolipid-cholesterol rich microdomains, also known as “lipid rafts”. These lipid rafts mediate receptor clustering at the plasma membrane, which facilitates signal transduction of extracellular and intracellular ligands (Cremesti, Goni, and Kolesnick 2002).

Apart from their structural roles in cellular membranes, several sphingolipid metabolites can also act as bioactive signaling molecules that regulate a variety of important cellular processes. CER, for example, has long been established as a pro-apoptotic signaling molecule and second messenger, while S1P has been shown to promote cell survival and proliferation (Bartke and Hannun 2009). Due to the opposing effects of these sphingolipids, their metabolism must be carefully regulated to ensure cellular homeostasis.

![Sphingolipid structure](image)

**Figure 2. Basic structure of a sphingolipid.**
All sphingolipids are made from sphingoid bases, which contain a polar amine head group attached to a single, long-chain hydrophobic tail. In mammals, the most common sphingoid base is sphingosine (SPH).
1.4.1 Sphingolipid metabolism

Sphingolipid metabolism is tightly regulated by a variety of enzymes. In this metabolic pathway, CER is the central lipid precursor whose breakdown gives rise to all other sphingolipid metabolites. CER can be produced \textit{de novo} at the endoplasmic reticulum (ER), beginning with the condensation of serine and palmitoyl-CoA catalyzed by the enzyme serine palmitoyl-CoA transferase (SPT). Alternatively, CER can be produced via the hydrolysis of sphingomyelin (SM) by sphingomyelin phosphodiesterase enzymes, also referred to as sphingomyelinases (SMases), which exist primarily in the endo-lysosome and at the plasma membrane (PM) (Bartke and Hannun 2009). SMases cleave the phosphodiester bond of SM, releasing phosphorylcholine and generating CER (Figure 3). The production of CER by SMases is triggered by a variety of stress stimuli (i.e. cytokines, environmental stresses, or chemo-therapeutic agents) (Schenck et al. 2007). Inhibition of SMase activity, either through the use of genetic models or inhibitory agents, has been shown to prevent stress induced apoptosis and cell death (Smith and Schuchman 2008; Yoshimura et al. 1999). These findings highlighted the bioactive role of CER as a pro-apoptotic second messenger in the cellular response to stress, as well as the importance of SMases in mediating this response.
Figure 3. Production of ceramide by sphingomyelinase enzymes (SMase).
SM is the most abundant sphingolipid in plasma membranes, and is believed to be the main source of CER. SMases cleave the phosphodiester bond of SM, releasing phosphorylcholine and generating CER.

In addition to CER, several downstream sphingolipid metabolites that are produced from its breakdown have bioactive signaling properties. CER is broken down by cerimidase (CDase) to produce SPH, which like CER, has been shown to mediate apoptosis. SPH is subsequently phosphorylated by sphingosine kinases 1 and 2 (SPHK1/2) to produce S1P. Unlike its precursors, S1P has distinct roles in promoting cell growth and survival, and is an inhibitor of CER mediated apoptosis (Cuvillier et al. 1996). In addition, S1P has been shown to be an important regulator of vasculogenesis and angiogenesis, as it promotes endothelial cell migration, survival, proliferation and permeability. Due to the absence of a long fatty acyl-CoA chain, S1P is not restricted to the membrane and can be secreted extracellularly. It is mainly secreted by red blood cells, endothelial cells and platelets, and is found in highest concentration in blood plasma (Proia and Hla 2015). Extracellular S1P binds to a family of five G-protein-coupled receptors: S1P₁-S1P₅. These S1P receptors are largely concentrated in endothelial cells and vascular smooth muscle cells (Proia and Hla 2015). S1P₁ null embryos die in utero around d12.5 due to a lack of smooth muscle remodeling of blood vessels and excessive bleeding (Allende, Yamashita, and Proia 2003; Liu et al. 2000). In addition, Sphk1/2 null embryos, which have no detectable levels of S1P, die at mid-gestation due to excessive hemorrhaging, defective smooth muscle coverage of blood vessels, and poor endothelial cell migration (Mizugishi et al. 2005). These findings solidified the importance of S1P in embryonic vascular development.
Figure 4. The sphingolipid metabolic pathway.

Sphingolipid synthesis starts with the production of ceramide (CER). Cer can be generated de novo from the condensation of serine and palmitoyl-CoA by the enzyme serine palmitoyl-CoA transferase (SPT), yielding 3-ketosphinganine which is further reduced to Sphinganine (Sa) by the enzyme 3-ketosphinganine reductase (KSR). Sa is then coupled to a fatty acyl-CoA chain by ceramide synthase (CerS) to produce dihydroceramide (DHCer), which is finally converted to CER by the enzyme desaturase (DES). Alternatively, CER can be generated by the hydrolysis of sphingomyelin (SM) by the action of sphingomyelinases (SMases). CER can be further broken down by ceramidases (CDase) to produce sphingosine (Sph). Sph is phosphorylated by sphingosine kinases (SPHK), yielding sphingosine-1-phosphate (S1P). These reaction are reversible (CS=ceramide synthase, SPP=sphingosine phosphate phosphatase). CER and SPH are pro-apoptotic signaling lipids, while S1P promotes proliferation and cell survival.
1.5 Acid Sphingomyelinase

1.5.1 Function and regulation

SM is the most abundant sphingolipid in cellular membranes and its hydrolysis by the action of SMase enzymes accounts for a significant portion of CER produced in the cell (Ueda 2015). Thus far, four types of SMases have been identified which can be categorized based on their subcellular location, pH optima and cation dependence. These include: ubiquitous lysosomal acid sphingomyelinase (A-SMase), Zn2+ dependent secreted A-SMase (present in serum), Mg2+ dependent neutral sphingomyelinase (N-SMase), and alkaline SMase (Marchesini and Hannun 2004). Of these SMases, the acidic and neutral forms are the most well studied due to their ability to mediate CER production in the cellular response to stress and apoptosis (Smith and Schuchman 2008; Yoshimura et al. 1999). Moreover, there has been an increased interest in the study of A-SMase because its deficiency results in the human lysosomal storage disorder Niemann-Pick disease A and B (NPDA&B) (Edward H. Schuchman 2007).

Acid sphingomyelinase was the first member of the SMase family to be cloned, and was thus designated sphingomyelin phosphodiesterase 1 (SMPD1). SMPD1 maps to chromosome 11p15.1-11p15.4 and contains 6 exons, spanning ~6 kb (E H Schuchman et al. 1992). A single mRNA gives rise to at least 3 splice variants, however transcript 1 is the only protein coding variant that generates the catalytically active enzyme. Post-translation modifications of A-SMase, including proteolytic cleavage, glycosylation, and phosphorylation regulate both the targeting of the enzyme to the lysosome and its catalytic activity. The biosynthesis of the enzyme begins with a 75kDa pre-proenzyme, which is rapidly cleaved to a 72kDa proenzyme in the ER/golgi. A minor portion of the 75kDa pre-proenzyme is also cleaved into a 57kDa isoform that has very little enzymatic activity (Quintern et al. 1987). The 72kDa isoform is heavily glycosylated by the addition of mannose-6-phosphate linked oligosaccharides, which facilitates its targeting to the lysosomal compartment (Ferlinz et al. 1997). Once in the lysosome, the 72kDa proenzyme is further processed to a 70kDa mature and active lysosomal A-SMase. This form has six potential N-linked glycosylation sites, and functional studies have shown that at least 5 of these sites are important for the function of the enzyme (Ferlinz et al. 1997; Newrzella and Stoffel 1996). Differential trafficking of the 72kDa proenzyme also produces a non-lysosomal A-SMase that is secreted extracellularly.
The catalytic activity of the mature A-SMase is influenced by several additional factors including pH and cation requirement. Lysosomal A-SMase requires a low pH for proper enzymatic activity (Callahan et al. 1983), while secretory A-SMase has been reported to function at a neutral pH. Additionally, both forms of A-SMase are metalloenzymes, which require zinc (Zn$^{2+}$) for proper functioning. Lysosomal A-SMase cleaves SM both in the endo-lysosomal compartment, and at the plasma membrane (PM). Physical translocation of the enzyme from the lysosomal lumen to the outer leaflet of the PM facilitates the generation of CER, causing membrane reorganization and the formation of CER-rich lipid rafts that mediate receptor clustering and promote signal transduction (Figure 5) (Cremesti, Goni, and Kolesnick 2002; Jin et al. 2008). Disturbance of lipid rafts by cholesterol depletion has been shown to reduce the translocation A-SMase to the cell surface, and impair signaling events (Lacour et al. 2004). Secretory A-SMase on the other hand, is believed to participate in SM hydrolysis extracellularly, specifically among lipoproteins in serum, however the mechanism is still not well understood (Schissel et al. 1998).

**Figure 5. Translocation of lysosomal A-SMase and formation of lipid rafts.**
Acid sphingomyelinase is most often found within lysosomes, anchored to the inner lysosomal membrane. Physical translocation of the enzyme to the plasma membrane (PM) allows for cleavage of PM-bound SM and production of ceramide (CER) enriched microdomains. These
microdomains fuse to form large lipid platforms (lipid rafts) that cluster receptors (depicted as blue lines) and facilitate signaling processes.

1.5.2 Niemann-Pick disease

Inherited deficiency of A-SMase results in the human lysosomal storage disorder known as Niemann-Pick disease A and B (NPDA&B) (Jones et al. 2008). Lysosomal storage disorders (LSD) are characterized by a progressive accumulation of undigested macromolecules within the cell due to lysosomal dysfunction. In the case of NPDA&B, deficiency of A-SMase results in an accumulation of unmetabolized SM within cells, eventually leading to impaired cellular function and in some cell types, increased death (Ledesma et al. 2011). Various mutations within the SMPD1 gene, including point mutations and splicing abnormalities, are known to contribute to the disease (Simonaro et al. 2002). The severity of symptoms depends on the type of mutation present, and its effect on levels of residual A-SMase activity. Two forms of NPD are classified with respects to symptoms presented—NPD type A (NPDA) and NPD type B (NPD) (Edward H. Schuchman 2007).

NPDA is characterized by a complete loss of A-SMase activity which leads to rapid neurodegeneration, hepatosplenomegaly, pulmonary failure and death at ~3 years of age in affected individuals. In contrast, NPDB is the non-neuronal, less severe form of the disease, in which some residual activity of A-SMase is retained. For this reason, NPDB is often compatible with survival into early adulthood, although hepatosplenomegaly, and pulmonary dysfunction are still present. The cause of these symptoms is mainly attributed to heightened levels of SM, which has been observed in the lungs, spleen, liver and skin fibroblasts from patients with NPDA&B. Patients will also often display several secondary metabolic defects, in addition to an accumulation of SM. Specifically, low platelet counts have been observed in NPDB patients, as well as a combination of heightened plasma triglycerides, high LDL and low HDL cholesterols (Mcgovern et al. 2009; McGovern et al. 2004). Interestingly, although NPDA&B is an autosomal recessive disease, some carriers have been reported to exhibit symptoms (Lee et al. 2003). Epigenetic modification of SMPD1, specifically hypermethylation of the paternal allele, has been reported in lymphoblasts and skin fibroblasts of patients affected with NPDB, which contributes to some of the clinical variability observed in carriers (Calogera M Simonaro et al. 2006).

While the common feature NPDA&B is an accumulation of SM, secondary effects on overall sphingolipid metabolism are less well known (Edward H. Schuchman 2007). In addition,
although neurodegeneration is observed in NPDA patients, a thorough analysis of this phenotype has been problematic in humans due to ethical and practical issues. A-SMase deficient mice that model human NPDA have thus been a useful tool in the study of the pathogenesis of the disease.

1.5.3 Acid Sphingomyelinase deficient (Smpd1<sup>−/−</sup>) mice

Murine A-SMase is encoded by <i>Smpd1</i>, which maps to chromosome 7 and contains 6 exons, spanning ~4.5 kb. A-SMase is highly conserved between human and mice, sharing approximately 81% sequence identity at the cDNA level, and 82% sequence identity at the amino acid level (Newrzella Dieter and Wilhelm 1992). Much like the human protein, murine A-SMase appears as a 70/72kDa protein that contains six N-glycosylation sites which are heavily glycosylated by high-mannose type oligosaccharides. In 1995, Horinouchi et. al created an A-SMase deficient mouse line (<i>Smpd1<sup>−/−</sup></i>) by the insertion of a replacement vector containing a PGK-neomycin (<i>neo</i>) expression cassette in exon 2 of <i>Smpd1</i>, thereby disrupting the coding sequence (Horinouchi et al. 1995). Using radiolabeling activity assays, Horinouchi et. al showed that <i>Smpd1<sup>−/−</sup></i> mice exhibit no detectable levels of A-SMase activity in the brain, heart, kidney, liver, lung or spleen, while heterozygote mice (<i>Smpd1<sup>+</sup>/−</i>) display approximately 50% of normal A-SMase activity.

