Regulation of Mitochondrial Fission by Ceramide-Induced Bcl-2-Related Ovarian Killer in Preeclampsia

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Sciences
University of Toronto

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Abstract

Mitochondrial fission (MF) is regulated by the dynamin-related protein 1 (DRP1), and its excess is implicated in a number of human diseases. Preeclampsia (PE) is a hypertensive disorder of pregnancy associated with elevated placental ceramide (CER), a bioactive sphingolipid implicated in cell death. We hypothesize that increased CER, as found in PE, increase MF.

We have found that PE placentae have increased expression of DRP1, and the mitochondrial compartment is enriched in CERs. JEG3 and primary isolated cytotrophoblast cells treated with CERs have increased pDRP1 expression and recruitment to the mitochondria. CER also increases pro-death Bcl-2 related ovarian killer (BOK) recruitment to the mitochondria in JEG3 cells, where it increases DRP1 expression. pDRP1 and BOK are recruited to the mitochondria associated ER membranes in primary cytotrophoblast cells treated with CER. In conclusion, increased CER augments MF via a mechanism involving BOK, and this may contribute to increased cell death in PE.
Acknowledgments

First and foremost I would like to thank my supervisor Professor Isabella Caniggia for welcoming me into her team. I have had an incredible journey into the depths of cell biology learning about the function of the critical organelle, the mitochondrion. I truly appreciate the opportunity to have learned from you, and for your support in helping me to achieve my dreams of becoming a scientist and clinician in Canada.

Next I would like to thank my committee members Dr. Post, Dr. Liu and Dr. Bocking for their interest in my project, and invaluable feedback that helped me to understand, in depth, the importance of this work.

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Contributors

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List of Abbreviations

A1    Bcl-2-related protein A1
AC    Acid ceramidase
ACOG  American College of Obstetricians and Gynecologists
ACTB  Beta-actin
AGA   Appropriate for gestational age
Akt   Protein kinase B
ANOVA Analysis of variance
Apaf1 Apoptotic protease activating factor 1
ASM   Acid sphingomyelinase
ATP   Adenosine triphosphate
BAK   Bcl-2 homologous antagonist/killer
BAX   Bcl-2-associated X protein
Bcl-w  Bcl-2-like protein 2
Bcl-xL Bcl-extra large
Bcl-2  B-cell lymphoma 2
BID/tBID BH3 interacting-domain death agonist
BH    Bcl-2 homology domain
C/S   Caesarean section
CER   Ceramide
CERase Ceramidase
CERT   Ceramide transfer protein
CerS  Ceramide synthase
DMEM  Dulbecco's modified eagle medium
Dnm1  Dynamin 1
Dox   Doxycycline
DR4/DR5 Death receptor 4/ Death receptor 5
DRP1  Dynamin-related protein 1
Erk2  Mitogen-activated protein kinase 1
ETC   Electron transport chain
FADD  Fas-associated protein with death domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>FADH2</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fis1</td>
<td>Mitochondrial fission protein 1</td>
</tr>
<tr>
<td>FUNDC1</td>
<td>FUN14 domain-containing protein 1</td>
</tr>
<tr>
<td>GDM</td>
<td>Gestational diabetes mellitus</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>GTPase</td>
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<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks cells</td>
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<tr>
<td>HIF1α</td>
<td>Hypoxia-inducible factor 1-alpha</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>JEG3</td>
<td>Human choriocarcinoma cells</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>MAPL</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Induced myeloid leukemia cell differentiation protein 1</td>
</tr>
<tr>
<td>Mdivi-1</td>
<td>Mitochondrial division inhibitor 1</td>
</tr>
<tr>
<td>MFF</td>
<td>Mitochondrial fission factor</td>
</tr>
<tr>
<td>MFN1/MFN2</td>
<td>Mitofusin 1/ Mitofusin 2</td>
</tr>
<tr>
<td>MI</td>
<td>Mitochondrial isolate</td>
</tr>
<tr>
<td>MiD49/51</td>
<td>Mitochondrial elongation factor 1</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal horse serum</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy 1</td>
</tr>
<tr>
<td>PARL</td>
<td>Presenilins-associated rhomboid-like protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>PE</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced kinase 1</td>
</tr>
<tr>
<td>PMS</td>
<td>Post-mitochondrial supernatant</td>
</tr>
<tr>
<td>PNS</td>
<td>Post-nuclear supernatant</td>
</tr>
<tr>
<td>PTC</td>
<td>Preterm control</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sEng</td>
<td>Soluble endoglin</td>
</tr>
<tr>
<td>SENP</td>
<td>SUMO1/sentrin/SMT3 specific peptidases</td>
</tr>
<tr>
<td>sFlt</td>
<td>Soluble fms-like tyrosine kinase 1</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for gestational age</td>
</tr>
<tr>
<td>SMase</td>
<td>Sphingomyelinase</td>
</tr>
<tr>
<td>SMS</td>
<td>Sphingomyelin synthase</td>
</tr>
<tr>
<td>SS</td>
<td>Scrambled sequence</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline and Tween®20</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TC</td>
<td>Term control</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of inner mitochondrial membrane</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR1</td>
<td>Tumor necrosis factor receptor 1</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of outer mitochondrial membrane</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TUBA</td>
<td>Alpha-tubulin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular-endothelial growth factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>Mitochondrial membrane potential</td>
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Chapter 1
Introduction

1 Introduction

The mitochondria are ancient organelles that originated 1.5-2 billion years ago from intracellular bacterial parasites [1, 2]. These dynamic organelles measure 0.5-1 um in diameter and are bounded by two layers – the inner (IMM) and outer mitochondrial membranes (OMM), each with a distinct set of functions required to maintain mitochondrial homeostasis [2]. In the IMM, it is well characterized that the mitochondria function as the powerhouse of the cell by producing adenosine triphosphate (ATP) through the process of oxidative phosphorylation [3], and in doing so generate reactive oxygen species (ROS); the OMM contain a number of enzymes and proteins including porins which form channels allowing molecules to diffuse in and out of the mitochondria [4]. The importance of mitochondrial fission and fusion, processes collectively known as mitochondrial dynamics is emerging, and studies have underscored their importance in a variety of cellular events in physiological and pathological conditions [5-7]. In particular, mitochondrial fission is associated with cell division, cell death processes such as apoptosis [8], and is required for mitophagy, the autophagic degradation of mitochondrial fragments [9]. Hence, the focus of this thesis is to investigate mitochondrial fission in the context of preeclampsia, a hypertensive disorder of pregnancy.
1.1 Mitochondria

1.1.1 ATP Synthesis: Fuel for the Cell

Energy production in the cell is complex, and starts with glycolysis in the cytoplasm and the tricarboxylic acid cycle (TCA) in the mitochondria [10]. Glycolysis involves an energy investment of two ATP molecules per glucose and produces four ATP and two pyruvate molecules. Cytosolic pyruvate is linked to the mitochondria via the pyruvate dehydrogenase complex. Pyruvate is required as the substrate for the TCA cycle that occurs in the mitochondria, and this cycle produces the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH$_2$)[11]. NADH and FADH$_2$ are used to execute oxidative phosphorylation, the terminal process by which ATP is produced using the electron transport chain (ETC) of the IMM[12]. There are four integral (and well characterized) enzyme complexes in the IMM that facilitate the ETC; the first complex involves the acquisition of electrons from NADH, the second accepts electrons from FADH$_2$ and these are subsequently shuttled through the remaining complexes, creating a proton gradient that is responsible for generating ATP by ATP synthase [10].

1.1.2 Mediators of Intrinsic Apoptosis

Apoptosis often referred as programmed cell death, is a process which is physiologically important for organogenesis and embryonic development [13]. There are two types of apoptosis – extrinsic and intrinsic. External ligands like tumor necrosis factor (TNF), Fas-ligand, or TNF-related apoptosis-inducing ligand (TRAIL), interacting with specific members of the TNF death-receptor family (Fas, TNF-receptor 1 (TNFR1), death receptor 4 and 5 (DR4/DR5)), results in the assembly of the death-inducing signaling complex, leading to caspase 8 activation and
 eventual caspase 3 activation, and is known as extrinsic apoptosis [14]. Intrinsic apoptosis is a mitochondrial event [8], and is regulated by a balance between pro-survival and pro-death members of the B cell lymphoma 2 (Bcl-2) family [13, 15]. Pro-survival proteins such as Bcl-2 (similarly named to the family with which it belongs) inhibit OMM channel formation and prevent pro-apoptotic cytochrome c release into the cytoplasm [16]. Apoptosis is blocked by increased Bcl-2 synthesis, increased Bcl-2 activation via Akt kinase, and inhibitors of apoptosis (IAPs) that block caspase activity in the cytoplasm [17]. Conversely, pro-apoptotic Bcl-2 family members are triggered by an number of stimuli, and result in the activation, oligomerization, and mitochondrial localization of Bcl-related X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK), which initiate mitochondrial outer membrane permeabilization (MOMP) followed by cytochrome c release to the cytoplasm, resulting in the formation of the apoptosome, and consequently activation of initiator caspases [18]. The initiator caspases (caspases 2, 8, 9 and 10) signal the effector caspases (caspases 3, 6, 7 and 14) which execute apoptosis leading to DNA fragmentation and condensation, membrane blebbing and cell shrinkage [13, 18]. The products of apoptosis are packaged into dense membrane covered apoptotic bodies which do not illicit an inflammatory response (Figure 1.1) [19].
Extrinsic apoptosis requires activation of the TNF death-receptor family by an external ligand. Once this activation occurs, Fas-associated protein with Death Domain (FADD) and pro-caspase 8 form the death-inducing signaling complex which activates caspase 8. Caspase 8 has the dual function of either activating the effector caspase 3, or for signaling BH3 interacting-domain death agonist (BID) to bind to its receptor tBID at the OMM. Intrinsic apoptosis responds to intracellular stress by activating BH3-only proteins which bind to OMM receptors and anti-apoptotic Bcl-2 family members resulting in the recruitment of BAX to the mitochondria, and the oligomerization of both BAX and BAK which induce MOMP and cytochrome c release. Cytochrome c is localized to the inner mitochondria, and upon its release into the cytoplasm it binds to apoptotic-protease activating factor 1 (APAF1) which together with pro-caspase 9 form the apoptosome, and activates caspase 9. Activation of effector caspases (like caspase 3) by caspase 9 (intrinsic apoptosis) and caspase 8 (extrinsic apoptosis) result in the morphological hallmarks of apoptosis including cell shrinkage, nuclear condensation and membrane blebbing.
1.1.3 Mitochondrial Dynamics

The mitochondria are mobile organelles that undergo a delicate balance between fission and fusion to maintain cell and tissue homeostasis [7]. In physiological circumstances, mitochondrial fusion forms healthier organelles, capable of enhanced oxidative phosphorylation, mitochondrial DNA (mtDNA) sharing [2], and typically occur between organelles with normal mitochondrial membrane potentials (Δψₘ) [20]. In stark contrast, mitochondrial fission isolates components of damaged mitochondria with diminished Δψₘ, and simultaneously down-regulates the fusion apparatus [7]. The pathogeneses of a number of human diseases are attributed to alterations in mitochondrial dynamics and can be due to inborn genetic mutations or acquired abnormalities. Optic atrophy [21], early-onset stroke [22] and Charcot-Marie Tooth disease type 2A all result from genetic alterations of key players of the fusion apparatus [23], whereas pulmonary arterial hypertension [24], lung cancer [25] and arterial restenosis are all acquired abnormalities of the fusion apparatus. Alternatively, genetic abnormalities of the fission apparatus result in congenital microcephaly and lactic acidosis, whereas acquired abnormalities lead to Parkinson’s disease, Huntington’s disease, patent ductus arteriosis [26], pulmonary arterial hypertension [24] and lung cancer [25].