<i>Smpd1<sup>−/−</sup></i> mice are viable, but begin to exhibit symptoms of NPDA at ~3-4 months of age including rapid neurodegeneration, ataxia, mild tremors, dehydration, weight loss and premature death at ~6-8 months of age. The inheritance pattern of the phenotype is autosomal recessive, with heterozygous mice (<i>Smpd1<sup>+/−</sup></i>) displaying no overt phenotype. Histological analysis of brains from <i>Smpd1<sup>−/−</sup></i> mice revealed a loss of Purkinje cells as well as atrophy of various regions, which largely explains the neurological phenotype observed in NPDA (Ledesma et al. 2011). However, unlike human NPDA, no sign of hepatosplenomegaly has been observed in <i>Smpd1<sup>−/−</sup></i> mice (Horinouchi et al. 1995). SM accumulation has been reported in the brain, liver, lungs, and eyecups from <i>Smpd1<sup>−/−</sup></i> mice. Interestingly, the degree of SM accumulation has been shown to be cell-type specific (Ledesma et al. 2011; Lozano 2001; Wu et al. 2015). This most likely reflects the amount of A-SMase activity required in certain cell types, and possible compensation by other SMases, such neutral SMase (N-SMase). For example, while N-SMase activity was found to be similar in <i>Smpd1<sup>−/−</sup></i> and WT kidneys, it was almost one-half decreased in brain tissue of <i>Smpd1<sup>−/−</sup></i> mice, indicating that although N-SMase may be able to compensate for the loss of A-SMase in the kidney, this may not be the case in the brain (Horinouchi et al. 1995).
Given the prominent neurological phenotype of NPDA, several studies have focused on analyzing the underlying molecular defects in brains and neurons from Smpd1−/− mice, which has provided a great deal of insight into the pathogenesis of the disease. Thin layer chromatography analysis of lipid extracts from lysosomal, Golgi and plasma membrane (PM) fractions of hippocampal neurons demonstrated that SM accumulates both within the lysosome and at the PM (Chen et al. 2007). Additional histological studies revealed an accumulation of enlarged lysosomes within Smpd1−/− neurons, which also contained elevated levels of cholesterol (Passini et al. 2005; Sarna et al. 2001). Cholesterol accumulation within lysosomes of macrophages isolated from Smpd1−/− mice has also been reported, and was shown to be a result of defective trafficking and efflux through the endo-lysosomal pathway. These studies suggested that the secondary metabolic defects observed in NPDA&B (i.e., cholesterol accumulation) may be due to defective lysosomal clearance, which could lead to a build-up of lipids and undigested macromolecules within the cell. Indeed, over the past several years there has been more evidence suggesting that cellular clearance pathways which heavily rely on lysosomes, such as autophagy, are impaired due to Smpd1 deficiency. Recently, Rodriguez et al. showed that neurons from Smpd1−/− mice and skin fibroblasts from NPDA patients exhibit features of defective autophagy, including accumulated autophagolysosomes and a build-up of undegraded material (Gabandé-Rodríguez et al. 2014). Similar findings were reported in a study by Wu et al., which revealed that autophagosomal markers are increased in eyecups from Smpd1−/− mice, as well as skin fibroblasts of NPDA patients (Wu et al. 2015). Moreover, growing evidence suggests a role for A-SMase in regulating autophagy, indicating that this pathway may be disrupted in NPDA&B and in Smpd1−/− mice (Perrotta et al. 2015).
1.6 Autophagy

Autophagy is an intracellular degradation and recycling process that plays a critical role in nutrient sensing and cellular homeostasis. In this pathway, cytoplasmic components such as long-lived proteins, lipids, nucleotides and organelles are engulfed by double-membrane-bound structures known as autophagosomes, and subsequently delivered to lysosomes for degradation (Figure 6) (Klionsky et al. 2011). These digested materials are then either recycled back into the cell where they can be used as building blocks for other complex molecules, or excreted from the cell. Three main forms of autophagy exist: macroautophagic, microautophagy and chaperone mediated autophagy (CMA). During macroautophagy, cytoplasmic cargo is delivered to the lysosome via an intermediate autophagosome, while in the case of microautophagy and CMA, cytoplasmic contents are taken up directly by the lysosome without an intermediary autophagosome. Because the process of macroautophagy has received more attention in the scientific community and has therefore been better characterized, it will be the focus of this thesis and will from herein be referred to as autophagy.

Autophagy is a highly conserved mechanism, as mammalian orthologues of the common autophagy related genes (Atg) found in yeast have been shown to function similarly across many species (Klionsky, Cuervo, and Seglen 2007). It occurs at low basal levels within a variety of cell types and increases during starvation/nutritional deprivation in order to restore the energy balance of the cell. Starvation induced autophagy is considered to be a non-selective, bulk degradation process (Feng et al. 2014). Other than its involvement in mediating nutritional status of the cell, autophagy is also involved in a variety of other physiological processes, including cellular differentiation, cell growth and migration, and innate immunity. Moreover, its dysfunction has been associated with several pathological conditions, including cancer and neurodegenerative disorders (Rubinsztein et al. 2005; White 2015).
Figure 6. Schematic of autophagy.
(a) Cytosolic proteins and organelles are sequestered and enclosed by a phagophore. (b) A double-membraned organelle called an autophagosome fully encloses the cytoplasmic material. (c) The outer membrane of the autophagosome fuses with lysosomes, and its contents are degraded by lysosomal proteases.

The process of autophagy begins with the formation of a membrane (phagophore), which elongates into a double-membraned autophagosome that engulfs cytoplasmic material. The autophagosome subsequently fuses with a lysosome where its cargo is digested by lysosomal proteases (Figure 6). One of the key upstream regulators of autophagy is the Ser/Thr kinase mammalian Target of Rapamycin (mTOR). mTOR is a nutrient sensing kinase that forms two structurally distinct complexes termed TOR complex 1 (mTORC1) and TOR complex 2 (mTORC2). These complexes function to coordinate cell growth, proliferation, and metabolism in response to the nutritional status of the cell. Of the two complexes, mTORC1 is exclusively sensitive to the inhibitory effects of rapamycin, and is the primary nutrient sensing complex that directly regulates autophagy (Rabanal-Ruiz, Otten, and Korolchuk 2017). Under nutrient poor conditions (i.e. insufficient glucose, amino acids and energy) mTORC1 becomes deactivated and
induces autophagy by suppressing (via dephosphorylation) downstream targets that enhance cell growth, such as S6 protein kinase 1 (p70S6K1), and activating targets that induce autophagy, such as transcription factor EB (TFEB) (Klionsky et al. 2011).

TFEB is a basic-helix-loop-helix leucine zipper transcription factor that is often described as the “master regulator” of lysosomal biogenesis. Upon translocation to the nucleus, TFEB positively regulates the expression of genes that belong to the Coordinated Lysosomal Expression and Regulation (CLEAR) network. This network includes genes which encode proteins required for lysosomal biogenesis, as well as several involved in autophagy (Settembre et al. 2011). Nuclear translocation of TFEB is regulated by mTOR, which integrates signals from the cell that reflect lysosomal status. Under normal/nutrient rich conditions, TFEB is phosphorylated by mTOR and retained at the lysosome and within the cytosol. However, during nutritional starvation mTOR becomes inactivated, resulting in dephosphorylation of TFEB and its nuclear translocation (Roczniak-ferguson et al. 2012) (Figure 7). Interestingly, the mechanism by which mTOR senses lysosomal status has been shown to be mediated by lysosomal vacuolar ATPases (V-ATPase), suggesting that the lysosome itself can directly regulate mTOR activity, and thus autophagy (Panic et al. 2011).
Figure 7. TFEB nuclear localization is regulated by mTOR.
Phosphorylation of TFEB by mTOR causes cytoplasmic localization. Nuclear translocation occurs when mTOR is deactivated and TFEB is dephosphorylated. TFEB positively regulated the expression of CLEAR network genes that encode for proteins involved in lysosomal biogenesis and autophagy.

After the autophagy pathway is activated, autophagosome formation is initiated. Several Atg proteins downstream of mTOR regulate the formation of autophagosomes. Among these proteins, the microtubules associated protein 1 light-chain 3 (LC3/Atg8) conjugation system is required for autophagosome elongation and maturation. The cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) to generate LC3-PE (LC3-II), which is recruited to the autophagosomal membrane and is used as a common marker of autophagosomes. In the final stages of autophagy, autophagosomes fuse with lysosomes to form autophagolysosomes. The proper degradation of materials in these vesicles is dependent on the function of proteolytic enzymes that require an acidic environment (low pH) to function. In addition to LC3-II, other
adaptor molecules that target cargo to the autophagosome are used as markers of autophagy. The ubiquitin binding molecule p62, also called sequestosome 1 (SQST1), targets polyubiquitinated proteins for degradation in the autophagolysosome by binding directly to LC3-II. Since p62 is itself degraded in the autophagolysosome, it is widely used a marker of autophagic activity (i.e. autphagic flux).

1.6.1 Autophagy in the placenta

Autophagy occurs during normal placental development, and its dysregulation has been associated with various placental disorders. Autophagic vacuoles, as well as markers of autophagy, have been observed in the villous trophoblast of placentas from normal pregnancies (Curtis et al. 2013; T.-H. Hung et al. 2013). While it has been postulated to play a protective role against starvation, elevated levels of autophagy have also been observed in placentas from complicated pregnancies, and may contribute to the pathogenesis of these disorders. Several studies have reported increased levels of autophagy in placentas from IUGR and preeclamptic pregnancies (Curtis et al. 2013; T. H. Hung et al. 2012; Melland-Smith et al. 2015). Specifically, accumulation of autophagic vacuoles, as well as heightened levels of LC3-II, p62 and lysosomal associated membrane proteins (LAMP), have been detected in villous trophoblast of affected placentas. In addition, reduced activity of mTOR has been reported in placentas from IUGR pregnancies, indicating that nutritional stress may be enhancing autophagy in these cases (Roos et al. 2007). However, whether dysregulated autophagy in vivo leads to placental dysfunction or vice versa, is still not understood. In any case, it is clear that this mechanism plays an important role in the placenta.
1.7 Objectives and Hypothesis

Although not thoroughly investigated, there is evidence to suggest a role for A-SMase (Smpd1) in regulating placental development and function. In 1985, Schoenfeld et al. analyzed the placentas from four NPDA fetuses that were terminated in the second trimester of pregnancy. They found a striking accumulation of SM within these placentas, which was shown to be concentrated among the chorionic villi (Klibansky et al. 1985). Moreover, ultrasonic and histological analysis revealed several morphological abnormalities, including an abnormally thickened chorionic plate and a highly vacuolated villous syncytiotrophoblast layer. This report suggests an essential role for A-SMase in the placenta, as early as the second trimester of pregnancy. While there have been no additional analyses performed on placentas from NPDA&F fetuses, placental abnormalities in other lysosomal storage disorders have been reported (Ferreira and Gahl 2017). More recently, a thorough analysis of sphingolipid regulation in placentas from preeclamptic (PE) pregnancies revealed significantly decreased levels of A-SMase enzymatic activity (Melland-Smith et al. 2015). In addition, abnormal levels of sphingolipid metabolites, which are regulated by A-SMase, have been observed in various placental disorders. Lipidomic analysis of third-trimester placentas from PE and IUGR pregnancies revealed increased levels of SM, the substrate of A-SMase, compared to age-matched controls (Baig et al. 2013; Brown et al. 2016). In another study, CER levels, the product of SM hydrolysis, were found to be reduced in placentas from severe IUGR pregnancies (Chauvin et al. 2015).

In light of this accumulating evidence, the objective of my study is to investigate the role of A-SMase in murine placental development. In this project, I aim to characterize the placental phenotype caused by A-SMase deficiency and determine if this phenotype is maternal or fetal in origin. Based on the relevant literature, I hypothesize that A-SMase deficiency leads to abnormal placental development due to:

1) Altered levels of sphingolipid metabolites (CER and S1P) required for normal placenta development.

2) Sphingomyelin accumulation which causes a poor lysosomal environment leading to a reduction in normal autophagy processes.
Chapter 2

2 Methods

2.1 Animals and Genotyping

The mice used in these experiments were maintained on a 12-h:12-h light-dark cycle with free access to food and water. Mouse experiments were performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines for the Use of Animals in Research and Laboratory Animal Care, under protocols approved by the animal care committees of the Toronto Centre for Phenogenomics (TCP).

2.1.1 Acid sphingomyelinase (Smpd1) mice

Wild type (Smpd1+/+ or WT) and mutant mice (officially known as 129.Smpd1\textsuperscript{tm1Esc} and referred to as Smpd1−/− or KO in this thesis) of mixed C57Bl/6 and 129/Sv background were obtained from the breeding colony of Dr. Schuchman (Horinouchi et al. 1995). Heterozygote breeders were mated from 6 weeks of age until they reached about 6 months, or stopped producing litters. The offspring were genotyped using DNA isolated from ear tissue. The tissue was first digested overnight at 55°C in lysis buffer (100mM Tris, 5mM EDTA, 200mM NaCl2, 0.2% SDS) supplemented with proteinase K (Roche Applied Sciences). The DNA was then purified using a standard alcohol precipitation protocol. PCR amplification (35 cycles, each consisting of 30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C) was performed using 2x PCR Master Mix with dye (Ambiogene). Two sets of primers were used to detect the WT Smpd1 allele and the PGKneo expression cassette of the mutated allele, respectively. WT primers: 5’-AGCCGTGTCTCTTCTCCTAC-3’ (fwd) and 5’-CGAGACTGTGGCCAGACATC-3’ (rev), and the PGKneo primers: 5’-AGCCGTGTCTCTCCTCTTTAC-3’ (fwd) and 5’-GGCTACCCGTTATTTGCTG-3’ (rev).

2.1.2 Smpd1 FLAG-tagged mice

Smpd1 FLAG-tagged mice (officially known as C57BL/6N-Smpd1\textsuperscript{tm1Tcp}/1 and referred to as Smpd1-FLAG in this thesis) were generated at the TCP using CRISPR/Cas9 technology. A
3xFLAG tag followed by a flexible linker (GGGGS) was inserted adjacent to the ATG codon of Smpd1, Chr7:

105554563_105554535insGATTACAAAGATCATGATGGCGACTACAAGGACCACGACAT CGACTACAAGGATGACGACGATAAGGGCGGAGAAGGAC. Genotyping by PCR amplification (35 cycles, each consisting of 15 sec at 95°C, 15 sec at 60°C, 15 sec at 72°C) was performed using 2x PCR Master Mix with dye (Ambiogene) and primers for the 3XFLAG sequence: 5’-CCTAAAGGGCTTCCATCAGTTCTCTG-3’ (fwd) and 5’-
CCTTATCGTCGTATCCTTGTAGTC-3’ (rev), and the WT allele: 5’-
GAGCTATCAGTCAACCACAACGAAG-3’ (fwd) and 5’-
GAGCAGTGAATAAGACTTTTCAGG-3’ (rev).

2.2 Tissue Collection

For collection of WT (Smpd1+/+) and KO (Smpd1−/−) placentas, females of approximately 6-8 weeks of age were mated with male studs of the identical genotype (i.e., WT♀xWT♂ or KO♀xKO♂). Gestational age was determined based on the presence of a vaginal plug, with the morning of detection designated as day 0.5 (d0.5). Pregnant dams were euthanized at d11.5, d13.5, d15.5, d17.5 and d18.5, and the number of conceptuses and resorptions were recorded. Fetuses and placentas were removed from the uterus, and their weights were recorded. In addition, kidneys from d17.5 pregnant dams were collected. All tissues were briefly washed in ice cold phosphate buffered saline (PBS) pH 7, before fixation in 10% formalin, or storage in -80°C.

2.3 Proteinuria

Proteinuria was assessed in non-pregnant females (NP) and in pregnant females at d17.5. Baseline proteinuria was measured by collecting urine from NP WT and KO females (7 weeks of age) on 3 consecutive days. Females were then set up with male studs of the identical genotype (i.e. WT♀xWT♂ or KO♀xKO♂). Urine was collected longitudinally at d17.5 from females that had successfully mated and became pregnant. All urine samples were collected between 9:00 AM and 11:00 AM and stored at -80°C until further processing. Protein concentrations were measured in the urine samples using the Bradford Reagent.
2.4 Embryo Transfer

WT and KO donor females of approximately 4-5 weeks of age were super-ovulated with 5 IU's of pregnant mare serum gonadotropin (PMSG) (Prospect), followed by 5 IU's of human chorionic gonadotropin (hCG) 48 hours later, administered intraperitoneally. Following hormonal priming, females were set up with stud males of identical genotype (i.e., WT♀xWT♂ or KO♀xKO♂), and plugs were checked the following morning to determine successful mating. With one day delay, recipient WT and KO females approximately 8-9 weeks of age were injected with 2.5 IU's of PMSG/hCG, and subsequently mated to vasectomized males to induce pseudo-pregnancy. At 3.5 days post coitum (dpc), donor females were euthanized by cervical dislocation and blastocysts were flushed from their uteri. The blastocysts (WT or KO) were collected with a sterile glass pipette, and transferred surgically into 2.5 dpc recipient WT or KO pseudo-pregnant females. Table 1 below summarizes the transfer experiments performed. During the procedure, the recipient females were anesthetized using isoflurane gas. A small incision (~1-1.5cm) was made on the back of the females, allowing access to the uterine horn. Once externalized, a small puncture hole was made just below the utero-tubal junction with a sterile needle, and donor embryos were transferred through this opening into the uterine cavity. Pregnant dams were euthanized at d15.5 and d17.5, and tissues were collected and weighed as described above.

<table>
<thead>
<tr>
<th>Embryo Genotype</th>
<th>Recipient Female Genotype</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>WT→WT</td>
</tr>
<tr>
<td>WT</td>
<td>KO</td>
<td>WT→KO</td>
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<td>KO</td>
<td>WT</td>
<td>KO→WT</td>
</tr>
<tr>
<td>KO</td>
<td>KO</td>
<td>KO→KO</td>
</tr>
</tbody>
</table>
2.4.1 Vasectomized Males

Vasectomy surgeries were performed by the staff at TCP, as well as by Dr. Han Li from the Jurisicova lab. ICR males 8-10 weeks of age were anesthetized with isoflurane gas and a 1.5 cm transverse incision was made at the lower abdomen, at a point level with the top of the hind legs. The vas deferens was exteriorized using fine blunt forceps, and placed on sterile gauze. Vasectomy was accomplished by cauterizing ~1cm of the vas deferens with red-hot forceps. Two weeks post-surgery, males were mated with females overnight and plugs were checked the following day. Plugged females were monitored until d9.5 of gestation to ensure no embryos were present, and thus vasectomizing was successful.

2.5 Paraffin Embedding and Sectioning

Fixed whole placentas and kidneys were dehydrated in an ascending gradient of ethanol, washed in xylene for 60 minutes, then submerged in hot paraffin wax for 90 minutes. Before embedding, placentas were cut at the midline (perpendicular to the chorionic plate), and kidneys were cut at the mid-transverse plane. Placentas and kidneys were embedded separately in wax. Then, using a microtome, placentas were sectioned serially at 5µm thickness, with three sections every 100µm apart (midline, 100µm, 200µm) collected. Kidneys were sectioned at 5µm thickness. The slides were allowed to dry overnight at room temperature.

2.6 Hematoxylin and Eosin Staining

The slides were incubated at 60°C for 20 minutes then washed three times in xylene, and subsequently rehydrated in a descending gradient of ethanol from 100% to 70%. They were then stained in filtered Hematoxylin (Sigma-Aldrich, #HHS16) for 12 minutes, rinsed in deionized water and then washed in tap water for 5 minutes. They were briefly dipped in 1% acid ethanol, and rinsed with warm tap water. Next, the slides were stained with Eosin (Sigma-Aldrich, #HT110316) for 30 seconds and washed in tap water for 1 minute. After staining, the slides were dehydrated in ethanol, and mounted with a coverslip using Permount solution. Hematoxylin stains the nuclei blue-purple, while eosin stains the cell cytoplasm and extracellular matrix varying shades of pink (Feldman and Wolfe 2014).
2.7 Semi-thin sections and Transmission Electron Microscopy

Pregnant WT and KO dams were euthanized at d17.5 and four placentas as well as one kidney from each dam were collected as described above. A ~1mm³ piece of labyrinth adjacent to the umbilical cord was dissected from each placenta, as well as a ~1mm³ piece of cortex from each kidney. Placenta and kidney pieces were fixed separately in 2% gluteraldehyde overnight, then dehydrated and embedded in plastic resins. Embedded labyrinth tissues were sectioned at 1μm thickness (semi-thin sections), and stained with toluidine blue for stereological analysis. Sections of 50nm thickness (thin sections) were viewed using a transmission electron microscope (Model CM100; FEI). Embedding and sectioning were performed by Doug Holmyard at the Nanoscale Biomedical Imagining Facility at Sickkids Hospital in Toronto, ON.

2.8 Stereology

2.8.1 Placenta proportions

Serial sections of d15.5 WT and KO placentas were stained with H&E, and viewed on a light microscope (BX61, Olympus KeyMed, United Kingdom) at 10x. The areas of individual placenta regions (decidua, junctional zone, labyrinth) were measured using the Cavalieri principle, which is a point counting method that superimposes a grid of cross hairs over an image, with each cross hair representing a defined area (mm²). The cross hairs were labelled either decidua (D), junctional zone (J) or labyrinth (L), if they fell on the respective region of the placenta. The total number of labels per region was determined and multiplied by the defined area of each cross hair to calculate the total area (mm²) of each individual layer. Total area counts were averaged among the midline, 100μm, and 200μm serial sections collected per placenta.

2.8.2 Interhemal Membrane thickness

Interhemal membrane thickness was measured using the orthogonal intercept method, on the NewCAST program from Visiopharm (Hoersholm, Denmark). Semi-thin labyrinth tissue sections were obtained as described above, and viewed at 60X magnification on a light microscope (BX61, Olympus KeyMed, United Kingdom). The NewCAST program superimposes a grid of equidistant parallel lines on the image. When a line intersects the trace of
a maternal blood space, the shortest linear distance to a fetal capillary is measured (Figure 8, red line). A total of 200 measurements from sections of each genotype were made. The interhemal membrane thickness is represented as a harmonic mean thickness ($T_h$), which is the reciprocal of the mean of the reciprocal intercept lengths. $T_h$ is also multiplied by $8/3\pi$ to correct for the plane of sectioning (Jensen, Gundersen, and Østerby 1979). The harmonic mean is often used to represent air-blood or interhemal barriers because it weighs thin barrier measurements more heavily, which has more physiological relevance (Hsia et al. 2010).

Figure 8. Measuring interhemal membrane thickness.
Semi-thin section of a toluidine blue stained d17.5 WT placental labyrinth. When a superimposed gridline (blue) intersects the trace of a maternal blood space (MBS), the shortest linear distance to a fetal capillary is measured (red line). Fetal capillaries (FC) can be distinguished by the presence of large, nucleated fetal red blood cells (black arrowhead), as well as the presence of fetal endothelial cells (FE). Maternal blood spaces (MBS) are characteristically larger, and contain classically biconcave red blood cells (*). Sinuisodal trophoblast giant cells (S-TGC) are large, mononuclear trophoblast cells that reside close to the MBS (†).
2.9 Immunohistochemistry

Slides were deparaffinized in xylene and rehydrated in a descending alcohol gradient as described above. Antigen retrieval was performed using 10 mM citrate buffer, pH 6.0 at 100°C for 10 minutes. Sections were allowed to cool down to room temperature, washed in PBS, and then blocked with 10% horse serum in PBS for 1-hr at room temperature. Sections were then incubated overnight at 4°C with primary antibodies diluted with 5% horse serum in PBS. The following day slides were washed in PBS with 0.1% Tween 20 (PBS-T), then incubated for 1-hr at room temperature with a biotinylated host-specific secondary antibody diluted in 5% horse serum. For detection of trophoblast cells, placenta slides were incubated with an anti-cytokeratin primary antibody (1:200, Z0622, rabbit anti-cytokeratin; Dako, Burlington, ON, Canada), followed by incubation with biotinylated anti-rabbit secondary antibody (1:200 dilution in 5% horse serum) from a VectaStain ABC kit (no. PK-4001, Vector Laboratories). The ABC solution was prepared according to the manufacturer’s instructions, and detection using DAB substrate followed by counterstaining with hematoxylin. After staining, the slides were dehydrated in ethanol, and mounted with a coverslip using Permount solution.

2.10 Immunofluorescence

Slides were processed as described in section 2.8. After antigen retrieval, slides were incubated in a solution of 0.1% Sudan Black in 70% ethanol for 15 minutes, and then washed in PBS. Sections were then blocked with 10% horse serum in PBS for 1-hr at room temperature and incubated overnight at 4°C with primary antibodies diluted with 5% horse serum in PBS. The following day, slides were washed in PBS-T, then incubated for 1-hr at room temperature with a host-specific secondary antibody conjugated with Alexa Flour dyes (Invitrogen Life Technologies), and counterstained with blue fluorescent 4’,6-diamidino-2-phenylindole (DAPI, sigma). To analyze syncytiotrophoblast morphology, sections were co-stained with syncytiotrophoblast-I marker Mct1 (1:500, chicken anti-mct1; AB12861, Abcam) and syncytiotrophoblast-II marker Mct4 (1:500, rabbit anti-mct4; AB3314P, Abcam). Lysosomes were assessed by staining for lysosomal membrane marker LAMP-1 (1:200, rat anti-lamp1; ID4B, Abcam), and TFEB (1:200, rabbit anti-TFEB; A303-673A, Bethyl Laboratories). The secondary antibodies used were conjugated to either red or green fluorophore, and included anti-rabbit IgG (594nm) (Alexa Fluor, Molecular Probes), and anti-rat IgG (499nm) (Bethyl).
2.11 Western Blotting

To assess protein expression in WT and KO placentas, three placentas from three separate dams of each genotype were collected at d15.5 and d17.5, and stored at -80°C. Upon thawing, whole placentas were homogenized in 1% RIPA buffer (1% Nonidet P40, 0.5% Sodium deoxycholate, 1% SDS in PBS), with 1x protease inhibitor cocktail (Sigma-Aldrich) and 1x phosphatase inhibitor cocktail (Sigma-Aldrich). Samples were sonicated for 5 minutes to shear DNA. Samples were then centrifuged at 18000 rcf for 30 minutes, and the supernatant containing protein was collected. Next, the total protein concentration of each lysate was determined using a BCA Protein Assay Kit (Cat# 23225, Thermo Fischer). Once an equal amount of protein was aliquoted per lysate, 4x loading buffer was added (100mM Tris, pH6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 5% β-mercaptoethanol) and the samples were boiled at 100°C for 5 minutes. Standard denaturing acrylamide gels of various concentration were prepared according the manufacturer’s instructions (BioRad). Proteins were transferred to a 0.2-m BioTrace nitrocellulose membrane (no. 66485; Pall, Mississauga, ON, Canada). Blots were blocked for 1-hr at room temperature with 5% skim milk in PBS with 0.1% Tween-20 (PBST), then incubated at 4°C with various primary antibodies (Table 2). Following primary antibody incubation, membranes were washed in 0.1% PBS-T, then incubated with a horseradish peroxidase (HRP) conjugated secondary antibody for 1-hr at room temperature. Next, the membranes were placed in enhanced chemiluminescence HRP substrate (Western Lighting Plus ECL, PerkinElmer, MA, USA) for 1 minute at room temperature, and imaged on a film. After imaging the membrane was washed in 0.1% PBS-T, and the steps outlined above were repeated with another primary antibody. Films were scanned on a STORM imager (Molecular Devices), and densitometric analyses was performed using ImageJ software.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Catalogue #</th>
<th>Dilution</th>
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<tbody>
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<td>sc-1616</td>
<td>1:500</td>
<td>various</td>
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<td>Source</td>
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<td>Dilution</td>
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<td>---------------------------------------------</td>
<td>----------------</td>
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<td>mTOR, rabbit polyclonal</td>
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<td>M2 FLAG-HRP, mouse monoclonal</td>
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<td>A8592</td>
<td>1:1000</td>
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</tbody>
</table>
2.12 Lipid profiles

WT and KO placentas were collected at d15.5 as described above. Tissue processing followed by sphingolipid extraction and tandem mass spectrometry was performed at the Mass Spectrometry facility at the Hospital for Sick Children, Toronto, Canada. This analysis detects a variety of sphingomyelin (SM) species comprised of different fatty acyl-CoA chain lengths, as well as ceramides (CERs), sphingosine (SPH), sphingosine-1-phosphate (S1P), Sphinganine (Sa) and dihydroceramides (DHCers). Thus, this technique allows for the measurement of sphingolipid metabolites originating from hydrolysis of SM by sphingomyelinases (SMases), as well as from the de novo synthesis pathway.

2.13 Acid Sphingomyelinase Activity Assay

Acid sphingomyelinase (A-SMase) enzyme activity was measured using the Echelon Acid Sphingomyelinase Assay Kit (K-3200, Echelon Bioscience) following the manufacturer’s protocol. Briefly, WT placentas were collected at d13.5, d15.5 and d17.5, cut in half at the umbilical cord, then stored immediately at -80°C. Individual placenta layers (labyrinth, junctional zone and decidua) were also collected at d15.5. Upon thawing, samples were homogenized in substrate buffer (K-3203), and a BCA protein assay was performed to determine the total protein concentration per sample. Approximately 7ug of total protein from each sample was used in the assay. The reaction was stopped after incubation with the A-SMase substrate (K-3202) at 37°C for 3 hours, and analyzed using a 96-well fluorescence microtiter plate reader (Tecan Infinite M200) at 360nm excitation and 460nm emission. Enzyme activity was determined from comparing sample RFU, to a linear curve generated from A-SMase standards provided by the manufacturer (K-3201). The specificity of the kit was tested by running KO (Smpd1−/−) brain tissue, which produced no detectable signal (data not shown).