1.1.3.1 Mitochondrial Fusion

The mitochondria are bounded by both an IMM and OMM, and a tightly coordinated process is required to carry out mitochondrial fusion [27]. Optic atrophy 1 (OPA1) is a dynamin-like guanosine trisphosphatase (GTPase) that is responsible for cristae remodeling [2], and IMM fusion [28]. One prerequisite for apoptosis is cristae remodeling, and in physiological circumstances, OPA1 protects the mitochondria from this, and in doing so preserves the
respiratory function of the organelle [29]. OPA1 undergoes alternative splicing generating up to eight long and short splice-variants, and fusion is dependent on long isoforms [30]. In the presence of apoptotic stimuli such as the downregulation of p53 in cancers, or in mitochondrion with $\Delta \psi_m$ or low ATP synthesis, proteolytic cleavage of long OPA1 isoforms by ATP-dependent metalloprotease YME1L1 occurs and fusion is abrogated [31]. Mutations in the gene encoding OPA1 results in autosomal dominant optic atrophy, and recently has been also linked to the pathogenesis of auditory neuropathy spectrum disorder [32]. Also, OPA1 has an important role in trophoblast cell differentiation and Waselewski et al. has demonstrated in cultured human choriocarcinoma BeWo cells, that mitochondrial fusion mediators are diminished when cytotrophoblast cells differentiate into syncytiotrophoblast cells and this associates with impaired steroidogenesis [33].

Mitofusins 1 and 2 (MFN1/2) are large GTPase’s required to tether the mitochondria together to initiate the process of fusion [34]. MFN1 is localized exclusively to the OMM, whereas MFN2 is resident in the OMM and the endoplasmic reticulum (ER) membranes [35]. Critical to MFN1/2-dependent mitochondrial fusion is an intact $\Delta \psi_m$, and Legros et al. have shown that even in the absence of a functional respiratory chain and impaired ATP production, mitochondrial fusion can proceed in human-derived cervical cancer HeLa cells and osteosarcoma 143B and 143B-p0 cells, but not when membrane potentials are diminished [20]. MFN2 is important for placental development as evident from Mfn2$^{-/-}$ mice which exhibit embryonic mid-gestation lethality that associated with diminished trophoblast giant cell quantity with smaller nuclei. Interestingly, in the same study, in contrast to Mfn2$^{-/-}$, Mfn1$^{-/-}$ mice did not result in placental defects [36]. Yu et al. have recently reported that MFN2 mRNA levels are decreased in preeclamptic (PE) placentae and this was associated with diminished ATP levels as assessed by a luciferase-luciferin assay.
[37]. MFN2 has an important function to bridge the ER to the mitochondria forming the mitochondria-associated ER membranes (MAM), a subcellular region of intense metabolic activity, discussed in detail in section 1.1.3.4 (Figure 1.2) [35, 38, 39].
Mitochondrial fusion is carried out by three key players. First, MFN1 and MFN2 act to bring the OMMs of adjacent mitochondria together through the formation of intermolecular anti-parallel coiled coils which tether the organelles and initiate lipid bilayer mixing. OPA1 is present in the IMM, and is cleaved by the action of PARL and AAA proteases. Long OPA1 isoforms are required for IMM fusion[6]. One prerequisite for mitochondrial fusion is an intact mitochondrial membrane potential ($\Delta \Psi_m$).

**Figure 1.2 Mechanism of mitochondrial fusion.**

1.1.3.2 Mitochondrial Fission Apparatus

Mitochondrial fission causes organelle fragmentation and is increased in apoptosis, cell division, as well as it is required for mitophagy [7]. Central to mammalian mitochondrial fission is the dynamin-related protein 1 (DRP1), a GTPase responsible for regulating the fission apparatus.
DRP1 is an 80kDa member of the dynamin family – a group of GTP binding proteins that have a variety of cellular functions [40]. This important player in mitochondrial fission was first discovered in the ER [41, 42], and shares homology with Dnm1 in the yeast *Saccharomyces cerevisiae*, where it regulates both mitochondrial morphology and cortical distribution [43]. DRP1 is a cytosolic protein, that once activated (described below) is recruited to the mitochondria where it interacts with OMM ‘receptor’ proteins mitochondrial fission factor (MFF), mitochondrial fission 1 protein (Fis1), and mitochondrial dynamics proteins 49 and 51 (MiD49/51) [44]. Fis1 has been described as the preferred receptor for Dmn1, the yeast homologue of DRP1, whereas MFF is the preferred receptor in mammals [43]. The preference of DRP1 to recruit to a specific OMM protein was evaluated by Loson *et al.* who demonstrated that MFF and Fis1 are both able to recruit DRP1 independent of each other using mouse embryonic fibroblast cells containing gene trap disruptions for MFF or Fis1. Furthermore, MiD49/51 was able to recruit DRP1 in the absence of both MFF and Fis1 [44], indicating that all four proteins are able to recruit DRP1 independent of each other.

**1.1.3.3 Activation of Dynamin-related Protein 1**

DRP1 is activated through a number of post-translational modifications including deSUMOylation[45-48], S-nitrosylation [49, 50], glycosylation [51], and importantly – phosphorylation events which are the most commonly characterized [7]. SUMOylation is an enzymatic process in which small ubiquitin-related modifier (SUMO) is tagged to a target protein to alter its function, whereas deSUMOylation involves SUMO1/sentrin/SMT3 specific peptidases (SENPs) that inactivate SUMOylation. Interestingly, both processes are vital for DRP1 activation. In cardiomyopathy, SENP5 is increased and deSUMOylates DRP1, resulting in increased apoptosis and mitochondrial fission [47]. Another study reports that the
translocation of SENP5 from the nucleoli to the mitochondria at the G2/M phase of the cell cycle results in decreased DRP1 SUMOylation and an increase in mitochondrial fragmentation [48]. However, Prudent et al. demonstrated that the mitochondrial-anchored protein ligase (MAPL) and mitochondrial E3 ubiquitin protein ligase 1 (MULE-1) driven SUMOylation of DRP1 was required for DRP1 association to the OMM and endoplasmic reticulum (ER) in a HeLa cell model [46]. Other work using in vitro models of primary cultured rat cortical neurons and HeLa cells have found that oxygen-glucose deprivation results in decreased SENP3, and corresponded to increased activation of DRP1 via SUMOylation [52].

S-nitrosylation of DRP1 (SNO-DRP1), by means of a reduction-oxidation reaction, results in mitochondrial fission. Brains from autopsy of human subjects who had Alzheimer’s disease (AD), a pathology characterized by the abnormal buildup of β-amyloid protein (Aβ), had uniformly increased SNO-DRP1 levels relative to controls. Exposure of cultured cerebro-cortical neurons to Aβ resulted in increase SNO-DRP1 levels underscoring the importance of nitrosylation in DRP1 activation and mitochondrial fission [49, 50]. Glycosylation of O-linked-N-acetyl-glucosamine in neonatal rat cardiomyocytes has been identified as crucial for DRP1 activation and recruitment to OMM, and this was dependent on its inhibition of phosphorylation of specific serine residues [51].

Phosphorylation of DRP1 is often described as balance between phosphorylation at pro-fission serine 616 (S616) and fission-inhibitor serine 637 (S637). Phosphorylation of S616 is carried out by cyclin B1-cyclin dependent kinase 1, which regulates ‘mitotic fission’ ultimately producing more mitochondrial fragments to distribute to daughter cells, and is exaggerated in hyperproliferative pathologies like cancer [53]. Recent work in pancreatic cancer biology has shown that mutations of RAS, a protein expressed in all mammalian cells, result in downstream
extracellular-regulated kinase 2 (Erk2) phosphorylation of DPR1 at S616, resulting in increased fission [54]. In glaucomatous optic nerve degeneration there has been an observed increase in mitochondrial fission that corresponds to elevated phosphorylation of DRP1 at S616 [55]. Patent ductus arteriosus (PDA) is a condition in which there is a failure to convert to adult circulation at birth and Hong et al. has reported that PDA is due to impaired DRP1 activation through phosphorylation at S616, resulting in failed vascular remodeling [26]. Alternatively, phosphorylation of S637 by protein kinase A inhibits DRP1 activation, thereby inhibiting its recruitment to the OMM and preventing fission [7]. Dephosphorylation of S637 has been attributed to increased intracellular calcium (Ca\(^{2+}\)) levels which activate calcineurin, a calcium-dependent serine-threonine phosphatase and activate fission [56]. Similarly, in renal tubular cells with depleted ATP, there is increased dephosphorylation at S637 corresponding to increased mitochondrial fission [57].

Once at the OMM, pDRP1 oligomerizes and provides the mechano-enzymatic force by which fragmentation occurs [58, 59]. The C-terminal GTPase effector domain has been identified as critical to formation of DRP1 oligomer complexes in COS-7 cells [60]. The hydrolysis of GTP by DRP1 provides the energy to divide the mitochondria (Figure 1.3) [61].
Figure 1.3 The balance of phosphorylation and dephosphorylation of DRP1.

The regulation of DRP1 activation is a balance between phosphorylation at alternating serine residues. DRP1 is activated by phosphorylation at S616 by certain kinases including cyclin-dependent kinase (cyclin B1), and DRP1 activation is inhibited by phosphorylation at S637 by the action of protein kinase A. Alternatively, in depolarized mitochondria, extrusion of Ca$^{2+}$ into the cytosol activates the phosphatase calcineurin which effectively dephosphorylates S637, activates DRP1, and leads to mitochondrial fission.
1.1.3.4 Mitochondria-associated Endoplasmic Reticulum Membrane

The mitochondria-associated ER membrane (MAM) is an area of close proximity between mitochondria and ER, that is vital for Ca\(^{2+}\) homeostasis, lipid metabolism, and is the microenvironment in which mitochondrial fission occurs [62]. The concentrations of intracellular Ca\(^{2+}\) differ amongst subcellular compartments such that high Ca\(^{2+}\) concentrations (around 100 nM) are found in the ER compared to low Ca\(^{2+}\) concentrations (around 0.5 nM) found in the cytoplasm [63]. Ca\(^{2+}\) shuttling to the mitochondria occurs at the MAM and Ca\(^{2+}\) is required for the functioning of key enzymes of glycolysis, the TCA cycle and oxidative phosphorylation by the ETC [64]. Excess Ca\(^{2+}\) in the mitochondria is a trigger for mitochondrial outer-membrane permeabilization, cytochrome c release and apoptosis [65]. Interestingly, ER Ca\(^{2+}\) is required to carry out the post-translational modifications of DRP1 including N-glycosylation and dephosphorylation at S637 residue [66].

Lipid synthesis occurs in both the mitochondria and the ER, and their interchange is reported to occur at the MAM [63]. The membrane structural lipids phosphatidylcholine and phosphatidylethanolamine are produced from precursor phosphatidylserine, via enzymes resident in the MAM including phosphatidylserine decarboxylase [67], phosphatidylserine synthases 1/2 [68], and phosphatidylethanolamine methyltransferase 2 [69]. Within the MAM, there are glycosphingolipid-enriched microdomains (GEM’s) that have been identified as the sites of Ca\(^{2+}\) mediated apoptotic signaling [70]. Ganesan et al. reported that the sphingolipid ceramide, produced in the ER, is trafficked to the mitochondria via the MAM [71], where it acts together with Bcl-2 family members to carry out apoptosis via ceramide channels [72]. In GM1-gangliosidosis (GM1), an inborn error that results in defective lysosomal β-galactosidase, there is
an accumulation of glycosphingolipid, GM1 at the MAM which results in apoptosis of neuronal cells [70].

In Cos-7 cells, it was shown that DRP1 recruitment and mitochondrial constrictions were always at the site where the ER crossed over the mitochondria, or immediately adjacent to it [73]. In yeast, the GTPase Sar1 has been shown to reduce the ER tubule-mitochondria contact area, thereby reducing mitochondrial fission [74]. In HeLa cells, the MAM compartment is enriched in DRP1 bound to Rab32, a protein that normally anchors protein kinase A preventing its phosphorylation of DRP1 at S637, and permitting mitochondrial fission to occur [75]. Mitofusin 2 (MFN2) is the GTPase responsible for tethering the ER and mitochondria together [39], and as described previously, is a key component of mitochondrial fusion [36]. Recently, the OMM protein Fun14-domain containing protein 1 (FUNDC1) was reported to accumulate in MAM’s of hypoxic HeLa cells where it recruited and bound to DRP1 through its cytosolic loop even when canonical OMM proteins MFF and Fis1 were silenced [76]. Taken together, it is clear that many of the signaling mechanisms required for mitochondrial fission reside in the MAM.
Figure 1.4 Mitochondrial fission occurs at the mitochondria-associated ER membranes

Mitofusin 2 is responsible for tethering the ER and mitochondria together to form the MAM. The MAM is an important subcellular communication between the ER and the mitochondria and is essential for calcium homeostasis, lipid trafficking, and importantly, mitochondrial fission. The ER crosses over potential sites of fission on the OMM, marking them for division by DRP1-oligomerization-driven mitochondrial fission.
1.1.3.5 Mitochondrial Fission in Disease

Much of our understanding about mitochondrial dynamics in embryonic development stems from murine models. In mice, Drp1 is vital to placental development, and complete Drp1−/− knockouts (KO) lead to absence of the trophoblast giant cell layer, and embryonic lethality between E10.5 and E12.5. In addition to the placenta, the hearts and angiogenic capabilities of these mice showed no significant differences from wild-type (WT) controls, indicating that DRP1 is critically linked to placental development/function [59]. Isihara et al. found that primary isolated neural-specific forebrain cells isolated from Drp1−/− mice have defective synapse formation [77]. Mitochondrial fission is required to maintain oocyte viability in Drp1−/− KO mice, where follicular maturation and ovulation were diminished in KO compared to WT controls, and this was found to be age-dependent [78].