2.14 Sphingosine Kinase Activity Assay

Sphingosine Kinase (SPHK) enzyme activity was measured using the Echelon Sphingosine Kinase Activity Assay Kit (K-3500, Echelon Bioscience). Briefly, WT and KO placentas were collected at d15.5, weighed, and stored immediately at -80°C. Upon thawing, placenta samples were homogenized in 1ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150
mM NaCl, 0.1% Lauryl sulfate, 0.5% Deoxycholic acid, 1% Igepal CA-630). Each sample was incubated with 100µM sphingosine and 10µM ATP at room temperature for 30 minutes in a 96-well microtiter plate. After incubation, 40µl of ATP detector was added to each well and the luminescent signal was analyzed using a 96-well luminescent microtiter plate reader (Tecan Infinite M200). RLU was normalized to the total amount of protein (µg), as determined by BCA assay. Echelon’s Sphingosine Kinase Activity Assay is an ATP depletion assay which quantifies the remaining ATP levels in solution following the kinase reaction. Thus, a lower RLU is proportional to higher Sphk activity, and vice versa. To test the specificity of the kit, samples were incubated with SPHK1 inhibitor (SKI-II) (10009222, Cayman Chemical), which produced no detectable signal (data not shown).

2.15 Quantitative Real-Time PCR

2.15.1 RNA Extraction

RNA was extracted from 8 WT and 8 KO placentas, from 4 separate litters each (2 placentas collected per litter). On the first day of RNA extraction, whole placentas were homogenized in a total of 2ml TRIzol (split into two tubes), then placed on ice for 15 minutes. Next, 200µl of chloroform was added to each sample, and the samples were centrifuged at 16,800 rcf for 15 minutes. After centrifugation, the top aqueous phase was collected, and 500µl of isopropanol was added to precipitate RNA. The sample were placed in -80°C overnight. The following day the samples were centrifuged for 15 minutes at 16,800 rcf to pellet the RNA. The supernatant was aspirated, and the pellet was washed with 500µl ice cold 70% ethanol-diethyl pyrocarbonate (DEPC). The samples were centrifuged at 6000 rcf and the ethanol was removed. Once dry, the pellet was suspended in 20µl of DEPC treated water. The quantity and purity of each RNA sample was assessed using a Nanodrop Spectrophotometer, before storage at -80°C.

2.15.2 Reverse Transcription and Quantitative real-time PCR (q-PCR)

After extraction of the RNA, 1µg of total RNA was treated with DNase-I (Sigma-Aldrich, #AMPD1) to remove DNA contaminants. The DNase treated RNA was then reverse transcribed into cDNA using a reverse transcription kit (ThermoFischer, #4368814), following the manufacturer’s protocol. The cDNA samples were diluted to 50ng/µl in DEPC treated water. For
each target gene, a master mix of Wisent Advanced qPCR master mix (with supergreen), forward and reverse primers (final concentration 300nM), and deionized water was made. Each well of a 96-well PCR plate (BioRad) was loaded with 4µl master mix and 1µl cDNA. For each target, primers were tested at a range of temperatures above and below the calculated Tm, in order to find the optimal annealing temperature which would provide the lowest Cq with no nonspecific amplification. Table 3 below provides a summary of the primer sequences, as well as the respective annealing temperatures used for each target gene. All PCR reactions were run using CFX96 Real-Time System (Bio-Rad C1000 Thermal Cycler) as follows: 95°C for 30 seconds, 60/61/62/62.5°C for 30 seconds, 72°C for 30 seconds, cycling for a total of 37 cycles. For comparisons between WT and KO placentas, expression levels were normalized to two housekeeping genes (β-actin and Pgk1), and ΔΔCT was determined. For analyzing Smpd1 expression in WT placentas across gestation, expression levels were normalized to three housekeeping genes (β-actin, Hprt1, Tbp) and ΔCT was determined.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
<th>Primer Eff. (%)</th>
</tr>
</thead>
<tbody>
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<td>Smpd1</td>
<td>Forward CAGCCGTGTCCCTCTTCCCTTA Reverse AGCAAAGTGGATCTGCACAGT</td>
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<tr>
<td>β-actin</td>
<td>Forward TCGTGCGTGACATCAAAGAGA Reverse GAACCGCTCGTTGCAATA</td>
<td>60°C</td>
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<tr>
<td>Pgk1</td>
<td>Forward CTGACTTTTGGACAAGCTGGACG Reverse GCAGCCCTGATCCTTTGGTTG</td>
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<tr>
<td>Hprt1</td>
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<td>Tbp</td>
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<td>Prl3d</td>
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<td>96.3</td>
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</tbody>
</table>
### 2.16 Microarray analysis

Two samples of d15.5 WT RNA and two samples of d15.5 KO RNA were sent for microarray analysis. Each sample contained RNA from 4 placentas, for a total of 8 combined samples per genotype. The RNA samples were submitted to The Princess Margaret Genomics Centre (Toronto, ON, Canada) for further processing. Quality of the RNA was examined using the Agilent 2100 BioAnalyzer. A total of 100ng of RNA from each sample was amplified using the WT Plus assay kit, and 5.5µg of fragmented, biotin labelled ss-cDNA was hybridized to MouseGene 2.0 ST arrays. Arrays were hybridized for 18-hrs at 45°C at 50 RPM, then washed using GeneChip Fluidics Station P450 fluidic station, and scanned with Affymetrix GeneChip Scanner 7G. Normalization was performed by Dr. Igor Jurisica. For genes that were significantly up- or down-regulated by a factor of 1.5 (p<0.05), we performed a gene ontology term analyses
(GO term) and phenotypic analysis using the MouseMine database (http://www.mousemine.org) setting a Holm-Bonferroni-corrected P value threshold of 0.05.

2.17 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (San Diego, USA). Data groups were first tested for normality, using the Shapiro-Wilk test. If the data was distributed normally, statistical analysis was performed using either an unpaired t-test for comparing two groups (with Welch correction if groups had unequal variance), and a one- and two-way ANOVA followed by Bonferroni post-test for comparison of multiple groups. In the case that data sets were not normally distributed, a Mann-Whitney Rank Sum Test was used to compare data sets with two groups, and the Kruskal-Wallis test followed by a Dunn’s multiple comparison test was used for comparison of multiple groups. All data are represented as mean SEM, with p<0.05 being defined as significant.
Chapter 3

3 Results

3.1 Expression and activity of acid sphingomyelinase (Smpd1) in the mouse placenta

Although there is evidence that sphingolipid metabolizing enzymes play a role in embryo development and pregnancy (Eliyahu et al. 2007; Kono et al. 2004; Melland-Smith et al. 2015; Mizugishi et al. 2007), the expression/activity of Smpd1 throughout placental development remains unknown. Thus, we aimed to determine the gene expression, protein expression and enzymatic activity of Smpd1 throughout normal placental development.

3.1.1 Acid sphingomyelinase gene (Smpd1) expression during normal placental development

*Smpd1* mRNA expression was analyzed in WT placentas at d11.5, d13.5, d15.5 and d17.5 of gestation. Overall, there was a trend towards increased *Smpd1* mRNA expression at d13.5-d15.5, however this increase did not reach statistical significance (Figure 9A). To get a better sense of the spatial expression of *Smpd1* in the placenta, we analyzed its expression in individual placental layers (i.e., decidua, junctional zone and labyrinth). We chose to analyze d15.5 placentas because at this time point all trophoblast subtypes are present, including glycogen trophoblast cells, which appear in the junctional zone at ~d12.5, and invade into the maternal decidua thereafter. Levels of *Smpd1* mRNA were significantly higher in the decidua of d15.5 placentas, compared to the junctional zone or labyrinth (p<0.05) (Figure 9B).

Since *Smpd1* was previously reported to be paternally imprinted in human skin fibroblasts and lymphoblasts, we measured its expression in heterozygous placentas (*Smpd1<sup>+/−</sup>* ) that had either a maternally or paternally inherited WT allele, to assess for potential preferential allelic expression of *Smpd1* in the placenta. Levels of *Smpd1* mRNA were significantly higher in heterozygous placentas that had a maternally inherited WT allele, compared to heterozygous placentas that had paternally inherited WT allele (p<0.05), suggesting preferential expression of *Smpd1* from the maternal allele (Figure 10).
Figure 9. *Smpd1* mRNA expression during normal placental development. 
(A) *Smpd1* mRNA expression did not change significantly between d11.5, d13.5, d15.5 and d17.5 of placental development. (B) *Smpd1* mRNA levels were significantly higher in the decidua (Dec) of d15.5 placentas, compared to junctional zone (JZ) and labyrinth (Lab) (p<0.05). Statistical significance was determined by a Kruskal-Wallis test, followed by Dunn’s post-hoc test. Numbers above bars represent the n-value (number of placentas) and different letters represent statistical significance. All data is represented as mean ± SEM.

Figure 10. Preferential expression of *Smpd1* from the maternal allele. 
Levels of *Smpd1* mRNA were significantly higher in placentas that had a maternal WT allele, compared to placentas that had a paternal WT allele (p<0.05). In addition, complete knock-outs
(Smpd1<sup>−/−</sup>) had significantly lower levels of Smpd1 compared to both heterozygous and WT placentas (p<0.001). Statistical significance was determined by a one-way ANOVA, followed by a Newman-Keuls post-hoc test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.

3.1.2 Acid sphingomyelinase (Smpd1) protein expression in the murine placenta

Due to a lack of reliable Smpd1 antibodies, we used a Smpd1 FLAG-tagged mouse line that allowed us to quantitate the protein expression of Smpd1 via FLAG detection in placental lysates. Smpd1 protein expression was analyzed in transgenic mice (Flg/Flg) at d11.5, d13.5, d15.5 and d17.5. Wildtype (WT) mice that did not express FLAG were used as a control. A FLAG-specific band was detected at ~75kDa, corresponding to the size of the precursor Smpd1 protein (non-lysosomal) (Figure 11A). While the mature lysosomal form of Smpd1 is ~70kDa, a non-specific band detected by our FLAG antibody hampered our ability to observe it. Overall, there was a trend towards increased protein expression of 75kDa Smpd1 at d13.5, but this did not reach statistical significance (Figure 11B).

**Figure 11. Smpd1-FLAG protein expression during normal placental development.**

(A) Representative immunoblot for Smpd1-FLAG expression in FLAG positive (Flg/Flg) and WT control placentas at d11.5, d13.5, d15.5 and d17.5 of development. A FLAG specific band was detected at 75kDa. (B) Densitometric analysis of Smpd1-FLAG protein expression normalized to β-actin. There was no statistically significant change in Smpd1-FLAG protein expression at d11.5, d13.5, d15.5 and d17.5 of development. Statistical significance was tested
with a Kruskal-Wallis test, followed by Dunn’s multiple comparison test. Numbers above bars represent the n-value (number of placentas). All data is represented as mean ± SEM.

3.1.3 Acid sphingomyelinase (Smpd1) enzyme activity in the murine placenta

Smpd1 undergoes several post-translation modifications (i.e. proteolytic cleavage, glycosylation) which have been shown to affect its catalytic activity. We therefore decided to assess the enzymatic activity of Smpd1 throughout placental development. There was no change observed in enzyme activity between d13.5-d17.5 (Figure 12A). However, when individual layers were assessed, Smpd1 activity was found to be significantly higher in the junctional zone (JZ) of d15.5 placentas, compared to the labyrinth region (p<0.05) (Figure 12B).

**Figure 12. Smpd1 enzyme activity during normal placental development.**

(A) Smpd1 enzyme activity did not change between d13.5, d15.5 and d17.5 placentas. (B) Enzymatic activity was significantly higher in the JZ of d15.5 placentas, compared to the labyrinth region (p<0.05). Statistical significance was determined by a Kruskal-Wallis test, followed by Dunn’s post-hoc test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.
3.2 Phenotypic changes of Smpd1-deficient (Smpd1−/−) fetuses, placentas and mothers during pregnancy

3.2.1 Intrauterine growth restricted (IUGR) phenotype of Smpd1−/− fetuses

One of the most common signs of placental dysfunction or insufficiency is a growth restricted fetus. We therefore analyzed the size and weight of WT and Smpd1−/− fetuses in utero from d13.5-d18.5, as well as neonates on the day of birth (DOB). Smpd1−/− fetuses exhibited a decline in birth weight starting at d17.5 (p<0.05), which became more significant towards the end of gestation (d18.5), and on the DOB (p<0.001) (Figure 13A). Figure 13B shows the difference in fetal size between a WT and Smpd1−/− litter at d18.5. Although fetal weights were reduced, there was no significant difference between WT and Smpd1−/− placental weights at d15.5 or d17.5 (Figure 13C). In addition, while there was no significant difference between WT and Smpd1−/− litter size (Figure 14A), there was a significantly increased number of resorptions in Smpd1−/− litters (p<0.05) (Figure 14B). The discrepancy between unchanged litter size and increased number of resorptions in Smpd1−/− litters was somewhat peculiar. A previous study that looked at the effects of Smpd1 deficiency in oocytes from this mousseline found that mutant females had a higher number of ovarian oocytes (ovarian hyperplasia) compared to WT females, which was reportedly due to a lack of normal oocyte death (Morita et al. 2000). Based on this report, we predicated that Smpd1−/− females may also produce more pre-implantation embryos. To investigate this possibility, we collected d3.5 embryos (passed morula stage) from WT and Smpd1−/− females that were super-ovulated and subsequently mated to males of identical genotype. Although there were no visible morphological differences between WT and Smpd1−/− d3.5 embryos, there was a significantly greater number of d3.5 embryos produced by Smpd1−/− females (p<0.05) (Figure 14C). This indicates that while more Smpd1−/− embryos are created and implanted, a smaller proportion reaches term.
Figure 13. Fetal growth restriction in Smpd1<sup>−/−</sup> mice.

(A) Smpd1<sup>−/−</sup> fetuses have significantly reduced weights starting at d17.5 of gestation (p<0.05). This trend becomes more significant at d18.5 (p<0.001) and in neonates measured on day of birth, DOB (p<0.001). Statistical significance was determined by a two-way ANOVA, followed by Bonferroni post-hoc test. (B) WT and Smpd1<sup>−/−</sup> fetal size at d18.5 of gestation. (C) There is no difference in placenta wet weight at d15.5 and d17.5. Statistical significance was determined by a Mann-Whitney test. Numbers above bars represent the n-value (number of litters), and different letters represent statistical significance. All data is represented as mean ± SEM.
Figure 14. *Smpd1*−/− litters have a greater number of early embryo resorptions.  
(A) There is no difference in the average litter size (number of pups per litter) between WT and *Smpd1*−/− litters. (B) There is a significantly higher number of resorbing fetuses in *Smpd1*−/− litters compared to WT (p<0.05). Data was collected for all litters from age-matched WT and *Smpd1*−/− dams (8-10 weeks of age). Statistical significance was determined with a Mann-Whitney test. (C) There is a greater number of d3.5 *Smpd1*−/− embryos produced per female compared to WT (p<0.05). Statistical significance was assessed using an unpaired t-test. Numbers above data represent the n-value (number of litters), and different letters represent statistical significance. All data is represented as mean ± SEM.
3.2.1 Maternal phenotype of Smpd1⁻/⁻ females carrying Smpd1⁻/⁻ fetuses

Since we observed a striking IUGR phenotype in Smpd1⁻/⁻ fetuses born to Smpd1⁻/⁻ mothers, we wanted to further examine the possibility of an abnormal maternal phenotype during pregnancy. We aimed to measure two common symptoms of maternal pre-eclampsia: proteinuria and gestational hypertension. Baseline measurements of total urinary protein were taken in 9 WT and 9 Smpd1⁻/⁻ females before pregnancy. The females were then set up to mate with a male of identical genotype, and urinary protein was measured again in those females that became successfully pregnant at d17.5 (5/9 WT females, 4/9 Smpd1⁻/⁻ females). While Smpd1⁻/⁻ mothers exhibited higher levels of total urinary protein at d17.5 compared to WT mothers (p<0.001), protein levels did not significantly increase due to pregnancy (Figure 15). In addition, protein levels in non-pregnant (NP) Smpd1⁻/⁻ females were significantly higher compared to WT NP females (p=0.032), indicating that kidney function may already be compromised in mutant mice before the onset of pregnancy. Renal dysfunction and proteinuria in NPD and other lysosomal storage diseases has been previously described (Fervenza, Torra, and Lager 2008; Grafft et al. 2009).

Kidney morphology was further examined in Smpd1⁻/⁻ pregnant females to determine the underlying cause of proteinuria. Figure 16 shows the morphology of the glomeruli and surrounding collecting tubules in WT (A) and Smpd1⁻/⁻ (B) H&E stained kidney sections collected from d17.5 pregnant females (black box=glomerulus, black arrow=collecting tubules). Smpd1⁻/⁻ kidneys exhibited an accumulation of what appeared to be large, empty vacuoles that were absent in kidneys from WT pregnant females. To further examine the morphology of the glomerulus, kidneys from WT and Smpd1⁻/⁻ pregnant females at d17.5 were analyzed with TEM (service provided by Sickkids Hospital). Figure 16C shows the morphology of a WT glomerulus (GC= glomerular capillary, P= podocytes). The most striking difference observed in the Smpd1⁻/⁻ glomerular network was the accumulation of large vacuoles (red arrows), which were found both within the capillary loops, and among the podocytes (Figure 16D, left). Furthermore, Smpd1⁻/⁻ glomeruli exhibited regions of capillary loops with flattened podocyte projections (red box), interrupting the underlying fenestrated endothelium that is responsible for normal filtration (Figure 16D, right). Interestingly, we found that the accumulation of these large vacuoles (presumably lysosomes) became much more prominent in older (8 months old) Smpd1⁻/⁻ non-pregnant females (Figure 16E), indicating that the kidney phenotype observed may be age related.
rather than pregnancy induced.

Unfortunately, due to repeated technical failures, we were unable to reliably measure blood pressure in our mice, and could therefore not assess the possibility of maternal hypertension.

**Figure 15. Proteinuria in Smpd1−/− females.**
Total urinary protein (mg/10µl of urine) was significantly increased in Smpd1−/− mothers carrying Smpd1−/− fetuses at d17.5, compared to WT counterparts (p<0.001). However, urinary protein did not increase significantly due to pregnancy in Smpd1−/− mothers. In addition, urinary protein was increased significantly in non-pregnant (NP) Smpd1−/− females compared to WT NP females (p=0.034). NP urine was collected in 6-7-week-old females for 3 consecutive days to obtain a baseline. Statistical significance was determined with a matched-pairs two-way ANOVA for females whose urine was measured longitudinally. An unpaired t-test was used to assess the significance of baseline urine between WT and Smpd1−/− NP females. Numbers above data represent the n-value (number of females), and different letters represent statistical significance. All data is represented as mean ± SEM.
Figure 16. *Smpd1*−/− pregnant females have increased urinary protein levels and damaged kidneys due to excessive lysosomal accumulation. (A) d17.5 WT kidney sections, stained with H&E, 40x magnification. (B) d17.5 *Smpd1*−/− kidney section, stained with H&E, 40x magnification. Black box=glomerulus, black arrow= collecting tubules. (C) Transmission electron micrograph (TEM) of d17.5 WT glomerular network at 5kx (left) and 10kx (right). (D) TEM of d17.5 *Smpd1*−/− glomerular network. P=podocyte, GC=glomerular capillary, red arrows=endo-lysosomes, red box= podocyte projections. (E) Kidneys from non-pregnant *Smpd1*−/− 8-month-old retired breeder, stained with H&E, 40x magnification.
3.2.2 Morphological defects of *Smpd1*\(^{-/}\) placentas

The presence of an IUGR phenotype in *Smpd1*\(^{-/}\) fetuses indicated that there may be developmental defects in the placenta. Since the IUGR phenotype appears at d17.5, placentas were analyzed at this gestational day first. Midline sections of d17.5 WT and *Smpd1*\(^{-/}\) placentas (Figure 17A and 17B, respectively) were analyzed to determine the distribution and relative proportions of the decidua (Dec), spongiotrophoblast (Spg) and labyrinth (Lab) regions. While the total area of WT and *Smpd1*\(^{-/}\) placentas was unchanged (Figure 17C), *Smpd1*\(^{-/}\) placentas did exhibit a greater proportion of spongiotrophoblast and a decreased labyrinth compartment (p<0.05) (Figure 17D). Upon further inspection, the labyrinth region of *Smpd1*\(^{-/}\) placentas showed striking morphological abnormalities compared to WT. Notably, the interhemal membrane which separates fetal capillaries (FCs) and maternal blood spaces (MBSs) was significantly thicker in the *Smpd1*\(^{-/}\) labyrinth (p<0.001), and there was a visible accumulation of dark vacuoles surrounding the FCs (Figure 18D, black arrows).

The interhemal membrane contains two layers of syncytiotrophoblast cells, ST-I and ST-II. ST-I resides adjacent to the maternal blood spaces (MBSs), and ST-II faces the fetal capillaries (FCs). MBSs and FCs can be distinguished based on their morphology, with the MBSs containing classically biconcave mature red blood cells (RBCs), while FCs contain large, immature fetal RBCs (refer to Figure 8 for more detail). Transmission electron micrographs of d17.5 WT and *Smpd1*\(^{-/}\) labyrinth sections revealed that both ST-I and ST-II appeared to be thicker in mutant placentas, and dark vacuoles accumulated within the fetal-facing ST-II (Figure 19C, black arrows). To further elucidate which ST layer might be most affected by *Smpd1* deficiency, placenta sections were stained with ST markers Mct1 and Mct4. Mct1 is localized to the apical membrane of ST-I cells (facing MBSs), and Mct4 is localized to the basal membrane of ST-II cells (facing FCs). In comparison to the thin and tightly juxtaposed pattern of Mct1/Mct4 staining observed in the WT labyrinth, the labyrinth of *Smpd1*\(^{-/}\) placentas had a noticeably diffused and disorganized Mct1/Mct4 staining pattern. Moreover, the *Smpd1*\(^{-/}\) labyrinth contained regions of increased or thickened Mct1 (green) and Mct4 (red) staining, indicating either undifferentiated or improperly fused ST cells (Figure 20B, yellow arrowhead).
Figure 17. Smpd1−/− placentas have a greater proportion of spongiotrophoblast and a decreased proportion of labyrinth. 

(A) d17.5 WT placenta, stained with H&E, 2x magnification. (B) d17.5 Smpd1−/− placenta, stained with H&E, 2x magnification. (C) Total placenta surface area was not significantly different between WT and Smpd1−/− d17.5 placentas. (D) The proportion of spongiotrophoblast (Spg) was increased, and the proportion of labyrinth (Lab) was decreased in Smpd1−/− placentas (p<0.05). Statistical significance was measured using a two-way ANOVA. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.
Figure 18. Increased interhemal membrane thickness in Smpd1−/− placentas. (A) d17.5 WT labyrinth at 10x magnification, stained with toluidine blue. (B) d17.5 WT labyrinth at 100x magnification, stained with toluidine blue. (C) d17.5 Smpd1−/− labyrinth at 10x, stained with toluidine blue. The labyrinth in Smpd1−/− placentas appears to be congested with less visible blood spaces. (D) d17.5 Smpd1−/− labyrinth at 100x, stained with toluidine blue. The cell layers in Smpd1−/− interhemal membranes exhibited an accumulation of vacuoles (black arrow), as well as disorganized syncytiotrophoblast structure. MBS=maternal blood space, FC= fetal
capillary, blue lines= superimposed parallel lines for interhemal membrane quantitation, red line= measured interhemal thickness. (E) Interhemal thickness was significantly increased in KO labyrinth (p<0.001). Statistical significance was measured with an unpaired t-test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.

Figure 19. Analysis of Smpd1−/− interhemal membrane. (A) Transmission electron micrograph of d17.5 WT interhemal membrane at 5kx and 11.5kx magnification. (B) Pseudo coloured transmission electron micrograph of a d17.5 WT interhemal membrane at 15kx magnification. (C) Transmission electron micrograph of d17.5 Smpd1−/− interhemal membrane at 5kx and 11.5kx magnification. (D) Pseudo coloured transmission electron micrograph of a d17.5 Smpd1−/− interhemal membrane at 15kx magnification. MBS=maternal blood space, FC= fetal capillary, black arrow=accumulated vacuoles (believed to be lysosomes), green pseudo colour= syncytiotrophoblast layer I (MBS facing), red pseudo colour= syncytiotrophoblast layer II (FC facing), black bar=1µm.
Figure 20. Analysis of Smpdl<sup>−/−</sup> interhemal membrane.
Expression of Mct1 (ST-I, green) and Mct4 (ST-II, red) were examined by immunofluorescence confocal microscopy in d17.5 WT (A) and Smpdl<sup>−/−</sup> placentas (B) at 20x magnification. Mutant placentas exhibit increased Mct4 staining, as well as diffuse and disorganized Mct4 staining (yellow arrowheads). Nuclei are stained with DAPI (blue).
3.3 Microarray analysis of d15.5 WT and Smpd1-deficient placentas

To further investigate the molecular phenotype associated with Smpd1 deficiency in the placenta, we performed a microarray analysis of d15.5 WT and Smpd1−/− placentas. RNAs were isolated from 8 WT and 8 mutant d15.5 placentae collected from 4 litters each (2 placentas collected from each litter), and pooled into two samples per genotype containing RNA extracts from 4 placentas (from 2 male and 2 female fetuses). Pooled samples were sent for labeling and hybridization on cDNA microarrays. We then listed genes that were found to have statistically significant changes in expression at 1.5-fold or greater (increase or decrease). Using this cut-off, we found only 29 genes to be differentially expressed between WT and Smpd1−/− placentas, most of which were down-regulated in mutant placentae (24/29 down-regulated) (Figure 21A, green=down-regulated in Smpd1−/−, red=up-regulated in Smpd1−/−). To better understand the biological features/meaning associated with our gene list we performed an over-representation analysis using the MouseMine database (www.mousemine.org). Gene ontology (GO) terms for mammalian phenotype (MP), biological processes and molecular functions showed very similar results, namely abnormal lipid and cholesterol efflux/transport (Figure 21B), indicating that abnormal lipid homeostasis is the predominant molecular phenotype in Smpd1−/− placentas.
Figure 21. Microarray analysis of d15.5 WT and Smpd1<sup>−/−</sup> placentas.

(A) List of genes differentially expressed between WT and Smpd1<sup>−/−</sup> placentas by a fold-change of 1.5 or greater. Green = down-regulated in Smpd1<sup>−/−</sup>, red = up-regulated in Smpd1<sup>−/−</sup>. (B) Gene
ontology (GO) enrichments for differentially expressed genes. MP= mammalian phenotype.

3.3.1 Trophoblast lineage-specific defects of Smpd1-deficient placentas

Since we have observed morphological changes in Smpd1−/− placentas, we wanted to also assess the expression of trophoblast lineage markers in order to get a better sense of how Smpd1 deficiency affects trophoblast development. We used our microarray data to guide our analysis. We generated a list of genes differentially expressed at 1.12-fold or greater, and found several which are common trophoblast markers. These included the labyrinth trophoblast markers SynA, Cts6 and Dlx3; spongiotrophoblast (SpT) markers Prl3a1 and CtsM; the glycogen trophoblast (GlyT) marker Prl7c1; markers that are found to be expressed in both GlyT and SpT, Prl4a1 and Prl8a9; and the functional marker for terminally differentiated parietal TGCs, Prl3d. We validated the expression of these markers in d15.5 WT and Smpd1−/− placentas by qRT-PCR.

The mRNA levels of the labyrinth syncytiotrophoblast marker SynA were significantly decreased in Smpd1−/− placentas compared to WT placentas (p=0.032) (Figure 22A). Other labyrinth markers (Cts6, Dlx3) were not significantly different between WT and Smpd1−/− placentas (Figures 22B,C). However, Smpd1−/− placentas did have a higher expression of SpT markers Prl3a1 (p<0.001), CtsM (p<0.001), Prl8a9 (p<0.01) compared to WT placentas (Figure 22D-F). Finally, Smpd1−/− placentas exhibited significantly decreased expression of the p-TGC marker, Prl3d (p<0.05) (Figure 22I). Overall, expression of SpT markers (Prl3a1, CtsM, Prl8a9) were elevated in mutant placentas, while markers of labyrinth trophoblast (SynA) and differentiated TGCs (Prl3d) were diminished, indicating that trophoblast differentiation may be impaired due to Smpd1 deficiency.
A. Syna

B. Cts6

C. Dlx3

D. Prl3a1

E. CtsM

F. Prl8a9

G. Prl7c1

H. Prl4a1

Relative expression
Normalized to actin, pgk1

WT   Smpd1^+/

0.0   0.5   1.0   1.5   2.0   2.5

(8)

(8)
Figure 22. Gene expression of trophoblast lineage markers.