Excessive DRP1-driven mitochondrial fission has been implicated in the pathophysiology of many human diseases. Fission can either serve a proliferative purpose, as seen in lung adenocarcinoma and pulmonary arterial hypertension (PAH), or participate in pathways leading to cell death, as seen in ischemia-reperfusion injury, Alzheimer’s disease, and heritable juvenile Parkinsonism [7].

Cancer is characterized by cellular evasion of apoptosis and exuberant proliferation [79]. Mitochondrial fission is enhanced during malignant cell proliferation to produce more mitochondria to distribute to daughter cells and is regulated by cyclin B1-cyclin dependent kinase-1 which initiates mitosis and activates DRP1 through phosphorylation at S616 [53]. In human lung cancer cell lines, DRP1 inhibition has been found to effectively reduce the proliferation of tumor cells, and using a xenotransplantation model in mice, Drp1 inhibition and Mfn2 gene therapy reduced tumor load [25]. PAH is characterized by elevated mean pulmonary
arterial pressure and strains the right ventricle leading to hypertrophy, heart failure and death [24]. Mitochondrial fission occurs in the pulmonary artery smooth muscle cells which proliferate and remodel excessively leading to elevated pulmonary arterial pressures [24].

Following myocardial infarction, ischemia-reperfusion injury remains a critical cause of cardiac arrest leading to increased mortality [7]. During cardiac arrest the damaged heart releases Ca\(^{2+}\) which activates calcineurin resulting in DRP1 dephosphorylation at S637 and increased mitochondrial fission. Sharp et al. found that this process could be rescued by inhibition of DRP1 by mitochondrial division inhibitor 1 (Mdivi-1) and therapeutic hypothermia, in a neonatal cardiomyocyte model [80]. Early onset Parkinson’s disease (PD) is characterized by a loss of the antioxidant glutathione and increased ROS which causes neuronal degeneration and impaired striatal dopamine production leading to mitochondrial fission and mitophagy [81]. In mice, supranigral injections of a recombinant adeno-associated virus to deliver a Drp1-K38A mutant effectively blocked mitochondrial fission, and in doing so attenuated neurotoxicity, restored striatal dopamine release [82], thus identifying new therapeutic targets for PD [83]. One study identified that 6-hydroxydopamine (6-OHDA) is increased PD, and this produced ROS and increased mitochondrial fission in neuroblastoma (SH-SY5Y) cell lines [84]. Manganese (Mn) induced Parkinsonism demonstrated increased DRP1 levels, and decreased OPA1 expression in rat astrocyte C6 cells [85]. In Alzheimer’s disease, excess S-nitrosylation of DRP1 in response to beta-amyloid leads to increased mitochondrial fragmentation, underscoring the importance of mitochondrial morphology in the pathology of a number of neurodegenerative diseases [49].

To summarize, there are a number of human diseases associated with excessive mitochondrial fission. Though the mechanisms of DRP1 activation may differ, there is a consensus that excessive mitochondrial fission negatively impacts on cell and tissue homeostasis.
1.1.3.6 Mitochondrial Fragments: Mitophagy

Mitochondrial fission produces fragments with impaired $\Delta \Psi_{m}$, that are efficiently disposed by means of mitophagy, the selective autophagic degradation of such by-products. Mitophagy is a physiological defense against ROS-induced oxidative stress and the propagation of mutated mtDNA [86]. In healthy mitochondria, the normal $\Delta \Psi_{m}$ permits PTEN-induced putative kinase 1 (PINK1) to be cleaved by presenilin-associated rhomboid-like serine protease (PARL). Once cleaved, PINK1 can be imported to the IMM via transport mechanisms known as the translocase of inner membrane (TIM) and translocase of outer membrane (TOM) complexes [87]. When the $\Delta \Psi_{m}$ is impaired, PARL fails to cleave PINK1 and it accumulates on the OMM, where its kinase domain acts as a ‘mitochondrial damage sensor’ recruiting and phosphorylating cytoplasmic Parkin, a protein coded on the PARK2 gene [88]. PINK1 directly phosphorylates serine 65 (S65) of the ubiquitin-ligase domain of Parkin, as well as the analogous S65 of ubiquitin – both of which are required for complete Parkin activation [89]. Once activated, Parkin builds chains of ubiquitin on the OMM which are recognized by the autophagy machinery which have ubiquitin-binding domains and LC3-interacting regions, to carry out the process of mitophagy [90].

Mitophagy and mitochondrial fission are described as parallel processes, but a point of conversion exists when mitochondrial damage is beyond cellular repair mechanisms [91]. In a rat hepatocyte model designed to simulate ethanol exposure, Eid et al. found that mitophagy was protective from hepatocyte apoptosis, and steatosis, and may represent a new pharmacological target (Figure 1.5) [92].
Figure 1.5 Mechanism of mitophagy.

In physiological conditions, PINK1 is cleaved by the action of PARL in the IMM; however, mitochondrial fragments with impaired $\Delta \psi_m$, mtDNA mutations, or the presence of increased ROS impair PINK1 shuttling through the TIM and TOM complexes resulting in its accumulation on the OMM (1). Once on the OMM, PINK1 autophosphorylates, and phosphorylates Parkin and ubiquitin at serine 65 resulting in a complex formation which fully activates Parkin (2). Activated Parkin creates chains of ubiquitin (3) which signal autophagy machinery to localize to the mitochondria (4).
1.2 Ceramide

The mitochondrion contain a number of lipids essential for structural integrity which are synthesized within the organelle, including cardiolipin, phosphatidylglycerol and phosphatidylethanolamine; however, other groups of lipids found in the mitochondria are imported, including sphingolipids, sterols and phosphatidylcholine [93]. Sphingolipids are bioactive signaling molecules once thought to function only as structural components of the cell [94]. One group of sphingolipids known as ceramides have numerous functions and have been identified as critical to growth regulation and apoptosis [95]. Many studies have linked elevated ceramides to the pathogenesis of metabolic diseases including atherosclerosis [96], and more recently, Summers et al. found that ceramides were an intermediate between free-fatty acids and insulin resistance in a mouse C2C12 myoblast cell line [97]. Faber disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency of ceramidases which lead to an accumulation of ceramides in the joints, liver and central nervous system [98]. Fabry disease is an X-linked recessive lysosomal storage disorder characterized by the deficiency of alpha-galactosidase A and an accumulation of globotriaosyl-ceramide in the cells of the body with resultant cardiac and renal failure, and a predisposition to stroke [99]. These two diseases underscore the toxic and detrimental nature of ceramide accumulation in different tissues of the body. At a molecular level, ceramide accumulation inhibits protein kinase B, also known as Akt, which is an important activator of a number of anabolic glycogen and protein signaling pathways, and an inhibitor of catabolic pathways [100]. Also, ceramide alters mitochondrial function leading to increased ROS and activation of apoptosis, as it is shuttled from the ER to the mitochondria via the MAM. Importantly, Shimeno et al. found ceramide regulatory enzymes in
the mitochondrial isolate of bovine hepatocytes [101], indicating that mitochondrial synthesis may contribute (though marginally) to the total levels of ceramide found in the mitochondria.

1.2.1 Ceramide Biosynthesis

Ceramide biosynthesis can be broadly divided into three pathways (Figure 1.6). The \textit{de novo} synthesis pathway involves a series of four enzymatic reactions, and begins with the production of an 18-carbon ‘backbone’ 3-ketosphinganine from palmitoyl CoA and serine by the action of serine palmitoyltransferase. The second reaction reduces 3-ketosphinganine to dihydrosphingosine by the action of 3-ketosphinganine reductase. Next, dihydrophingosine is converted to dihydroceramide by the action of dihydroceramide synthase, and finally ceramide is produced from dihydroceramide via dihydroceramide desaturase [102].

The hydrolysis pathway involves the interconversion of sphingomyelin and ceramide such that ceramide is produced by the action of sphingomyelinase (SMase), and sphingomyelin is produced by action of sphingomyelin synthase (SMS) [103]. The salvage pathway exists because ceramide cannot traffic out of the acidic environment of the lysosomes. Therefore, complex sphingolipids are broken down into ceramides which are converted to sphingosine by the action of acid ceramidase (AC), which can exit the lysosomes to be interconverted between ceramide in the ER by the action of ceramide synthases (CerS) and ceramidases (CERase) [104]. Sphingosine can also be interconverted with sphingosine-1-phosphate by the action of sphingosine kinases/phosphatases. This is of particular importance as sphinosine-1-phosphate is associated with a cell-survival function, contrary to the more predominant role of ceramide in cell death which is explored in this thesis [105].
Ceramide synthesis is instigated by a number of cellular stressors. High levels of saturated fatty acids cause an inflammatory reaction in obesity that activate the toll-like receptor 4 resulting in increased ceramide synthesis and insulin resistance [97]. Using a cardiomyocyte model in mice, Azzam et al. found impaired dihydroceramide desaturase in hypoxia and a resultant decrease in ceramide levels, with an accumulation of dihydroceramide [106]. Ultimately, increased synthesis is linked to substrate availability and membrane composition, where increased palmitoyl CoA increases de novo ceramide synthesis (Figure 1.6) [104].

1.2.2 Ceramide Accumulation in the Mitochondria

Ceramide is produced in the ER, golgi, plasma membrane, lysosomes and to a lesser extent the mitochondria [103]. Many studies have shown that ceramides are present in the glycosphingolipid-enriched microdomains, or ‘lipid rafts’ where they protect the plasma membrane from harmful environmental insult, and are critical for cell recognition and signaling [107]. Interestingly, Babiychuk et al. found in stress-induced Jurkat-T cells, ceramide produced in the plasma membrane invaginates the mitochondria – the so called ‘kiss-of-death’, which leads to OMM permeabilization and apoptosis [108]. One study has shown that ceramide synthesized in the ER vesicles can transfer to the mitochondria by way of the MAM in Sprague-Dawley male rat hepatocytes, and that this is associated with transient OMM permeability [109]. The ‘mitochondrial ceramide-rich macrodomains’ are present in irradiated HeLa cells which increase ceramide synthase-mediated ceramide production and optimize pro-apoptotic Bcl-2 family member insertion in the OMM with eventual MOMP and apoptosis (Figure 1.6)[110].
Figure 1.6 Ceramide biosynthesis and subcellular localization.

Ceramide is synthesized from palmitoyl CoA and serine in the ER by means of the de novo pathway involving a series of four enzymatic reactions. In the lysosomes, the salvage pathway uses ASM to convert sphingomyelin to ceramide which is converted to sphingosine by AC. Sphingosine can exit the lysosome for conversion to ceramide by the action of CerS, and back to sphingosine by CERase. Sphingomyelin hydrolysis occurs in the plasma membrane and golgi and is catalyzed by SMS, and SMase. Ceramide from the de novo pathway in the ER can be transported to the mitochondria via the MAM. Plasmalemmal ceramide from the plasma membrane is transported to the mitochondria via ceramide transporter protein (CERT).
1.2.3 Ceramide Interacts with Pro-Apoptotic Bcl-2 Family Members

The permeabilization of the OMM is largely dependent on the action of Bcl-2 family members. This may involve the formation of ceramide channels, which allow the passage of pro-apoptotic proteins into the cytoplasm [111], irreversibly initiating cell death [95]. In HeLa cells, increased neutral-sphingomyelinase produces more ceramide which induces BAX oligomerization by increased BID (a BH3-only protein), binding to its receptor tBID on the OMM [112]. UV irradiation of HeLa cells has been found to increase ceramide 16:0 (CER16), forming the ‘mitochondria ceramide-rich macrodomains’ on the OMM which favour BAX insertion and MOMP [110]. CER16 has been found to regulate a conformational change in BAX structure which is required for MOMP, and interestingly, pro-survival Bcl-2 prevents this from occurring in a HeLa cell model [113]. Pro-apoptotic BAX and BAK have been found to act synergistically with ceramide to form protein-permeable channels [72, 114]; whereas, anti-apoptotic B-cell lymphoma-extra large (Bcl-xL) has been found to inhibit CER16 induced MOMP [71, 115]. One interesting clinical application is the elevated ceramide content in malignant cells treated with vincristine, a commonly used chemotherapy [116]. This drug suppressed tumor growth by induction of apoptosis via elevated ceramides – presumably through formation of ceramide channels or pro-apoptotic Bcl-2-induced MOMP [116, 117]. Functionally, ceramides can also act indirectly through inhibition of complex III of the electron transport chain, with subsequent release of ROS, and eventual MOMP – processes that precede cytochrome c release, and apoptosis [118]. In the human placenta, our group has recently reported that excessive oxidative stress, as found in the pregnancy disorder preeclampsia, increases ceramide biosynthesis resulting in excessive trophoblast cell death and autophagy [119].
1.2.4 Ceramide is Associated with Mitochondrial Fission

Ceramide has been linked to mitochondrial fission in a handful of studies. Smith et al. found that ceramide increased DRP1 mRNA expression in C2C12 myotubes following a 4 hours treatment with ceramide 2:0, and this was associated with impaired complex II respiratory function and could be prevented by pre-treatment with the fission inhibitor Mdivi1 [120]. In cultured neonatal rat cardiomyocytes, ceramide 2:0 treatment stimulated mitochondrial fragmentation, and this was associated with an increase in the mitochondrial content of DRP1 and Fis1 [121]. In yeast, one study found that ceramide signaling targets Sit4p, a ceramide activated protein phosphatase, and Hog1, a mitogen-activated protein kinase to regulate mitochondrial dynamics and mitophagy [122]. In human UM-SCC-22A cells, when DRP1 was knock-down using short-pin RNA, ceramide 18:0 treatment was unable to induce fission or mitophagy [123]. Taken together these studies implicate ceramide as a vital stimulus of mitochondrial fission, and that may be exerting its effect through members of the Bcl-2 family which are discussed in further detail in the subsequent section.

1.3 B-Cell Lymphoma 2 (Bcl-2) Family

The Bcl-2 family is broadly divided into anti-apoptotic and pro-apoptotic proteins which determine cell fate as inducers or inhibitors of apoptosis. The most characterized anti-apoptotic members of the Bcl-2 family are Bcl-xL, Bcl-2, B-cell lymphoma-2-like protein 2 (Bcl-w), B-cell lymphoma-2-related protein A1 (A1) and induced myeloid leukemia cell differentiation protein 1 (Mcl-1) [124]. The anti-apoptotic proteins are opposed by pro-apoptotic Bcl-2 family members in what Skommer et al. describes as ‘rugby players trying to block each other’s’ moves’[125]. Pro-apoptotic Bcl-2 family members are sub-divided based on their structure,
where BAX, BAK and Bcl-2-related ovarian killer (BOK) contain many Bcl-2 homology (BH) domains, and BID, Bcl-2-like protein 11 (BIM), Bcl-2-associated death promoter (BAD), Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), p53 upregulated modulator of apoptosis (PUMA) and Mcl-1 ubiquitin ligase E3 (MULE) contain only the BH3 domain [124].

### 1.3.1 Interactions Between Bcl-2 Family Members

Anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL, Bcl-w, A1, and Mcl-1) stabilize the OMM, inhibit pro-apoptotic Bcl-2 family members, and block cytochrome $c$ release into the cytoplasm, preventing the formation of the apoptosome from APAF-1-mediated caspase-9 activation [126]. The protein structures of anti-apoptotic Bcl-2 family members contain four BH domains, BH1-BH4, with most containing an additional transmembrane domain (TMD) [127]. Interestingly, pro-apoptotic BAX, BAK and BOK are structurally similar to their anti-apoptotic counterparts and contain up to four BH domains, though their function is lethal to the cell [16].

Classically, apoptotic signaling has been thought of as a rheostat where cell fate is determined by the relative abundance of pro-death versus pro-survival proteins [16]. Inhibition of pro-death caspases by pro-survival Bcl-2 protein in a cell-free Xenopus egg extract model, [17], as well as genetic knockout of $Bcl-2$ in mice resulting in increased apoptosis, support the rheostat model [128]. Another model describes the inhibition of anti-apoptotic proteins by their binding to BH3-only Bcl-2 family members, and that apoptosis only proceeds when all anti-apoptotic proteins are functionally neutralized [16]. In accordance with this model, BH3-only proteins must have specific affinities for anti-apoptotic proteins, such that BIM, PUMA and tBID have a global affinity for all anti-apoptotic Bcl-2 proteins, whereas, NOXA binds only to A1 and Mcl-1, and BAD binds only to Bcl-2, Bcl-w and BCL-XL [129, 130]. The neutralization model brings to the forefront the dynamic role of BH3-only proteins, as in addition to suppressing the activity
of pro-survival Bcl-2 family members, they also directly activate BAX and BAK, which oligomerize, create pores in the OMM, and initiate apoptosis [127]. It should be noted that the BH3-domain is found in all Bcl-2 family members and is required for binding and oligomerization, an important requirement by pro-apoptotic BAX for its channel formation on the OMM [131]. The importance of the Bcl-2 family in human disease is clear in colorectal cancer cell models which show that anti-apoptotic Bcl-2 protein promotes cell migration and invasion [132], whereas pro-apoptotic BAX and BAK suppress cell invasion and inhibit ROS production [133].

1.3.2 Bcl-2 Related Ovarian Killer

BOK is a 23 kDa, multi-domain containing pro-apoptotic Bcl-2 family member that was discovered in the late 1990’s [134]. The mRNA expression of BOK was assessed in human and mouse tissues in earlier studies by Northern blot and is localized to the liver, brain, appendix and lymphoid tissues in humans, and at E15, in the liver, thymus, lung, intestinal epithelium, and follicles of the wiskers in mice [134]. Hsu et al. reported that BOK expression was restricted to the reproductive tissues such as the testis, ovary and uterus and that it heterodimers only with anti-apoptotic Mcl-1 and BHRF1 through the BH3 domain in accordance with the rheostat model (previously described) [135]. One study observed that MEF cells lacking Mcl-1 were more vulnerable to the apoptotic effects of BOK, compared to WT [136]. The pro-apoptotic mechanisms of BOK are of recent interest and Carpio et al. reported that in Bok−/− mice, there was a reduced activation of BAK/BAX activation following ER stress hindrances [137]. BOK is degraded by ubiquitination and proteosomal degradation, and in HCT116 cells lacking BAX and BAK, one group found that apoptosis was able to ensue due to BOK stabilization by proteasome
inhibition [138]. This indicates that BOK can induce directly MOMP and consequently apoptosis in the absence of downstream canonical BAX/BAK activation [138].

Though there is agreement that BOK is a BH multi-domain protein, one earlier study reported that it contained BH1-BH4 [134], while another reported that it only contained BH1-BH3 [135]. The literature have reached a consensus that BOK contains four BH domains in its full-length form (BOK-L), and that there is a splice variant (BOK-S) which lacks the 3rd exon spanning a portion of the BH1 and BH3 domains; however, BOK-S still retains the ability to induce apoptosis, but not the ability to heterodimerize with anti-apoptotic Bcl-2 family members in CHO cells [139]. In BOK-L, the BH3 domain is critical for BOK oligomerization at the OMM, and heterodimerization to anti-apoptotic Mcl-1 and BHRF1 [129]. The BOK-BH3 domain correlates to the 65th-82nd base pairs of the structure of BOK, and is functionally analogous with other pro-apoptotic Bcl-2 family members, where BAX-BH3 deletion attenuates dimerization and reduces apoptosis [131].

Our lab has discovered one novel splice variant of BOK known as BOK-P, which is highly expressed in placentae, and lacks exon 2, resulting in loss of 15 out of the 18 base pairs of the BH3 domain [140]. Interestingly, BOK-P is increased in hypoxic placental pathologies such as PE, and retains its apoptotic functions through two alpha-helices (αH5 and αH6) and its C-terminal transmembrane domain (TMD) [140], which is vital for BOK intracellular targeting [136]. The importance of the TMD upon the subcellular localization of BOK has been reported by Echeverry et al. who deleted the TMD and found that BOK remained in the cytosol and nucleus, whereas WT BOK was localized to the mitochondria, ER and golgi [136]. In JEG3 cells, full length BOK (BOK-L) localized to the nucleus in proliferating cells, and to the mitochondria following oxidative stress by means of sodium nitroprusside hindrance [141].
Alternatively, another study reported that nuclear translocation of BOK in HEK-293T cells correlated to increased apoptosis[142]. Interestingly, Gao et al. found that OMM localization of BOK was triggered by apoptotic stimuli, in particular, the BH3-only protein BNIP3 [143]. Our group has identified that BOK and pro-survival Mcl-1 exist in a rheostat where oxidative stress and hypoxia tilt this balance toward BOK accumulation, resulting in increased trophoblast cell death [144]. Taking into account the importance of BOK in the human placenta, and the recent literature implicating BOK as a cause of increased autophagy in PE [144] which is dependent upon elevated ceramides [119], we thought to investigate the roles of ceramides and BOK upon mitochondrial fission in PE.
Figure 1.7 Protein structure of full length BOK.

BOK protein consists of BH1-BH4 in its full length form (upper). The BH3 domain is required for binding to other Bcl-2 family members and spans 17 base pairs and is indicated by the color red in the 3-dimensional protein structure of BOK (lower). The TMD is required for the intracellular targeting of BOK to the mitochondria, ER and golgi and is indicated by the color blue (lower). Apoptosis has been shown to progress in the absence of the BH3 domain in BOK-P through the αH5 and αH6 helices indicated by the color green (upper). (3D model adapted from NESG model, available on www.uniprot.org)
1.3.3 Bcl-2 Family Members and Mitochondrial Fission

There is scarce literature describing the relationship between Bcl-2 family members and mitochondrial fission. One study describes that mitochondrial fragmentation is increased during apoptosis through enhanced activation of DRP1, and its recruitment to the OMM where it interacts with MFF, Fis1 and MiD49/51 [44]. Clerc et al. finds that DRP1 is not required for apoptosis, but does alter the kinetics of cytochrome c release and regulates alterations in respiration in MCF10A and fibroblast cells [145]. In HeLa cells, it has reported that BAX and BAK promote the SUMOylation of DRP1, and its recruitment to the OMM during apoptosis [146]. Interestingly, overexpression of the Bcl-2/adenovirus E1B 19-kDa interacting protein 1 (BNIP1), a pro-apoptotic BH3-only protein resulted in increased DRP1 expression and mitochondrial fragmentation [147]. One study postulates that DRP1 stimulates BAX oligomerization through tBID activation, leading to apoptosis - a function which is independent of its GTPase activity [148]. Mitochondrial division inhibitor (mdivi-1), a selective inhibitor of DRP1, has been found to not only block self-assembly at the OMM, but also to retard pro-apoptotic Bcl-2 family regulated MOMP – indicating that DRP1 has a fission-independent role in apoptosis [149]. Alternatively, pro-apoptotic Bcl-2-like protein 13 has been reported to increase phosphorylation of DRP1 at S637, thereby inhibiting its activation and oligomerization [150].
1.4 The Human Placenta and Preeclampsia

1.4.1 Development of the Human Placenta

The human placenta is an essential and transient organ that provides the interface between the maternal and fetal vasculature, providing both gas and nutrients to nourish the fetus and the means to remove wastes. Additionally, the placenta has an endocrine function, and in early pregnancy is required for the synthesis of glycogen, cholesterol, and fatty acids which are vital for embryonic growth [151]. The 7th post-conception day represents the implantation of the blastocyst into the uterine decidua basalis and the outer cell layer, known as the trophoectoderm gives rise to the placenta [152]. From the trophoectoderm the fetal trophoblast cells differentiate along both extravillous and villous pathways of development to form the respective uteroplacental circulation of the intervillous space, and the fetoplacental circulation of the villous placenta [153]. From the anchoring cell columns of the early placenta arise invasive extravillous trophoblasts (EVTs), which separate and progressively invade the decidua and up to the first third of the myometrium. Their principal aim is to remodel the uterine spiral arteries thus increasing maternal blood flow for gas and nutrient exchange to the fetal circulation. This involves replacement of the arterial endothelial cells with EVT, and leads to a dilation and loss of vasocontrol, which increase uterine blood flow to the intervillous space [154]. In the 20 week placenta, the stem villi contain branching intermediate villi, and terminal villi by which the majority of maternal-fetal exchange occurs. The mature villi contain fetal capillaries, connective tissue, and cytotrophoblast cells which replenish the overlying syncitiotrophoblast layer [155].