Normalized gene expression of a subset of trophoblast lineage specific markers in d15.5 WT and Smpd1<sup>−/−</sup> placentas. (A-C) mRNA levels of SynA were significantly decreased in Smpd1<sup>−/−</sup> placentas (p=0.032), while other labyrinth specific markers (Cts6 and Dlx3) not reach statistical significance. (D, E) mRNA levels of Prl3a1 and CtsM, which are both markers of spongiotrophoblast, were significantly increased in Smpd1<sup>−/−</sup> placentas (p<0.001). (F) Prl8a9, a marker of both spongiotrophoblast and glycogen trophoblast, was significantly increased in Smpd1<sup>−/−</sup> placentas (p<0.01). (G) Glycogen trophoblast marker Prl7c1 trended upwards in Smpd1<sup>−/−</sup> placentas, but did not reach statistical significance (p=0.09). (H) Levels of Prl4a1, which is also a marker of both spongiotrophoblast and glycogen trophoblast, did not change significantly between WT and Smpd1<sup>−/−</sup> placentas. (I) TGC marker Prl3d was significantly decreased in Smpd1<sup>−/−</sup> placentas. Statistical significance was measured with an unpaired t-test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.
3.4 Altered sphingolipid metabolism in Smpd1-deficient placentas

In order to better understand how sphingolipid metabolism is affected by Smpd1 deficiency in the placenta specifically, we analyzed sphingolipid levels in d15.5 WT and Smpd1−/− placentas by liquid chromatography linked to tandem mass spectrometry (MS/MS) to. MS/MS analysis revealed that sphingomyelin (SM) levels, were increased in Smpd1−/− placentas. Specifically, SM species with a 12-carbon and 18-carbon fatty acyl-CoA chain (SM12:0 and SM18:0) were significantly increased in Smpd1−/− placentas (p<0.01), while levels of SM16:0, SM18:1, SM24:0 and SM 24:1 trended upwards but did not reach statistical significance (Figure 23).

Interestingly, levels of ceramide (CER) did not change significantly between WT and Smpd1−/− placentas (Figure 24). Since CER can also be synthesized via a de novo synthesis pathway (Smpd1-independent), we examined levels of the de novo intermediates sphinganine (Sa) and dihydro-ceramide (DHCer), to determine whether this pathway is altered in Smpd1−/− placentas. Levels of sphinganine (Sa), the primary intermediate of the de novo CER synthesis pathway, were significantly increased in Smpd1−/− placentas (p<0.01) (Figure 25A). This suggested that there may be compensation from the de novo pathway to increase CER production in the absence of Smpd1-mediated CER synthesis. However, levels of the immediate precursor to CER, dihydro ceramide (DHCer) were unchanged in Smpd1−/− placentas (Figure 25B).

Finally, levels of downstream CER metabolites, sphingosine (SPH) and sphingosine-1-phosphate (S1P) were examined. Levels of SPH trended upwards in Smpd1−/− placentas (p=0.057), while S1P levels were decreased ~3-fold in Smpd1−/− placentas (p<0.05) (Figure 26A and B). This suggested inefficient conversion of SPH into S1P by sphingosine kinase (SPHK). To determine whether SPHK activity may be affected by Smpd1 deficiency, we measured the activity of SPHK in d15.5 WT and Smpd1−/− placentas. SPHK activity was significantly reduced in Smpd1−/− placentas (p<0.05) (Figure 27). These results indicate that Smpd1 deficiency leads to increased levels of SM and decreased levels of S1P in the placenta, which together may contribute to the phenotype observed in Smpd1−/− conceptuses.
Figure 23. SM levels in WT and Smpd1−/− d15.5 placentas.
Levels of SM measured by MS/MS showed a significant increase of SM12:0 and SM18:0 in Smpd1−/− placentas (p<0.01). SM16:0, SM18:1, SM24:0 and SM24:1 trended upwards in Smpd1−/− placentas, but did not reach statistical significance. Numbers indicate fatty acyl-CoA chain length. Statistical significance was measured with a two-way ANOVA, followed by Bonferroni post hoc test. The n-value represents the number of placentas, and different letters represent statistical significance. All data is represented as mean ± SEM.

Figure 24. CER levels in WT and Smpd1−/− d15.5 placentas.
Levels of CER measured by MS/MS showed no significant change between WT and Smpd1−/− placentas. Numbers indicate fatty acyl-CoA chain length. Statistical significance was measured
with a two-way ANOVA, followed by Bonferroni post hoc test. The n-value represents the number of placentas, and different letters represent statistical significance. All data is represented as mean ± SEM.

**Figure 25. Levels of de novo pathway intermediates, sphinganine and dihydro ceramide, in WT and Smpd1⁻/⁻ d15.5 placentas.**

(A) Sphinganine levels were significantly increased in Smpd1⁻/⁻ placentas (p<0.01). Statistical significance was measured with an unpaired t-test. (B) Levels of dihydro ceramide did not change significantly between WT and Smpd1⁻/⁻ placentas. Numbers indicate fatty acyl-CoA chain length. Statistical significance was measured with a two-way ANOVA, followed by Bonferroni post hoc test. The n-value represents the number of placentas, and different letters represent statistical significance. All data is represented as mean ± SEM.

**Figure 26. Levels of CER derivatives, sphingosine and sphingosine-1-phosphate, in WT and Smpd1⁻/⁻ placentas.**

(A) Levels of sphingosine trended upwards in Smpd1⁻/⁻ placentas but did not reach statistical significance (p=0.057). (B) Sphingosine-1-phosphate was significantly decreased in Smpd1⁻/⁻ placentas (p<0.05). Statistical significance was measured with a Mann-Whitney test. The n-value represents the number of placentas, and different letters represent statistical significance. All data
Figure 27. Sphingosine kinase enzyme activity in WT and Smpd1<sup>−/−</sup> d15.5 placentas. Activity of SPHK was measured with an ATP depletion assay, where RLU is directly proportional to the amount of ATP remaining and is inversely proportional (1/RLU) to the kinase activity. Sphingosine kinase (SPHK) activity was significantly reduced in Smpd1<sup>−/−</sup> placentas (p<0.05). Statistical significance was measured with a Mann-Whitney test. The n-value represents the number of placentas, and different letters represent statistical significance. All data is represented as mean ± SEM.
3.5 Impaired autophagy in Smpd1−/− placentas

Recent studies have highlighted the importance of Smpd1 in regulating autophagy; an intracellular lysosomal degradation and recycling process required for maintaining cellular homeostasis and survival. Given that acid sphingomyelinase is a lysosomal enzyme, we first set out to determine how its deficiency affects the function and integrity of lysosomes in the placenta. Western blot analysis revealed a significant increase in the protein expression of the commonly used lysosomal marker, LAMP1, in Smpd1−/− placentas (p<0.05) (Figure 28A, B). In addition, immunofluorescence staining for LAMP1 revealed a drastic accumulation of enlarged lysosomes in the Smpd1−/− placental labyrinth (Figure 28D). To further analyze the lysosomal phenotype in Smpd1−/− placentas, we assessed the protein level of ATP6V1B2, a subunit of the V-ATPase that is responsible for acidification of lysosomes. Western blot analysis revealed that the protein expression of ATP6V1B2 was significantly reduced in d15.5 Smpd1−/− placentas (p<0.01) (Figure 29A,B). This suggests that lysosomal acidification, and therefore activity, may be impaired in Smpd1−/− placentas. Decreased catalytic processing of lysosomal proteases is another common sign of lysosomal dysfunction. We analyzed the expression of Cathepsin D (CtsD), a ubiquitous lysosomal protease, and found no difference in the levels of its mature forms between WT and Smpd1−/− placentas (Figure 29C,D).

Autophagosome formation and subsequent fusion with lysosomes are key steps in autophagy. To investigate whether autophagosome formation and clearance is impaired in Smpd1−/− placentas, we analyzed the levels of the autophagosome marker LC3-II and its binding protein p62/SQSTM1. Levels of LC3-II (Figure 30A,B) and p62 (Figure 30C,D) were significantly increased in Smpd1−/− placentas, suggesting either increased autophagy or impaired autophagosome clearance.

An accumulation of lysosomal and autophagosomal markers in Smpd1−/− placentas could be due to either increased biogenesis (enhanced autophagy), or defective clearance (impaired autophagic flux). To determine whether lysosomal and autophagosomal production is enhanced, we analyzed the protein expression of TFEB, a transcription factor that positively regulates the expression of genes which encode proteins involved in lysosomal biogenesis and autophagy. Under normal conditions, TFEB is retained at the lysosome and within the cytosol in its phosphorylated state, which is maintained by mTOR (Settembre et al. 2011). Under conditions of nutritional deprivation or lysosomal stress, TFEB is dephosphorylated and translocates to the
Western blot analysis revealed that levels of phosphorylated TFEB (p-TFEB) were significantly reduced in Smpd1<sup>−/−</sup> placentas (Figure 31A,B). This trend was stage-dependent, and most obvious at d15.5, where almost no p-TFEB was observed in the mutant placentas. In concurrence with our western blot findings, immunofluorescent staining revealed more nuclear localization of TFEB in Smpd1<sup>−/−</sup> labyrinth trophoblast, primarily among sinusoidal trophoblast giant cells (S-TGCs) (Figure 32A,B). Moreover, we found less apparent co-localization of TFEB with lysosomes in Smpd1<sup>−/−</sup> S-TGCs, as indicated by TFEB and LAMP-1 co-staining (Figure 33).

Finally, protein levels of mTOR, the key upstream regulator of autophagy, as well as its downstream target p70S6 kinase were assessed. Levels of phosphorylated or active mTOR (Ser2448) were significantly increased in Smpd1<sup>−/−</sup> placentas (Figure 34 A,B). In addition, levels of the downstream mTOR target and translational activator p70S6 kinase were elevated, indicating increased mTOR activity (Figure 34 C,D). Since activated mTOR is believed to inhibit autophagy, this finding could not explain the decreased phosphorylation and observed nuclear localization of TFEB in mutant placentas. However, it is possible that TFEB is regulated by other kinases, or that mTOR activity in Smpd1-deficient lysosomes is insufficient to phosphorylate TFEB.
Figure 28. Analysis of lysosomal phenotype in Smpd1⁻/⁻ placentas.
(A) Immunoblot for LAMP1 in WT and Smpd1⁻/⁻ d17.5 placentas. (B) Densiometric analysis of LAMP1 protein expression normalized to β-actin. LAMP1 protein level is significantly increased in Smpd1⁻/⁻ placentas (p<0.05). Statistical significance was measured with a Mann Whitney test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM. (C) Expression of LAMP1 (green) was examined by immunofluorescence confocal microscopy in d17.5 WT and Smpd1⁻/⁻
placentas. (D) Smpd1−/− placental labyrinth exhibits a greater number and increased size of lysosomes (white arrow) compared to WT placentas.

Figure 29. ATP6V1B2 and CtsD protein expression in Smpd1−/− placentas.
(A) Immunoblot for ATP6V1B2 in WT and Smpd1−/− d15.5 placentas. (B) Densiometric analysis of ATPV1B2 protein expression normalized to β-actin. ATPV1B2 protein level is significantly decreased in Smpd1−/− placentas (p<0.01). (C) Immunoblot for CtsD in WT and Smpd1−/− d15.5 placentas. (D) Densiometric analysis of CtsD protein expression normalized to β-actin. CtsD protein levels were unchanged between WT and Smpd1−/− placentas. Statistical significance was measured with a t-test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.
Figure 30. LC3 and p62 protein expression in *Smpd1*<sup>−/−</sup> placentas.

(A) Immunoblot for LC3 in WT and *Smpd1*<sup>−/−</sup> d15.5 placentas. (B) Densiometric analysis of LC3-II levels relative to LC3-I. LC3-II:LC3-I ratio is significantly higher in *Smpd1*<sup>−/−</sup> placentas (p<0.05). (C) Immunoblot for p62 in WT and *Smpd1*<sup>−/−</sup> d15.5 placentas. (D) Densiometric analysis of p62 protein expression normalized to β-actin. P62 protein level is significantly increased in *Smpd1*<sup>−/−</sup> placentas (p<0.05). Statistical significance was measured with a t-test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.
Figure 31. TFEB protein expression in Smpd1\(^{-/-}\) placentas.

(A) Immunoblot for TFEB in WT (+/+) and Smpd1-deficient (-/-) d13.5, d15.5 and d17.5 placentas. (B) Densimetric analysis of phospho-TFEB (p-TFEB) and total TFEB protein expression in d15.5 WT and Smpd1\(^{-/-}\) placentas, normalized to β-actin. P-TFEB protein level is significantly decreased in Smpd1\(^{-/-}\) placentas (p<0.05). Although slightly elevated in WT placenta, there was no significant change in total TFEB levels. Statistical significance was measured with a t-test. The n-value represents the number of placentas, and different letters represent statistical significance. All data is represented as mean ± SEM.
Figure 32. TFEB localization in Smpd1<sup>−/−</sup> placentas.

Immunofluorescence analysis of TFEB localization examined by confocal microscopy in d15.5 WT and Smpd1<sup>−/−</sup> placentas at 40x. (A) TFEB (red) localizes to the cytoplasm of WT placental labyrinth. (B) TFEB localizes to the nucleus of labyrinth trophoblast in the Smpd1<sup>−/−</sup> placental labyrinth (yellow arrow). Nuclei are stained with DAPI (blue). (C) Quantitation of TFEB localization in sinuisodal and syncytiotrophoblast. Smpd1<sup>−/−</sup> placentas have a higher percentage of labyrinth trophoblast which have nuclear TFEB staining, compared to WT (p<0.05). Statistical significance was measured with a t-test. The n-value represents the number of placentas, and different letters represent statistical significance. All data is represented as mean ± SEM.
Figure 33. TFEB and LAMP1 localization in Smpd1−/− placentas.
Immunofluorescence analysis of TFEB and LAMP-1 localization examined by confocal microscopy in d15.5 WT and Smpd1−/− placentas at 60x. (A) TFEB (red) exhibits dotty staining and localizes to the cytoplasm and lysosomes (LAMP-1, green) of S-TGCs and ST cells in WT placental labyrinth (white arrow=S-TGC, yellow arrow=TFEB and LAMP1 co-localization). (B) TFEB exhibits a more diffuse pattern in Smpd1−/− placentas, and localizes primarily to the nucleus of S-TGCs in the Smpd1−/− placental labyrinth. Very little colocalization occurs with lysosomes in Smpd1−/− S-TGCs (yellow arrow=lysosomes). Nuclei are stained with DAPI (blue).
Figure 34. mTOR and p70S6 kinase protein expression in Smpd1−/− placentas.