Placental development in the first trimester occurs in an environment of relative hypoxia (pO₂ 12 mmHg) which stimulate cytotrophoblast proliferation and inhibit differentiation and invasion
In this low-oxygen environment, antioxidant defenses are absent. When the placenta enters the second trimester, there is an increase in the blood flow into the maternal intervillus space and the pO\(_2\) rises to 60 mmHg at 16 weeks gestation [157].

1.4.2 Bioenergetics in the Human Placenta

Energy production is primed in the human placenta, an extraordinarily metabolic organ that consumes high levels of oxygen and glucose to feed the glycolytic and tricarboxylic acid (TCA) pathways [158]. The endocrine functions of the placenta – including the production of human chorionic gonadotropin (hCG) and human placental lactogen also require an abundance of ATP [159]. Interestingly, the placenta functions in a physiologically hypoxic environment despite requiring oxygen for aerobic respiration, and studies have shown that immediately after delivery, mitochondrial ATP stores are rapidly depleted when the maternal blood supply is disrupted [158, 160]. This concept provides the imperative for rapid collection of placentae used in our experiments. Interestingly, lipid fractions are not altered based on time of placental collection, except for negligible reductions in polyunsaturated fatty acids and glycerol phosphate [160].

1.4.3 Preeclampsia

Preeclampsia is clinically characterized by the American College of Obstetricians and Gynecologists (ACOG) as the onset of hypertension after 20 weeks gestation accompanied with either proteinuria, or additional symptoms including visual disturbances, headache, epigastric pain, thrombocytopenia or compromised liver function [161]. There is no effective treatment, prevention, or early diagnostic test for preeclampsia and delivery of the fetus early is often the only management. The pathophysiology of this devastating disorder is classically divided into initial placental insult, with an exaggerated maternal immune response [162] which is decreased
with prior exposure to paternal antigens [163]. Abnormal placentation results in placental hypoxia, which induces trophoblast cell death, and releases syncytial debris and anti-angiogenic factors like soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) [164] which oppose vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) [165], preventing the establishment of a functioning vascular supply to the fetus, and resulting in disturbed maternal vascular tone (hypertension) and permeability (edema and proteinuria) [166]. Abnormal trophoblast differentiation in preeclamptic placentae leads to impaired infiltration of the maternal spiral arteries and failure to create high capacitance, low resistance vessels conducive to maternal-fetal exchange [167].

The generation of ROS by hypoxia in PE, leads to mitochondrial dysfunction in trophoblast cells, which further produce oxidative stress in a feed-forward mechanism [168]. Increased ROS causes mitochondrial DNA mutations coding for integral membrane proteins leading to loss of membrane potential, diminished ETC function and loss of ATP production [169]. The importance of ROS in the pathophysiology of PE has been demonstrated by numerous studies which have shown that selenium, an antioxidant, is diminished in PE patients and that supplementation may reduce oxidative stress [170]. Cytotrophoblast cell death through apoptosis ensues when antioxidant defenses are unable to cope with the oxidative load. It is particularly important to point out that uncontrolled apoptosis in the syncitiotrophoblast layer would be catastrophic, as the syncytium lacks cell membranes and would spread without control, however, in vivo, this layer is somewhat immune to programmed-cell death [171]. The cytotrophoblast layer is sensitive to apoptotic stimuli, and Hung et al. have reported that term placental villi subjected to repeated hypoxia-oxygenation injury had increased protein expressions of pro-apoptotic BAX and BAK, and decreased mRNA expression of anti-apoptotic BCL-2 [172].
BOK has been recognized as an important player in placental dysfunction such that under preeclamptic conditions of hypoxia, both BOK-L and BOK-P expression levels are elevated leading to mitochondrial apoptosis of the trophoblast [140]. Soleymanlou et al. describe an altered balance between anti-apoptotic MCL-1 and pro-apoptotic BOK, where hypoxia in preeclampsia favour BOK-induced trophoblast cell death which can be rescued by \textit{MCL-1} overexpression in JEG3 cells[173]. The altered MCL-1/BOK rheostat in favour of BOK has been further implicated as the regulator of placental autophagy, in which silencing of \textit{MCL-1} increases autophagy marker LC3B-II in JEG3 cells [144]. Furthermore, our group has recently discovered the important regulation of ceramide biosynthesis enzymes in the hypoxic environment of preeclamptic placentae. Oxidative stress was found to significantly decrease the expression of acid ceramidase in placental explants and JEG3 cells treated with sodium nitroprusside leading to an accumulation of ceramide 16:0 and ceramide 18:0 and resultant trophoblast autophagy due to BOK accumulation and decreased Mcl-1 expression [119].

1.5 Summary

The mitochondria exist in altering states of fission and fusion – processes collectively known as mitochondrial dynamics. These antagonistic processes act in response to the ever changing physiological demands of the cell [7]. Mitochondria are found as networks of organelles that have functions in energy production and cell death pathways. The process of fusion enhances the metabolic activity of the cell and helps to dissipate energy; whereas, fission isolates irrelevant factions of the organelle in more quiescent cells [56]. The discovery of mitochondrial dynamics can be dated to Lewis and Lewis’s 1914 observations of fusion and fission, and today have been causally linked to a number of neurological and cardiometabolic pathologies [7].
The dynamin-related protein 1 (DRP1) is a GTPase from the dynamin ‘superfamily’ that is central to the fission apparatus[174]. When DRP1 is activated, it is recruited from the cytosol to the OMM where it interacts with resident proteins such as mitochondrial fission factor (MFF). DRP1, once recruited to the OMM, oligomerizes and creates a ring like structure that gives the mechanical-enzymatic force to fragment the organelle [56]. Activation of DRP1 is regulated by the alternating effects of phosphorylation at two separate serine residues; phosphorylation at S616 increases DRP1 activity, whereas phosphorylation at S637 decreases DRP1 activity [7]. There are a number of alternate pathways and post-translational modifications that also regulate DRP1 activity - including dephosphorylation at S637 by calcium-dependent calcineurin [56]. The mitochondria-associated ER membranes have been identified as the microenvironments in which mitochondrial fission occurs [73].

Ceramide is a bioactive sphingolipid produced through either the de novo synthesis pathway or as a product from either sphingosine (via ceramide synthase) or sphingomyelin (via acid sphingomyelinase) [119]. This thesis project focuses on the effect of ceramide upon mitochondrial fission in PE, a disorder of pregnancy clinically defined as hypertension and proteinuria in pregnancy, or in the absence of proteinuria - new onset hypertension with thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or cerebral or visual symptoms[161]. To support our endeavor, recent data from our lab have shown that ceramides are found in excess in PE placentae where it propagates cell death and autophagy via a mechanism that involves BOK [119], a pro-apoptotic protein that is highly expressed in the human placenta [140].

In summary, this project will evaluate mitochondrial fission in PE, and the role of ceramide-induced BOK-regulation of DRP1. Furthermore, though the biological relevance of increased
fission in PE is yet to be determined, this project will investigate the role of mitophagy, the selective autophagic degradation of mitochondrial fragments, as a possible mechanism by which PE placentae attempt to evade oxidative stress produced by excess fission in PE.
1.6 Rationale, Hypothesis and Objectives

Mitochondrial fission and fusion are physiologically balanced in healthy cells and are required for embryonic development. Central to mammalian development is the placenta, which is the interface between the maternal and fetal circulations. Complete Drp1 knockouts in mice result in the absence of trophoblast giant cells and embryonic lethality at E10.5-12.5 [59]. Interestingly, the giant cell layer in Mfn2−/− mice was also reduced, and the few cells that remained had smaller nuclei, though 86% of these embryos were not viable at E11.5 [36]. Thus, it is clear that mitochondrial dynamics are required for placental viability and embryonic development.

Preeclampsia is a potentially devastating hypertensive disorder of pregnancy which is characterized by a hypoxic placenta which exhibit accelerated trophoblast cell death rates, and extrudes syncytial debris into the maternal vasculature thereby initiating a generalized inflammatory response. At a mitochondrial level, hypoxic insults lead to impaired complex III-IV of the electron transport chain, thereby accumulating toxic reactive oxygen species [175], leading to damaged mitochondrial proteins, mtDNA and impaired mitochondrial membrane potentials – all of which trigger mitochondrial fission and Bcl-2-mediated apoptosis. Ceramide, a bioactive signaling sphingolipid is increased in human preeclamptic placentae [119], and complete knockout of sphingolipid regulatory enzyme sphingosine kinase in mice results in increased sphingosine (a substrate for ceramide synthesis via ceramide synthase), leading to impaired decidualization, implantation and early pregnancy loss [176]. Importantly, ceramide induces mitochondrial fission in human squamous cell carcinoma cells, and when DRP1 is silenced, ceramide alteration of mitochondrial morphology and function is diminished [123]. Furthermore, ceramide acts in close concert with pro-apoptotic Bcl-2 family members like BAX, BAK and importantly BOK, to execute mitochondrial apoptosis [72]. In extrapolating these facts
to the preeclamptic placenta, the mitochondria appear to be an important organelle for the interplay between ceramide and Bcl-2 family members which participate readily in mitochondrial fission and cell death.

We hypothesized that increased ceramides found in preeclamptic placentae, tilt the mitochondrial dynamics balance towards mitochondrial fission.

Hence, our objectives were to determine the presence of mitochondrial fission machinery in the human placenta in physiological and pathological conditions, and to investigate the mechanism by which ceramide regulates mitochondrial fission using in vitro models.
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Placental Tissue Collection

Informed consent was obtained from all clinical subjects, and placental collection was conducted in accordance with the ethical guidelines of the University of Toronto Faculty of Medicine and Mount Sinai Hospital by the Placenta BioBank, Mount Sinai Hospital, Toronto. All experiments were in agreement with the Helsinki Declaration of 1975, including its current 7th revision in 2013. PE subjects (n=33) were selected based upon the American College of Obstetrics and Gynecology (ACOG) criteria of maternal hypertension and proteinuria, or in the absence of proteinuria – thrombocytopenia, impaired liver function, pulmonary, renal, or cerebral disease. PE patients were further stratified according to the weight of their baby as either appropriate for gestational age (AGA), or small for gestational age (SGA) as defined as <3rd percentile when plotted on a fetal growth chart obtained from the Public Health Agency of Canada website (http://www.phac-aspc.gc.ca/rhs-ssg/bwga-pnag/index-eng.php). Normotensive age-matched PTC (n=30) were selected based on the absence of placental disease with AGA fetuses.

Placental tissue was sampled according to the standard operating procedures of the Research Center for Women’s and Infant’s Health Placenta BioBank (http://biobank.lunenfeld.ca). In short, placentae were obtained within 10 minutes of delivery from the labour rooms or operating theatres of Mount Sinai Hospital, Toronto. Using sterilized surgical dissection scissors and forceps, four 2x2 cm trans-placental cores were obtained from each quadrant. These cores were washed 2 times in 4°C phosphate buffered saline (PBS) to remove red blood cell contamination,
after which they were further dissected into 0.5x0.5 cm pieces, placed in cryotubes and snapped-frozen using liquid nitrogen. The clinical parameters of PE and PTC subjects are listed in Table 3.1.

2.2 Mitochondrial Isolation

Approximately 200 mg of frozen tissue from PE and PTC placentae were partially thawed on ice, cut into smaller pieces, rinsed with isotonic saline (0.9% NaCl solution) and suspended in 500µL of ice-cold buffer A (0.25 M sucrose, 0.001 M EDTA, 10 mM Tris-HEPES, pH 7.4). The tissue was subjected to two, one-minute homogenizations: one at low (setting 1), and the next at medium speed (setting 2) (Homogenizer: VWR®, West Chester, Pennsylvania). The homogenate was centrifuged at 1300g for 5 minutes at 4°C with and the supernatant corresponded to the post-nuclear supernatant (PNS) which was separated into graduated 1.5 mL microtubes (Scientific Specialities, Inc., Lodi, CA) and further centrifuged at 12000g for 15 minutes at 4°C. The pellet corresponded to the mitochondrial isolate (MI), and the supernatant corresponded to the post-mitochondrial supernatant (PMS). The MI was resuspended in 100µL of RIPA. The purity of all three fractions (PNS, PMS, MI) were validated by WB using TOM20, a marker of the outer mitochondrial membrane, and β-Actin, a cytoskeletal protein marker. The MI was assessed biochemically by Western Blot (WB) for p-DRP1, and a portion was used for ceramide analysis using high powered liquid chromatography linked to tandem mass spectrometry (LC-MS/MS).

2.3 Mitochondria-Associated ER Membrane (MAM) Isolation

Subcellular fractionation and isolation of the MAM was carried out as described by Azzo et al. [177]. In short, the mitochondrial pellet of PE and PTC tissue was isolated as described above,
and resuspended in 2 mL of EMEM media, and subsequently placed on a 30% Percoll gradient and centrifuged at 95000 g for 30 minutes at 4°C. The Percoll gradient was separated into the heavy fraction (HF) containing the mitochondria, and the light fraction (LF) containing the MAMs. The LF was centrifuged at 6300 g for 10 minutes at 4°C, and the supernatant further centrifuged at 100000 g for 1 hour at 4°C. The resulting pellet was the MAM isolate and was validated by TOM20 and calreticulin enrichment. DRP1 expression was evaluated by WB, and an aliquot was used for ceramide analysis by LC-MS/MS.

2.4 High Powered Liquid Chromatography Linked to Tandem Mass Spectrometry

MI and MAM isolates from PE and PTC placental tissues were processed for lipid extraction. 25 mg of tissue was lyophilized and homogenized in 2 mL of water and methanol prior to mixture with internal standard sphingolipids (ceramide C17 (d18:1/17:0), sphingosine (d17:1), sphingosine-1-phosphate (d17:1), and sphingomyelin 17:0 (d18:1/17:0) obtained from Avanti Polar Lipids (Alabaster, Alabama). Samples were then combined with 2 mL of chloroform, vortexed for 1 minute. The samples were then kept on ice for 10 minutes, after which they were centrifuged for 5 minutes at 1000g. By removing the chloroform layer, the lipids were extracted and dried in the presence of nitrogen. To prepare the samples for LC-MS/MS, they were reconstituted in 100 µL of EtOH acidified with 0.2 µL of formic acid and placed in siliconized minivials.

Samples were sent for LC-MS/MS at the Analytical Facility for Bioactive Molecules (AFBM), The Hospital for Sick Children, Toronto, which was performed on an Agilent 1200 Series binary pump (Agilent Technologies Canada Inc., Mississauga, Ontario) linked to an AP14000 triple-
quadruple mass spectrometer (AB SCIEX, Vaughan, Ontario). First, reverse phase high-powered liquid chromatography (HPLC) was conducted using a Kinetex C18 Column (Phenomenex, Torrance, CA) which had a mobile phase with the following two solvents containing 0.05% formic acid. Solvent A: dH$_2$O/acetonitrile/ methanol; and Solvent B: tetrahydrofuran/acetonitrile/methanol. The solvents were delivered using the solvent-delivery system pump at a flow of 400 µL per minute with varying ratios of solvents A:B. The first 4.5 minutes before sample injection and the 2.5 minutes post injection had a solvent ratio of 60:40 (A:B). For the next 13 minutes (post sample-injection) the solvent ratio was progressively changed to 15:85 (A:B) and maintained there for 15 minutes, and then gradually returned to 60:40 over the next 17 minutes. The sample injection at 4.5 minutes contained 1-5 µL of lipid extract as described above. Positive electrospray ionization (5000 volts) was employed for mass spectrometry analysis with a source temperature of 400°C and nitrogen as the collision-induced dissociation gas. A standard curve was made for each sphingolipid species and samples were plotted against their respective curves.

2.5 Transmission Electron Microscopy

PE (n=8) and PTC (n=7) placental tissue were collected within 10 minutes of delivery and prepared for TEM analysis. Primary isolated cytотrophoblast cells from term placentae (n=5) were treated with synthetic ceramide 16:0 (CER16) or EtOH vehicle, and HEK-293 cells were stably transfected with BOK and induced with doxycycline (Dox) or dH$_2$O vehicle (n=3). Tissue and cell samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for up to 24 hours at 4°C. The samples were processed by the Advanced Bioimaging Centre, Mount Sinai Hospital, Toronto. Placental tissue was processed into 90 nm sections, picked up on copper grids and stained with uranyl acetate and lead citrate. For cells grown on coverslips, they were first
washed three times with 0.1M cacodylate buffer, then fixed in cacodylate buffer containing 1% osmium tetroxide and 1.25% potassium ferrocyanide in for 1.5 hours. Three successive 0.1M cacodylate buffer washes were followed by two washes with distilled H$_2$O (dH$_2$O), and the coverslips were stained for 30 minutes in 4% aqueous uranyl acetate. After two washes with dH$_2$O, the coverslips were dehydrated with EtOH. The samples underwent infiltration with Quetol-651 for 2 hours, and Quetol resin for two, two-hour durations. The coverslips were embedded in Quetol resin at 65°C overnight, then warmed to 90°C and the cells were dissected out. They were further cut into 90 nm sections, picked up on copper grids and stained with uranyl acetate and lead citrate. Imaging for both tissue and cells was conducted on a FEI Technai 20 Transmission Electron Microscope (Hillsboro, Oregon).

### 2.6 Mitochondrial Fission Quantification

TEM images of cytotrophoblast cells from PE (n=8) and PTC (n=7) placentae were obtained. For each placenta three cytotrophoblast cells were identified, the number of mitochondria per nucleus was counted, and an internal mean was generated. This process was blinded and verified by three separate individuals not related to this project, who had an understanding of mitochondrial identification. Mitochondrial width was measured using ImageJ® 1.49v software [178], where the minimum short-axis was recorded for each mitochondrion in three cytotrophoblast cells from all PE (n=8) and PTC (n=7) subjects. Statistical analysis was conducted as described in section 2.12.
2.7 Cell Culture and Analysis

2.7.1 JEG3 Human Choriocarcinoma Cells

JEG3 cells (ATCC, Manassas, VA, USA) were cultured in 6-well plates or coverslips in 20% O₂ at 37°C in Eagle’s Minimum Essential Medium (EMEM) (Visent Inc., Montreal, Quebec). The EMEM was composed of 445 mL media, 50 mL of fetal bovine serum (FBS), and 5 mL of penicillin-streptomycin (penicillin 10000U/mL + Streptomycin 10000ug/mL; Gibco, TFS, Waltham, MA, USA). Once the cells attained 80% confluency, they were treated with CER16 20 µM or EtOH vehicle for 6 hours, and either collected in RIPA for protein analysis by western blot (WB), or fixed with 4% paraformaldehyde for IF.

2.7.2 Human Embryonic Kidney-293 Cells

HEK-293 cells (ATCC®, Manassas, Virginia) were cultured in high glucose DMEM media (Lunenfeld-Tanenbaum Research Institute, Toronto, Ontario) at 20% O₂ at 37°C to a confluency of 60-80%. Cells were used for transfection to silence (siRNA) and overexpress BOK, and to overexpress BOK ΔBH3 (described below). HEK-293 cells were stably transfected with GFP-hBOK using a Flp-In-T-Rex-293 cell line (ThermoFisher Scientific®, Waltham, Massachusetts) as previously described. GFP-hBOK cells lines included wild type (WT) and those with the following deletions: ΔαH4, ΔαH5, ΔαH6, ΔTMD. BOK was induced in the transfected cell lines by doxycycline at 1.5 or 2.5 ng/mL for 36 hours. The vehicle for Dox is dH₂O.

2.7.3 Synthetic Ceramide 16:0 (CER16) Treatment

JEG3 cells were cultured in 6-well culture plates (Life Technologies, Carlsbad, CA, USA) and incubated at 37°C at 20% O₂ until 80% confluent (2 x 10⁵ cells/well). Primary isolated
cytotrophoblast cells were seeded onto 60.1 cm² petri dishes (6 x 10⁶ cells/petri dish) and incubated for 24 hours at 37°C in 8% O₂. Cells were treated with 20 µM synthetic CER16 dissolved in EtOH (Enzo Life Sciences Inc., Farmingdale, New York) for 6 hours. Optimal CER16 treatment concentration was ascertained from a concentration gradient experiment (Figure 3.7 A).

2.8 Isolation of Primary Cytotrophoblast from Term Placentae

Whole, term placentae (n=5), from normotensive, otherwise healthy women undergoing elective C/S for fetal malpresentation or previous C/S, were obtained within 10 minutes of delivery (Table 3.2). Approximately 60 grams of placental tissue was dissected, avoiding areas of calcification and large vasculature. The tissue was cut into smaller pieces, and subjected to a series of washes in Hank’s balanced salt solution without Ca²⁺ or Mg²⁺ (HBSS⁻/⁻) to remove red blood cell (RBC) contamination, and rinsed once in HBSS⁺/⁺ (with Ca²⁺ and Mg²⁺). The tissue was placed in a 1 litre Erlenmeyer flask filled with digestion buffer (200 mL Dulbecco’s Modified Eagle Medium (DMEM), 0.25g trypsin, 0.03g DNase) for three serial digestions (30, 40 and 30 minutes) in a rocking waterbath at 37°C. The supernatants from the last two digestions were strained, pooled together, and centrifuged for 20 minutes at 2500 rpm (soft start, brake off) in a Percoll gradient. Gradients corresponding to cytotrophoblast cells were washed in HBSS⁺/⁺, and subjected to differential attachment to remove fibroblast contamination. The cells were counted using Trypan blue and a haematocytometer, and cultured at a confluency of 1 x 10⁶ cells per 35 mm well, on coverslips or not, in DMEM F:12 media containing FBS and penicillin-streptomycin (Gibco®, Waltham, Massachusetts). Cells were left in culture for 24 hours at 8% O₂ (physiological oxygen tension for term placentae) and subsequently treated with CER16 (20 µM) or EtOH vehicle for 6 hrs prior to collection for WB analysis in radioimmunoprecipitation
assay buffer (RIPA, 150mM NaCl, 50mM Tris, 1% NP-40, pH 7.5), or fixation for immunofluorescence (IF) in 4% paraformaldehyde.

2.8.1 Transient Transfections

Transient transfections were conducted using HEK-293 cells to maintain consistency with our established stable, inducible cell lines.

2.8.1.1 BOK Silencing

HEK-293 were cultured as described above, and when a confluency of 60-80% was attained, cells were transfected with either 30 nM of Silencer® select siRNA targeted against the mRNA of BOK (Ambion, Waltham, Massachusetts), or scrambled siRNA sequences as a control, using a jetPRIME® protocol (Polyplus Transfection®, New York, New York). The jetPRIME® protocol involved three key steps. First, 2 µg/35 mm well of DNA was diluted in sterile filtered jetPRIME® buffer (Cat n°712-60), vortexed for 10 seconds and centrifuged for 1 minute at 8000g. Second, 200µL of jetPRIME® reagent (Cat n°114-07) was added to the 2µL of diluted DNA, vortexed for 10 seconds and centrifuged for 1 minute at 8000g and left at room temperature for 10 minutes to allow for optimal mixing. The transfection mix was added to DMEM media and placed on cells which were cultured at 37°C and collected 24 hours later in RIPA for protein analysis by WB.

2.8.1.2 WT-BOK Overexpression, and BOK-BH3 Deletion

HEK-293 cells were cultured as described above to a confluency of 60-80% and then transfected with 2 µg/35 mm culture well of pcDNA BOK-L (WT BOK), pcDNA BOK-ΔBH3 and pcDNA3.1
(empty vector) (ThermoFisher Scientific®, Waltham, Massachusetts) using a jetPRIME® protocol (explained in section 2.4.4.1). The plasmids were constructed by identifying open reading frames coding for BOK-L, which were cloned in pcDNA3.1/Hygro(+) (ThermoFisher Scientific®). The forward primer was: 5’-CCCGGTACCACCATGATCCGGCCTCTAC-3’ and the reverse primer was 5’-CCCGGATCCGGGTCATCTCTGGCAACAA CAGGAA-3’. The deletion of the BOK-ΔBH3 plasmid was designed based on the published sequence which identified the BH3 domain as base pairs 65-82 [140] (Figure 2.1). Cells were transfected with the BOK-L and BOK-ΔBH3 plasmids and protein was collected in RIPA after incubation at 37°C for 24 hours for WB analysis.