(A) Immunoblot for phospho-mTOR (Ser2448) and mTOR in WT and Smpd1−/− d15.5 placentas. (B) Densiometric analysis of p-mTOR and mTOR protein expression normalized to β-actin. P-mTOR protein level is significantly increased in Smpd1−/− placentas (p<0.05). (C) Immunoblot for phospho-p70S6K (Thr389) and p70S6K in WT and Smpd1−/− d15.5 placentas. (D) Densiometric analysis of p-p70S6K and p70S6K protein expression normalized to β-actin. Statistical significance was measured with a t-test. Numbers above bars represent the n-value (number of placentas), and different letters represent statistical significance. All data is represented as mean ± SEM.
3.6 IUGR phenotype of \textit{Smpd1}^{-/-} fetuses cannot be rescued in a WT uterine environment

In order to determine whether the IUGR phenotype observed in \textit{Smpd1}^{-/-} fetuses is embryonic versus maternal in origin, we performed embryo transfer experiments wherein we transferred WT and \textit{Smpd1}^{-/-} blastocysts (d3.5 embryos) into recipient females of the opposite genotype. As a control, WT blastocysts were transferred into WT mothers (WT$\rightarrow$WT transfer), and \textit{Smpd1}^{-/-} blastocysts into \textit{Smpd1}^{-/-} mothers (KO$\rightarrow$KO transfer). All recipient females were between 7-9 weeks of age. Weights of WT fetuses grown in \textit{Smpd1}^{-/-} mothers (WT$\rightarrow$KO transfer) and \textit{Smpd1}^{-/-} fetuses grown in WT mothers (KO$\rightarrow$WT transfer) were measured at d17.5, since this is the gestational day where we observed the onset of an IUGR phenotype in naturally derived \textit{Smpd1}^{-/-} fetuses. Fetal weights of KO$\rightarrow$WT embryos were significantly reduced compared to WT$\rightarrow$KO embryos ($p<0.01$), and WT$\rightarrow$WT control embryos ($p<0.01$) (Figure 35A). In addition, weights of KO$\rightarrow$WT embryos were similar to that of KO$\rightarrow$KO controls. Figure 35B shows the reduced size of KO$\rightarrow$WT fetuses at d17.5.

Placentas from reciprocal embryo transfer pregnancies were collected and analyzed histologically. Placentas from \textit{Smpd1}^{-/-} (KO) embryos grown in a WT uterus (KO$\rightarrow$WT) appeared to have a smaller labyrinth area (Figure 36). Further inspection of the labyrinth revealed that the \textit{Smpd1}^{-/-} labyrinth appeared much more congested, with a noticeably thicker interhemal membrane (depicted by red line, Fig 36). In addition, there is an accumulation of dark foci (believed to be lysosomes) in the \textit{Smpd1}^{-/-} labyrinth (red arrow heads).

Finally, placentas were stained with pancytokeratin to determine the localization of trophoblast cells within the maternal compartment (i.e. invasiveness of trophoblast). Placentas derived from \textit{Smpd1}^{-/-} embryos (KO$\rightarrow$WT) exhibited fewer cytokeratin-positive cells within the mesometrium compared to placentas from WT embryos (WT$\rightarrow$KO), indicative of impaired trophoblast invasion (Figure 37). Taken together, these results demonstrate that the IUGR phenotype observed in \textit{Smpd1}^{-/-} fetuses is attributable to embryonic/placental defects, rather than maternal.
Figure 35. Fetal growth restriction of *Smpd1<sup>−/−</sup>* (KO) embryos cannot be rescued in a WT uterine environment.

(A) *Smpd1<sup>−/−</sup>* (KO) embryos grown in a WT uterus (KO→WT) had significantly reduced weights at d17.5 compared to WT embryos grown in either a WT or *Smpd1<sup>−/−</sup>* uterus (WT→WT, WT→KO) (p>0.01). In addition, *Smpd1<sup>−/−</sup>* embryos grown in a WT uterus (KO→WT) had similar weights to *Smpd1<sup>−/−</sup>* embryos grown in a *Smpd1<sup>−/−</sup>* uterus (KO→KO). Statistical significance was determined by a one-way ANOVA, followed by Bonferroni post-hoc test. Numbers above bars represent the n-value (number of pups), and different letters represent statistical significance. All data is represented as mean ± SEM. (B) Reduced size of *Smpd1<sup>−/−</sup>* embryos grown in a WT uterus (KO→WT) at d17.5, compared to WT and *Smpd1<sup>−/−</sup>* embryos grown in either a WT or *Smpd1<sup>−/−</sup>* uterus (WT→WT, WT→KO).
Figure 36. Placental phenotype of Smpd1<sup>−/−</sup> (KO) embryos cannot be rescued in a WT uterine environment.  
(A) d17.5 WT placenta grown in a Smpd1<sup>−/−</sup> uterus (WT→KO), stained with H&E, 2x magnification.  
(B) d17.5 Smpd1<sup>−/−</sup> placenta grown in a WT uterus (KO→WT), stained with H&E, 2x magnification. Outlined region represents increased spongiotrophoblast, indicated by black arrowheads.  
(C) d17.5 WT→KO labyrinth at 100x magnification, stained with toluidine blue.  
(D) d17.5 KO→WT labyrinth at 100x magnification, stained with toluidine blue.  
MBS=maternal blood space, FC= fetal capillary, red line= interhemal thickness.
Figure 37. *Smpd1*<sup>−/−</sup> trophoblast exhibit poor invasion into maternal decidua and mesometrium.

Immunohistochemistry (IHC) for pan-cytokeratin (brown stain) shows localization of trophoblast cells in d17.5 placentas. (A) d17.5 WT placenta grown in a *Smpd1*<sup>−/−</sup> uterus (WT→KO). (B) d17.5 *Smpd1*<sup>−/−</sup> placenta grown in a WT uterus (KO→WT). Black arrows point to cytokeratin positive trophoblast cells surrounding maternal arteries. Nuclei are stained with hematoxylin (blue).
3.7 Lysosomal accumulation in Smpd1\(^{-/-}\) placentas cannot be rescued in a WT uterine environment

We also analyzed lysosomal accumulation and levels of TFEB in d15.5 KO→WT placentas to determine whether autophagy defects are predominantly embryonic/placental or maternal in origin. Levels of phosphorylated TFEB (pTFEB) were marginally rescued (i.e. increased) in KO→WT placentas compared to KO→KO placentas, but were not statistically significant (Figure 38A,B). Levels of LAMP1 remained elevated in KO→WT placentas, however, this change did not reach statistical significance (Figure 38C). Immunofluorescent staining of KO→WT placentas confirmed an accumulation of enlarged lysosomes (Figure 38E), compared to WT→WT controls (Figure 38D). Together, these findings demonstrate that while phosphorylation of TFEB is partially influenced by maternal factors, the accumulation of lysosomes in Smpd1\(^{-/-}\) placentas is an intrinsic cellular defect that cannot be rescued in a WT uterine environment.
Figure 38. Lysosomal accumulation in *Smpdl^−^" placenta cannot be rescued in a WT uterine environment.

(A) Protein expression of TFEB and LAMP1 in d15.5 WT→WT, KO→WT, and KO→KO placentas. Densiometric analysis of TFEB (B) and LAMP1(C) protein expression. Statistical significance was determined by a Kruskal-Wallis test, followed by Dunn’s post-hoc test. The n-value represents the number of placentas, and different letters represent statistical significance. All data is represented as mean ± SEM. Immunofluorescent staining for LAMP1 (green) in WT→WT(D) and KO→WT(E) placental labyrinth. Nuclei are stained with DAPI (blue).
Chapter 4

4 Discussion

Placental defects are associated with two of the most common and serious disorders of human pregnancy—maternal preeclampsia and fetal/intrauterine growth restriction (IUGR). Although these disorders have been studied for decades, their etiology is still poorly understood. In the present study, we demonstrate that acid sphingomyelinase (A-SMase, Smpd1), an enzyme involved in sphingolipid metabolism, is critical for normal placental development and fetal growth. Smpd1-deficient (Smpd1−/−) fetuses born to Smpd1−/− mothers exhibited a decline in birth weight starting at d17.5, which became more significant towards the end of gestation (d18.5), and resulted in a ~20% reduction in neonatal weight. Moreover, Smpd1−/− placentas displayed several striking morphological abnormalities, including an increased spongiotrophoblast region and a reduced labyrinth compartment. Further inspection of the labyrinth revealed a significant thickening of syncytiotrophoblast cells (ST) in the interhemal membrane, which may lead to impaired exchange of oxygen, nutrients and waste between the mother and fetus. In addition, analysis of sphingolipid metabolites in mutant placentas revealed a ~3-fold decrease in sphingosine-1-phosphate (S1P), a sphingolipid important for promoting angiogenesis.

We also observed several hallmarks of lysosomal storage disease in Smpd1−/− placentas, including defective autophagy and a drastic accumulation of dilated lysosomes, which occurred primarily within the labyrinth trophoblast. Furthermore, abnormal placentation and impaired growth of Smpd1−/− fetuses could not be rescued in a WT uterine environment, indicating that the phenotype observed is primarily embryonic, rather than maternal in origin.
4.1 Analysis of acid sphingomyelinase gene and protein expression throughout normal placental development

In order to better understand where deficiencies in acid sphingomyelinase could result in the most obvious placental/fetal phenotype, we examined the gene expression and enzymatic activity of acid sphingomyelinase (A-SMase, Smpd1) throughout normal murine placental development. We did not observe a significant change in Smpd1 gene expression from d11.5-d17.5 of placental development, and no change was observed in A-SMase enzymatic activity from d13.5-d17.5. However, there was an increase in Smpd1 gene expression in the decidual layer of d15.5 placentas. Unexpectedly, we found A-SMase enzymatic activity to be elevated in the junctional zone (JZ) of d15.5 placentas. The discrepancy between the spatial distribution of Smpd1 gene expression versus its enzymatic activity could be due post-translation modifications of the enzyme, or due to unknown mechanisms that regulate its activity. Proteolytic cleavage, as well as high mannose type glycosylation, are two important modifications required for the proper lysosomal targeting of A-SMase. Changes in these processes, such as disruption of glycosylation sites, have been shown to alter its catalytic activity.

4.2 Phenotype of Smpd1−/− placentas

The IUGR phenotype of Smpd1−/− fetuses was evident at d17.5, at which point morphological abnormalities in Smpd1−/− placentas were also readily noticeable. Routine histological analysis of d17.5 Smpd1−/− placentas revealed an increased spongiotrophoblast region, and a decreased, visibly compacted labyrinth compartment. In agreement with our histological findings, microarray analysis revealed a trend towards increased expression of spongiotrophoblast genes in Smpd1−/− placentas, and decrease in labyrinth genes—albeit, at a relatively small fold change. Validation of trophoblast lineage markers with RT-qPCR showed that the expression of spongiotrophoblast genes Prl3a1, Prl8a9, Prl4a1 and CtsM were significantly increased in Smpd1−/− placentas, while the labyrinth trophoblast marker SynA was decreased. Moreover, there was a one-half decrease in levels of the parietal trophoblast giant cell (P-TGC) marker Prl3d in Smpd1−/− placentas. It is known that during early placental development some spongiotrophoblast (SpT) cells differentiate into secondary TGCs. The increased levels of SpT cell markers and decreased levels of TGC marker Prl3d in Smpd1−/− placentas may therefore indicate impaired trophoblast differentiation.
TGCs are highly invasive cells that play several important roles in the placenta, including facilitating implantation, initiating attachment to the maternal vasculature, regulating decidualization of the maternal endometrium, and producing hormones which support pregnancy (including Prl3d). Insufficient P-TGC differentiation, indicated by lower levels of Prl3d in Smpd1<sup>−/−</sup> placentas, may result in a reduction in these important processes. However, it should be noted that lower levels of Prl3d could reflect either a decrease in the total number of P-TGCs, or a reduced amount of transcript produced per cell. Further quantification (by counting) of the total P-TGC number in Smpd1<sup>−/−</sup> placentas would need to be performed to address this question.

### 4.2.1 Labyrinth Phenotype

The labyrinth layer of the mouse placenta is the site of fetal-maternal exchange, and its proper development and function is critical for embryo survival. Throughout placenta development, trophoblast cells fuse and differentiate, creating a thin interhemal membrane between fetal capillaries and maternal blood sinuses, consisting of two layers of multinucleated syncytiotrophoblast—syncytiotrophoblast layer I (ST-I) and syncytiotrophoblast layer II (ST-II). This fusion is regulated by the fusogenic retroviral envelope genes SynA and SynB, which have been shown to be expressed exclusively in the placenta in ST-I and ST-II, respectively. In Smpd1-deficient placentas, we observed defective differentiation/fusion of ST cells, indicated by the prominent expansion of both ST-I and ST-II layers and reduced expression of SynA. We did not however, observe any changes in SynB expression between WT and Smpd1<sup>−/−</sup> placentas, as indicated by our microarray analysis. Interestingly, genetic studies in mice suggest that the role of SynA in regulating ST differentiation and fusion seems to be more important than SynB. Homozygous null SynA embryos are severely growth restricted and die in utero at approximately d14.5 (Dupressoir et al. 2009). The placentas from these mutants exhibit a thickened interhemal membrane with defective formation of ST-I, which subsequently impairs the formation of ST-II. Interestingly, while SynB deficient embryos exhibit impaired formation of ST-II, affected fetuses display only a mild growth impairment and do not die in utero (Dupressoir et al. 2011). This suggests that SynA may be the ultimate regulator of ST fusion. Expression of the human homolog of SynA, Syncytin 1 (Syn1), has also been shown to be decreased in placentas from IUGR and preeclamptic pregnancies (Ruebner et al. 2013). Moreover, thickening and abnormal morphology
of ST cells are evident in several cases of IUGR, suggesting that impaired development of ST cells may contribute to the pathophysiology of this disorder.