![Figure 2.1 BOK-BH3 domain deletion.](image)

Plasmid sequence for BOK showing in yellow the 65th-82nd deleted base pairs corresponding to the BH3 domain.

### 2.9 Western Blot Analysis (WB)

PE and PTC snap frozen tissue was pulverized in liquid nitrogen and homogenized in RIPA buffer for 1 minute at setting 2. The homogenate was centrifuged for 10 mins at 8000 g, and the supernatant transferred to a new tube. Cultured cells were collected in 40 µL of RIPA per well.
(of a 6 well plate) and placed on ice for 1 hour, centrifuged and the supernatant transferred to a new tube. The protein content of tissue and cell samples was assessed by Bradford protein assay (Bio-Rad®, Mississauga, Ontario).

For WB, tissue and cell samples contained 30 µg of protein, 8 µL of sample buffer, and a total volume of 32 µL supplemented with RIPA. Samples were run on a sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) in running buffer (25 mM Tris base, 192 mM glycine, 0.1% (SDS)) until the target band was well separated. The gels were transferred onto methanol-hydrated polyvinylidene fluoride (PVDF) membrane, in ice-cold transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol). The membranes were then blocked in 5% non-fat milk dissolved in tris-buffered saline with Tween® 20 (TBST) (ThermoFisher Scientific®, Waltham, Massachusetts) for 1 hour at room temperature, and left overnight in primary antibody dissolved in 5% non-fat milk at 4°C on a rocker. The next day, the membranes were washed three times for 15 minutes in TBST, and secondary antibody (horseradish peroxidase (HRP)-conjugated polyclonal antibody) dissolved in milk was added for 1 hour at room temperature on a rocker. Blots were imaged using chemiluminescence ECL-plus reagent (PerkinElmer Inc., Waltham, Massachusetts) and x-ray film (GE Healthcare, Chicago IL).

To assess loading control, PVDF membranes were stripped in buffer (0.2 M glycine, ddH2O, pH 2.2) for 30 minutes, two times. They were subsequently blocked in 5% non-fat milk dissolved in TBST for 1 hour and probed for cytoskeletal proteins (β-Actin, α-Tubulin). The membranes were subsequently imaged according to the above mentioned protocol.
2.10 Densitometric and Statistical Analysis

WB densitometric analysis was conducted using ImageQuant® 5.0 software. Volumes of protein expression were obtained, corrected for background, and normalized to either βActin, αTubulin, or Ponceau S. volumes. Statistical analysis was performed using GraphPad Prism 5 software, where comparison of two means utilized an unpaired Student’s t-test, and comparison of multiple means used a one-way analysis of variance (ANOVA) with a Tukey post-test to compare two variables where applicable. Significance was denoted as *P<0.05, **P<0.01, and ***P<0.001.

2.11 Immunofluorescence (IF)

Following cell treatments, JEG3, HEK-293 and primary isolated cytotrophoblast cells were fixed with 4% paraformaldehyde (Canemco®, Gore, Quebec) for 15 minutes at 37°C. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes, rinsed with PBS and blocked with 5% normal horse serum (NHS) (Sigma®, St. Louis, Missouri) for 1 hour at room temperature. Primary antibodies were diluted in antibody diluent (0.4% sodium azide, 0.625% gelatin) and 5% NHS, and placed on cells for incubation overnight at 4°C covered from any light in aluminum foil. The primary antibody was replaced with either normal-rabbit IgG (sc-2027) or normal-goat IgG (sc-2028) (Santa Cruz Biotechnology, Dallas, Texas), for negative controls corresponding to the primary antibodies being used. Following three PBS washes, HRP-conjugated secondary antibodies were diluted in antibody diluent and applied for one hour at a concentration of 1:2000 in complete darkness at room temperature, after which three additional PBS washes was carried out. Cells were treated with 4’,6-diamino-2-phenylindole (DAPI) for 5 minutes to detect the nucleus, prior to fixation to 25x75x1 mm glass slides with Immuno-Mount™ (ThermoFisher Scientific®, Waltham, Massachusetts). IF images were obtained using the DeltaVision.
deconvolution microscope (GE Healthcare, Baie d’Urfe, Quebec). Live cell staining of the mitochondria in JEG3 cells was conducted using 100 nM MitoTracker® red (ThermoFisher Scientific®), which was added for 5 minutes prior to fixation.

2.12 Antibodies

2.12.1 Primary Antibodies

Commercially available primary antibodies were obtained for WB and IF analyses. Antibodies against DRP1 (H-300, sc-32898, rabbit [WB 1:1500]), MFF (T-14, sc-168593, goat [IF: 1:200, WB 1:1000]), MFN2 (H-68, sc-50331, rabbit [IF: 1:200, WB 1:1000]), BOK (H-151, sc-11424, rabbit [IF: 1:200, WB 1:1000]), TOM20 (FL-135, sc-11415, rabbit [WB 1:1000]), α-Tubulin (P-16, sc-31779, goat [WB 1:2000]), and β-Actin (I-19, sc-1616, goat [WB 1:2000]) were purchased from Santa Cruz Biotechnology, Dallas, Texas. Antibodies against p-DRP1 (S616) (3455S, rabbit [IF: 1:500, WB 1:1000]) and DRP1 (4E11B11, 14647, mouse [WB 1:1000]) were purchased from Cell Signalling Technology®, Danvers, Massachusetts. OPA1 (612607, mouse [WB 1:1000]) was purchased from BD Biosciences®, San Jose, California; PINK1 (BC100-494, rabbit [WB 1:1000]) was purchased from Novus Biologicals®, Littleton, Colorado; and Parkin (AB9244, rabbit [WB 1:500]) was purchased from MilliporeSigma®, Darmstadt, Germany.

2.12.2 Secondary Antibodies

Secondary antibodies include goat anti-rabbit IgG-HRP (sc-2054 [WB: 1:2000]), donkey anti-goat IgG-HRP (sc-2056 [WB: 1:2000]), and goat anti-mouse IgG-HRP (sc-2005 [WB: 1:2000]) and were purchased from Santa Cruz Biotechnology, Dallas, Texas. For IF, Alexa Fluor® 488 donkey anti-rabbit IgG (A21206), Alexa Fluor® 594 donkey anti-rabbit IgG (A21207), Alexa
Fluor® 488 donkey anti-goat IgG (A11055), Alexa Fluor® 594 donkey anti-goat IgG (A11058), and Alexa Fluor® 594 donkey anti-mouse IgG (A21203) were all purchased from ThermoFisher Scientific®, Waltham, Massachusetts.
Chapter 3
Results

3 Results

Preeclampsia (PE) is a serious disorder that complicates 2-8% of pregnancies worldwide and represents a significant cause of maternal and fetal morbidity and mortality [180]. Notably, the involvement of mitochondrial dynamics in PE remains elusive. PE is typically characterized by excessive trophoblast cell death, which, in turn, generates syncytial debris that is aberrantly extruded into the maternal circulation where it exerts a generalized maternal inflammatory response clinically manifesting as hypertension[181, 182]. Interestingly, women who experienced PE and deliver preterm have an increased risk of developing chronic hypertension, hypertriglyceridemia and metabolic syndrome 5-10 years post-partum [183].

We have recently reported that excessive cell death and autophagy in PE are in part dependent on a build-up of ceramides (CERs), a group of bioactive sphingolipids[119]. The majority of CER is produced through the de novo pathway from serine and palmitoyl CoA, via a series of enzymatic reactions culminating in the conversion of dihydroceramide to ceramide by dihydroceramide desaturase. Alternate pathways involve the inter-conversion of CER from sphingosine via ceramide synthase, and from sphingomyelin via acid sphingomyelinase[100, 184]. The accumulation of CER in PE has been shown to increase the expression of Bcl-2 Related Ovarian Killer (BOK), a pro-apoptotic Bcl-2 family member, leading to increased trophoblast autophagy and death[119, 144]. In particular, BOK exerts its autophagic activity directly, or indirectly by disrupting the recruitment of BECN1 to MCL1, a pro-survival protein, which functions by sequestering Bcl-2 homology domain (BH3)-containing proteins in response to apoptotic stimuli [185]. The altered Mcl-1/BOK balance towards pro-death BOK has been
implicated in the pathogenesis of preeclampsia [140, 144], though, to date, this has not been 
evaluated in the context of mitochondrial fission.

Herein, I report increased expression of key regulators of mitochondrial fission in PE.
Furthermore, we attributed CER accumulation as a regulator of increased mitochondrial fission, 
through a mechanism involving BOK protein. Finally, we localized mitochondrial fission events 
to the MAM and verify that the degradation of mitochondrial fragments is occurring by 
PINK1/Parkin-mediated mitophagy.
3.1 Mitochondrial fission is Increased in Preclampsia

3.1.1 DRP1 Expression is Increased in PE vs. PTC

DRP1 is the key regulator of mitochondrial fission and its excess is associated with increased rates of apoptosis and mitophagy [7]. Hence, we first examined the expression of DRP1 in placental tissues from PE and normotensive age-matched control pregnancies. The clinical parameters of the clinical subjects are detailed in Table 3.1. Western blot (WB) analysis revealed significantly increased DRP1 protein expression levels in PE placentae relative to preterm controls (PTC) (Fig. 3.1 A). Following its activation, DRP1 is recruited to mitochondrial fission factor (MFF), a 35 kDa OMM resident protein [44]. Immunoblotting showed no changes of MFF expression in PE relative to PTC (Fig. 3.1 B). Mitochondrial dynamics involve a balance between fission and fusion, thus, we next examined the expression of OPA1, the key regulator of IMM fusion, and a GTPase responsible for cristae remodeling. WB analysis demonstrated a significant decrease in OPA1 protein expression levels in PE compared to PTC (Fig. 3.2A).
Table 3.1 Clinical parameters of the study population.

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Preterm Controls</th>
<th>Preeclampsia</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n=30</td>
<td>n=33</td>
</tr>
<tr>
<td>Gestational Age at Delivery (weeks)</td>
<td>29.7±2.3</td>
<td>29.3±3.0</td>
</tr>
<tr>
<td>Neonatal Weight (grams)</td>
<td>1719±282.2</td>
<td>1004±372.0</td>
</tr>
<tr>
<td>Neonatal Weight (Percentile)</td>
<td>0% of fetuses ≤ 3rd</td>
<td>53% of fetuses ≤ 3rd</td>
</tr>
<tr>
<td>Neonatal Sex</td>
<td>40% F, 60% M</td>
<td>33% F, 67% M</td>
</tr>
<tr>
<td>Blood Pressure (mmHg)</td>
<td>S: 114±12.1†***</td>
<td>S: 170±17.5†***</td>
</tr>
<tr>
<td></td>
<td>D: 80±8.3†***</td>
<td>D: 102±11.5††***</td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>Absent</td>
<td>3.6±0.85</td>
</tr>
<tr>
<td>Mode of delivery (%)</td>
<td>54% VD, 46 % C/S</td>
<td>12.5% VD, 87.5% C/S</td>
</tr>
<tr>
<td>C/S Labour vs. C/S Non-Labour</td>
<td>60% L, 40% NL</td>
<td>29% L, 71% NL</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (SD). Abbreviations: F, female; M, male; S, systolic; D, diastolic; VD, vaginal delivery; C/S, Caesarean section; L, labour; NL, non-labour.

†, systolic blood pressure PTC vs. PE (unpaired Student’s t-test, ***P<0.0001)
††, diastolic blood pressure PTC vs. PE (unpaired Student’s t-test, ***P<0.0001)
Figure 3.1 DRP1 and MFF protein expression in PE and PTC placentae.

Representative WB of (A) DRP1 and (B) MFF expression in PE vs. PTC placentae. Densitometric analysis normalized to α-Tubulin (TUBA) and expressed as a fold change relative to PTC. DRP1 was significantly increased in PE relative to PTC, but not MFF (PE, n=30; PTC, n=22; unpaired Student’s t-test ***P<0.001).
Figure 3.2 OPA1 protein expression in PE and PTC placentae.