Although we observed a decrease in SynA gene expression, no significant changes in the expression of labyrinth markers Cts6 or Dlx3 were detected. Cts6 has been shown to be expressed in sinusoidal TGCs (Huh 2000), and cell counts of S-TGC in WT and Smpd1−/− placentas revealed no differences in cell number (data not shown). Moreover, Dlx3, a placental specific transcription factor, has been shown to be most highly expressed at d9.5 with diminishing expression in later gestation. Thus, changes in its expression may be difficult to detect at d15.5 (Berghorn et al. 2005).

### 4.3 Altered lipid metabolism in Smpd1−/− placentas

Previous studies have indicated that sphingomyelin (SM) accumulation within Smpd1−/− tissues is cell type specific due to increased activity of compensatory pathways (Lozano 2001). In Smpd1−/− placentas we observed a general accumulation of SM, with significantly elevated levels of SM12:0 and SM18:0 specifically. Other than exhibiting different kinetic properties within cellular membranes, the roles of individual SM species in cellular physiology are still largely unknown. Interestingly, previous mass spectrometry analysis of lipid extracts from Smpd1−/− brain tissue revealed an exclusive accumulation of SM18:0, with only mild increases in other SMs (SM12:0 was not evaluated), indicating that acid sphingomyelinase may preferentially cleave SM18:0 in the brain (Ledesma et al. 2011). Moreover, an exclusive accumulation of SM18:0 has been observed in both IUGR and preeclamptic (PE) placentas, with mild to no differences observed in the levels of other SM species (Baig et al. 2013; Brown et al. 2016). More recently, SM18:0 was found to be enriched within lipid rafts of syncytial membranes in PE placentas, as well as circulating exosomes in PE mothers (Ermini et al. 2017). Thus, elevated levels of SM18:0 may be a pathological feature of PE and other placental disorders.

Although we observed increased levels of SM in Smpd1−/− placentas, we found no significant changes in overall levels of ceramide (CER) between Smpd1−/− and WT placentas. Unaltered levels of CER have also been observed in eyecups and total brain extracts from Smpd1−/− mice (Wu et al. 2015). The most affected sphingolipid metabolite in Smpd1−/− placentas was S1P, which was found to be decreased ~3-fold. This decrease can be partially attributed to the significantly lower activity of sphingosine kinase (SPHK)—the enzyme responsible for
converting SPH into S1P— in Smpd1<sup>−/−</sup> placentas. S1P is a proangiogenic signaling lipid that is an important regulator of embryonic vascular development. It has been shown to promote migration and subsequent <i>de novo</i> blood vessel formation in <i>in vitro</i> mouse allantois explants (Argraves et al. 2004). The insufficiency of S1P in Smpd1<sup>−/−</sup> placentas may therefore also result in poor vascularization of the placenta, contributing to the defects observed in the labyrinth region. In addition to its proangiogenic role, S1P has also been shown to promote <i>in vitro</i> extravillous trophoblast cell migration and invasion, suggesting that it may be required for the proper function of migratory trophoblast cells such as TGCs and glycogen trophoblast cells <i>in vivo</i> (Yang, Li, and Pan 2014).

In addition to an accumulation of SM, our microarray analysis revealed that the main genes differentially regulated in Smpd1<sup>−/−</sup> placentas were those related to lipid transport. Over-representation analysis of differentially expressed genes revealed enrichment of mammalian phenotype terms such as “decreased HDL cholesterol” and “abnormal HDL cholesterol levels”. Likewise, the most significant gene ontology terms related to biological processes included “phospholipid efflux” and “cholesterol efflux”. This was not surprising considering that most of the genes found to be differentially regulated are involved in lipid metabolism (i.e. albumin, apolipoprotein, alphafetoprotein). Moreover, given that the accumulation of lipids and cholesterol are hallmarks of NPDA&B and have been observed in various tissues from Smpd1<sup>−/−</sup> mice, this phenotype was expected.
4.4 Autophagy in Smpd1−/− placentas

4.4.1 Defective autophagy and lysosomal impairment

The secondary metabolic defects observed in NPDA&B, as well as Smpd1−/− mice (i.e. cholesterol accumulation) have been attributed to impaired lysosomal function, which results in defective cellular clearance, leading to a build-up of cholesterol, lipids and other undigested molecules. Cellular clearance pathways that are highly dependent on lysosomes, such as autophagy, have been observed to be deregulated in various cell types from Smpd1-deficient mice. We found several hallmarks of defective autophagy and lysosomal impairment in Smpd1−/− placentas, which may lead to poor cholesterol efflux and lipid accumulation. Smpd1−/− placentas displayed a drastic accumulation of lysosomal marker LAMP1, as well as an accumulation of the autophagosome marker LC3-II and its binding protein p62. An accumulation of lysosomal and autophagosomal markers in Smpd1−/− placentas may be due to impaired autophagosome-lysosome fusion, or inefficient lysosome degradation (Mizushima et al. 2008). A previous study that looked at autophagy in Smpd1−/− neurons found that autophagosome-lysosome fusion occurred normally, and that accumulation of these organelles was triggered by increased levels of sphingomyelin, which blocks autolysosomal efflux and also results in lysosomal damage. Although this may not be the case in trophoblast cells, it is possible that the enlarged lysosomes we observed within the labyrinth are late autolysosomes that cannot be cleared from the cell due to accumulated SM blocking efflux. Additional in vitro tracing experiments would be needed to address this possibility.

Our data indicates that lysosomal function in Smpd1−/− placentas may also be impaired. We observed a decrease in protein levels of ATP6V1B2, a subunit of the vacuolar-type H+ ATPase (V-ATPase), in Smpd1−/− placentas. Proper acidification of lysosomes via V-ATPase proton pumps is essential for the function of lysosomal hydrolytic enzymes, and deletion of ATP6V1B2 has been shown to result in reduced lysosomal acidification (Yuan et al. 2014). Thus, lower levels of ATP6V1B2 may lead to decreased lysosomal acidification, and impaired lysosomal function. Interestingly, we observed no difference in the protein levels of mature CtsD, a lysosomal protease which is cleaved to its mature form (34kDa) in the lysosome by hydrolytic enzymes. Contrary to our first claim, this suggests that lysosomal function may not be impaired in Smpd1−/− placentas. However, the lysosome contains over 50 hydrolytic enzymes and the degree of lysosomal acidification or dysfunction may affect certain enzymes more so than
others. In their study, Rodriguez et al. showed that lysosomal damage in Smpd1−/− neurons resulted in excess cytosolic release of cathepsin B (CtsB), while no cytosolic release of CtsD was observed. Therefore, alterations in the processing and activity of certain lysosomal proteases might occur under specific lysosomal conditions.

4.4.2 Upstream regulators of autophagy

It is possible that the accumulation of lysosomes and autophagosomes in Smpd1−/− trophoblast is due to increased synthesis of these organelles, rather than defective clearance. We found more nuclear translocation of TFEB, a transcription factor that positively regulates the expression of genes which promote lysosomal biogenesis and autophagy, in the labyrinth trophoblast of Smpd1−/− placentas compared to WT placentas. TFEB has previously been shown to be expressed exclusively in the placental labyrinth (Steingrimsson et al. 1998). More importantly, embryos lacking TFEB die in utero at d11.5 due to impaired labyrinth development, characterized by reduced vascularization (Steingrimsson et al. 1998). Whether this phenotype is accompanied by altered autophagy has not been investigated.

Unexpectedly, we found heightened activity of mTOR in Smpd1−/− placentas, indicated by increased phosphorylation of mTOR and its downstream target p70S6K, which would suggest inhibition of autophagy. Active mTOR has been shown to suppress autophagy by inhibiting nuclear translocation of TFEB via phosphorylation. This however, did not reflect the decreased phosphorylation and increased nuclear localization of TFEB observed in Smpd1−/− placentas. It is however possible that TFEB is regulated by other kinases irrespective of mTOR. For instance, it has been shown that extracellular signal-related kinases 1/2 (ERK1/2) can phosphorylate TFEB on Ser142, resulting in cytosolic retention (Settembre et al. 2011). Moreover, depletion of ERK was shown to result in nuclear localization of TFEB. In addition to alternative regulatory pathways, it is possible that mTOR activity in Smpd1−/− lysosomes is insufficient to phosphorylate TFEB due to excessive lipid accumulation within lysosomes, which could interfere with normal signaling processes.
4.5 IUGR phenotype of $Smpd1^{-/-}$ fetuses is embryonic vs. maternal in origin

Our embryo transfer experiments revealed that the IUGR phenotype of $Smpd1^{-/-}$ fetuses could not be rescued in a WT uterine environment. Likewise, WT fetuses that develop in a $Smpd1^{-/-}$ uterus did not show signs of IUGR, therefore eliminating the possibility that maternal $Smpd1$ deficiency contributes to IUGR. Moreover $Smpd1^{-/-}$ placentas grown in WT uteri (KO→WT) still exhibited placental abnormalities, namely abnormal labyrinth architecture characterized by an apparent increased interhemal membrane, as well as poor trophoblast invasion. Interestingly, while lysosomal accumulation was still evident in KO→WT placentas, TFEB dephosphorylation was partially rescued. This may indicate that nutritional availability from the mother (WT uterus) is able to reduce some aberrant TFEB signaling. We will also assess protein levels of mTOR in these placentas to further address this possibility. Still, the question as to whether IUGR of $Smpd1^{-/-}$ fetuses is due to embryonic versus placental causes is unclear. To address this, we will perform tetraploid rescue experiments, where WT embryonic stem cells are aggregated with a $Smpd1^{-/-}$ tetraploid blastocyst. In this case, the fetus will be WT while the placenta is $Smpd1$-deficient, allowing us to more specifically assess whether IUGR is due to embryonic vs. placental defects.
Figure 39. Graphical summary of results.
This graphical representation summarizes the main phenotypes observed in Smpd1−/− fetuses and placentas at day 17.5 of gestation. Smpd1−/− (KO) embryos remain growth restricted in a WT uterus, and placentas exhibit an increased interhemal membrane (black double-headed arrow), with both syncytiotrophoblast (ST) layers, ST-I (orange) and ST-II (yellow), enlarged. ST cells in KO placentas contain an accumulation of dilated lysosomes, which also cannot be rescued in a WT uterine environment, suggesting that lysosomal accumulation is an intrinsic cellular defect caused by Smpd1 deficiency. Parts of this figure were created using images provided by Les Laboratoires Servier at: https://smart.servier.com.
4.6 Future Directions

We experienced some limitations working with *in vivo* placental tissue. For example, although we observed several signs of lysosomal dysfunction in Smpd1⁻/⁻ placentas, the ideal method to assess lysosome quality would be to directly measure their acidity. This can be accomplished *in vitro*, by the use of various lysosomal probes (LysoSensor), which change in fluorescent intensity/color under varying degrees of acidity (i.e. pH). We have already established *in vitro* trophoblast stem (TS) cell lines derived from Smpd1⁻/⁻ blastocysts, and will use them as a tool to answer many of our questions regarding lysosomal function/autophagy regulation, which could not be done efficiently in *in vivo* placental tissue.

In Smpd1-deficient placentas we observed signs of increased autophagy (i.e. nuclear translocation of TFEB) and reduced autophagic flux (i.e. accumulation of lysosomal and autophagosomal markers) within the labyrinth. To better address whether the observed accumulation of lysosomes and autophagosomes is due to increased production versus defective clearance, we will assess levels of autophagosomal and lysosomal markers in Smpd1⁻/⁻ TS cells under basal and nutrient deprived conditions. Nutrient deprivation stimulates autophagy via deactivation of mTOR, resulting in the nuclear translocation of TFEB, and increased autophagosome/lysosome biogenesis. We will determine whether Smpd1⁻/⁻ TS cells can respond normally to nutritional cues, and whether autophagosome-lysosome biogenesis versus clearance is affected. Lysosomal and autophagosomal accumulation may also be due to impaired autophagosome-lysosome fusion. We will assess the rate of autophagosome-lysosome fusion in Smpd1⁻/⁻ TS cells using a tandem fluorescent-tagged LC3 marker, in which LC3 is engineered with both a red fluorescent protein (mRFP) and green fluorescent protein (EGFP). This allows for the labelling of autophagosomes with a combined red and green fluorescent signal (appears yellow). When autophagosomes fuse with acidic lysosomes the EGFP is quenched, and the subsequent autophagolysosomes fluoresce red. The ratio of red vs. yellow foci represents the amount of autophagosome-lysosome fusion. Reduced fusion may indicate lysosomal impairment and reduced lysosomal acidity.

Analysis of trophoblast lineage markers indicated impaired differentiation of trophoblast cells in Smpd1⁻/⁻ placentas. To investigate this possibility more directly, we will examine the expression of these markers in TS cells differentiated *in vitro*. TS cells can be differentiated *in vitro* into trophoblast giant cells (TGCs) by passing through an intermediate spongiotrophoblast
(SpT) phase. Thus, in vitro differentiation parallels the differentiation of trophoblast cells into secondary TGCs in vivo. Since we observed a decrease in Prl3d expression in Smpd1−/− placentas (marker of secondary P-TGCs), measurement of this marker in cultured Smpd1−/− TGCs would allow us to more specifically analyze defects in TGC differentiation. Unfortunately, differentiation of TS cells into labyrinthine trophoblast lineages in vitro is currently not easily achieved.

Lastly, we observed a striking decrease in levels of sphingosine-1-phosphate (S1P) in Smpd1−/− placentas. In addition to playing a role in promoting angiogenesis, S1P has recently been shown to regulate autophagy in neurons (Moruno Manchon et al. 2015), as well as trophoblast invasion/migration. We will further explore the role of S1P in mediating these processes in Smpd1−/− TS cells in vitro. Furthermore, we will determine whether S1P administration during pregnancy by intravenous injections could rescue the Smpd1−/− IUGR phenotype and placental defects observed in vivo.

In this thesis, we summarized a role for A-SMase (Smpd1) in placental development. We propose Smpd1-deficient mice as a novel model of IUGR, which can aid in the understanding of molecular events that lead to IUGR and other placental pathologies in humans.
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