Representative WB of OPA1 expression in PE vs. PTC. Densitometric analysis normalized to α-Tubulin (TUBA) and expressed as a fold change relative to PTC. OPA1 was significantly decreased in PE relative to PTC (PE, n=10; PTC, n=13; unpaired Student’s t-test *P<0.05).
3.1.2 DRP1 is Activated in the Mitochondria of PE Placentae

Activation of DRP1 by phosphorylation at S616 residue is required for its recruitment to the mitochondria where it triggers fission events[186]. Hence, we isolated the mitochondrial compartment from PE and PTC placentae and examined DRP1 activation using a specific antibody that recognizes phosphorylated DRP1 (p-DRP1). Western blotting revealed a significant increase in pDRP1 in the mitochondrial isolate (MI) from PE placenta relative to that harvested from PTC. Phosphorylated DRP1 protein levels were normalized to TOM20, a 20 kDa protein marker of the OMM (Fig. 3.3 A). The post-nuclear supernatant (PNS) was collected for comparison, and no changes in pDRP1 expression were found in the PNS of both PTC and PE (Fig. 3.3 B). These findings confirm that active p-DRP1 is found in excess in PE, where it localized to mitochondria. CER is a cell death inducer in PE leading to aberrant trophoblast autophagy [119]. Thus, we examined the ceramide content of MI from PE and PTC placentae using LC-MS/MS. A significant enrichment in CER 16:0 and CER 18:0 was observed in MI from PE placenta relative to PTC (Fig. 3.4).
Figure 3.3 p-DRP1 expression in mitochondrial isolate, post-mitochondrial supernatant, and post mitochondrial isolate of PE and PTC placentae.

(A) Representative WB analysis of p-DRP1 expression in the mitochondrial isolate of PE vs. PTC placentae. p-DRP1 is significantly increased in the mitochondrial compartment of PE placentae compared to PTC. Densitometric analysis normalized to TOM20, an OMM marker, and expressed as a fold change relative to PTC. (PE, n=4; PTC, n=4; unpaired Student’s t-test \( **P<0.01 \)). (B) Representative WB of the post-nuclear supernatant of PE vs. PTC placentae. Loading control was the cytoskeletal marker β-Actin (ACTB).
Figure 3.4 Ceramide analysis of mitochondrial isolates from PE and PTC placentae.

Ceramide analysis of mitochondrial isolate from PE/PTC placentae as assessed by LC-MS/MS. CER 16:0 and CER 18:0 are significantly increased in PE mitochondria compared to PTC. Ceramide levels normalized to cholesterol (PE, n=4; PTC, n=4; one-way ANOVA, *P<0.05, **P<0.01).
3.1.3 Mitochondrial Morphology is Altered in Preeclamptic Cytotrophoblasts

Transmission electron microscopy (TEM) was employed for qualitative observation of mitochondrial morphology in PE and PTC placentae. Mitochondrial fission was identified by clear contact points between adjacent organelles and smaller globular mitochondrial fragments, in contrast to the elongated ovular morphology typical of healthy mitochondria, which exist in branching networks [187]. Augmented mitochondrial fission events were observed in cytotrophoblast cells from PE placentae compared to PTC (Fig. 3.5, i. vs. ii.). Although to a lesser extent, mitochondrial fission was also seen in the syncitiotrophoblast and endothelial cells (data not shown). A quantitative analysis of mitochondrial number from TEM images of cytotrophoblast revealed a significant two-fold increase in the number of mitochondria per cell in PE compared to PTC (Fig. 3.5, iii. vs. iv.; Fig. 3.6 A). In addition, mitochondria from PE exhibited a significantly smaller mitochondrial width when compared to PTC (Fig 3.5, v. vs. vi.; Fig. 3.6 B). Taken together, this provides evidence that mitochondrial morphology in PE reflect predominance towards fission, as evident by increased mitochondrial fragments that are produced by this process.
Figure 3.5 Transmission electron microscopy images of cytotrophoblast from PE and PTC placentae.

Representative transmission electron microscopy images of cytotrophoblast cells from PE and PTC placentae collected at 29 and 30 weeks gestation (PE, n=8; PTC, n=7). i) Canonical mitochondrial morphology in PTC is identified by white arrows (Scale bar: 500 nm), and ii) Mitochondrial fission events in PE are denoted by white stars (N, nucleus; scale bar: 500 nm). iii/iv) Mitochondria in PE and PTC are indicated by white arrows (N, nucleus; scale bar: 1 μm). v/vi) Mitochondrial width is denoted by the white lines (scale bar: 100 nm).
Figure 3.6 Quantification of mitochondrial number and width from PE and PTC cytотrophoblasts.

(A) Densitometric analysis of mitochondrial number in PE vs. PTC. The number of mitochondria is increased in PE compared to PTC (PE, n=8; PTC, n=7, unpaired Student’s t-test *P<0.05). (B) Densitometric analysis of minimum mitochondrial width in PE vs. PTC. Minimum mitochondrial width is decreased in PE compared to PTC (PE, n=8; PTC, n=7; unpaired Student’s t-test **P<0.01).
3.2 Ceramide Increases Mitochondrial Fission

3.2.1 Ceramide Increases DRP1 Expression and Activation in JEG3 Cells

The presence of increased CER 16:0 and CER 18:0 in the MI of PE placentae prompted us to investigate the involvement of CERs in mediating mitochondrial fission. Treatment of choriocarcinoma JEG3 cells, an established in vitro model of trophoblastic origin, with synthetic CER 16:0 at concentrations ranging from 0 to 50 µM (Fig. 3.7A), identified an optimal experimental concentration of 20 µM. Both DRP1, and active p-DRP1 expression were significantly increased in cells following treatment with 20 µM synthetic CER 16:0 relative to EtOH control vehicle (V), as assessed by WB and densitometric analyses (Fig. 3.7B). Similarly to PE placentae, no significant changes in MFF expression were observed following CER 16:0 exposure (Fig. 3.7B). Interestingly, CER 16:0 hindrance resulted in a marked decrease in OPA1 expression in JEG3 cells (Fig. 3.7C). Immunofluorescence (IF) analysis showed a striking redistribution and co-localization of p-DRP1 to the mitochondria (Mitotracker® Red) in JEG3 cells treated with CER 16:0 relative to EtOH control vehicle (Fig. 3.8). Of note, mitochondria from CER 16:0-treated JEG3 cells displayed a fragmented, globular morphology consistent with increased mitochondrial fission, when compared to cells treated with EtOH vehicle demonstrating mitochondria networks radiating away from the nucleus (Fig. 3.8, Mitotracker®).
Figure 3.7 Ceramide concentration gradient and protein expression of DRP1, P-DRP1, and MFF in JEG3 cells.

(A) Representative WB of DRP1 expression in a CER 16:0 concentration gradient in JEG3 cells. This experiment identified the 20 µM concentration as optimal. Representative WBs of (B) DRP1, p-DRP1, MFF and (C) OPA1 expression in JEG3 cells treated with CER 16:0 or EtOH vehicle for 6 hours. Both DRP1 and p-DRP1 were significantly increased, and OPA1 significantly decreased following CER 16:0 hindrance compared to vehicle. Densitometric analysis normalized to TUBA and expressed as a fold change relative to EtOH vehicle (V, n=10; CER 16:0, n=10; unpaired Student’s t-test, *P<0.05, ***P<0.001).
Figure 3.8 Immunofluorescence of JEG3 cells stained with Mitotracker® and probed for P-DRP1.

IF analysis of JEG3 cells treated with CER 16:0 or EtOH vehicle and live stained with Mitotracker® Red and probed for p-DRP1 (green). p-DRP1 was recruited to the mitochondria more after CER 16:0 treatment as evident by increased colocalization (white arrow). The mitochondria of CER 16:0 treated JEG3 cells were more punctate in appearance (white star), indicating the presence of increased mitochondrial fission. Nucleus was stained with DAPI (blue). Magnification 100X.
3.2.2 Primary Isolated Cytotrophoblast Cells Isolated from Term Placentae are Vulnerable to Ceramide Hindrance

To assess if CER 16:0 had the same effect in a more physiological model, we used primary trophoblast cells isolated from term placentae. The clinical parameters of the subjects used in these experiments are listed in Table 3.2. CER 16:0 hindrance in primary isolated trophoblasts resulted in a significant increase in both DRP1 and p-DRP1 expression relative to control vehicle (Fig. 3.9, upper and middle panels), and this associated with a significant decrease in OPA1 expression (Fig. 3.9, lower panel). Furthermore, following CER 16:0, phosphorylated DRP1 was recruited to MFF on the OMM of primary isolated trophoblasts treatment as assessed by IF (Fig. 3.10). TEM analysis in sections from primary isolated cytrophoblasts treated with CER 16:0 established the presence of increased globular mitochondria fragments and fission events, when compared to the long, network-like mitochondria observed in vehicle-treated cells (Fig. 3.11). Taken together our data indicate that CER is a positive regulator of fission and particularly of DRP1 expression and activation in both JEG3 and primary isolated cytotrophoblast cells.
Table 3.2 Clinical parameters of term placentae used for primary cytotrophoblast isolation.

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Term Placentae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=8</td>
</tr>
<tr>
<td>Gestational Age at Delivery (weeks)</td>
<td>39.14±0.83</td>
</tr>
<tr>
<td>Neonatal Weight (g)</td>
<td>3575±607.2</td>
</tr>
<tr>
<td>Neonatal Sex</td>
<td>33% F, 67% M</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>Normotensive</td>
</tr>
<tr>
<td>Mode of delivery (%)</td>
<td>100% C/S NL</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (SD). Abbreviations: F, female; M, male; S, systolic; D, diastolic; VD, vaginal delivery; C/S, Caesarean section; L, labour; NL, non-labour.
Figure 3.9 Protein expression of DRP1, P-DRP1 and OPA1 in primary cytotrophoblast cells isolated from term placentae.

Representative western blot of DRP1, p-DRP1 and OPA1 expression in primary isolated cytotrophoblast cells treated with CER 16:0 or EtOH vehicle for 6 hours. Densitometric analysis normalized to TUBA and expressed as a fold change relative to EtOH vehicle. Both DRP1 and p-DRP1 are significantly increased, and OPA1 is significantly decreased in CER16 treated cells relative to EtOH vehicle control (V, n=5; CER 16:0, n=5; unpaired Student’s t-test, *P<0.05, **P<0.01).
Figure 3.10 Immunofluorescence of P-DRP1 and MFF in primary isolated cytotrophoblast cells.

IF analysis of p-DRP1 (green) and MFF (red) in primary isolated cytotrophoblast cells treated with CER 16:0 or EtOH vehicle. Nucleus was stained with DAPI (blue). CER treatment increases colocalization of p-DRP1 to MFF (white arrows). Magnification 100X.
Figure 3.11 Transmission electron microscopy images of primary isolated cytotrophoblast cells treated with CER16 and EtOH vehicle.

Representative transmission electron microscopy of primary cytotrophoblast cells isolated from term placentae treated with CER 16:0 or vehicle (n=5). **i/iii** Canonical mitochondrial morphology in vehicle is identified by white arrows (scale bar: i) 500 nm; iii) 2µm). **ii/iv** Mitochondrial fission events and fragments in CER 16:0 treated cells are identified by white stars (scale bar: ii) 500 nm; iv) 2µm).
3.3 Ceramide augments BOK-induced DRP1 expression

We have previously reported that CER-induced BOK is responsible for elevated trophoblast cell death and autophagy in PE [119]. Hence, we next sought to examine the role of BOK in mitochondrial fission. CER 16:0 treatment triggered the expression and recruitment of BOK to the mitochondria (Fig. 3.12). To further investigate the involvement of BOK on mitochondrial fission, we conducted a series of gain and loss of function studies. We first used an established HEK-293 Flp-In T-Rex cell system that allowed for the controlled expression of BOK upon doxycycline (Dox) stimulation [141]. Induction of BOK using Dox resulted in a significant increase in both DRP1 and BOK expression as assessed by WB analysis (Fig. 3.13). We subsequently knockdown BOK using a commercially available siRNA sequence in HEK-293 cells and found a significant decrease in DRP1 expression by WB, and as anticipated, this was accompanied by decreased BOK protein levels (Fig. 3.15). Electron microscopy of GFP-BOK HEK-293 expressing cells revealed the presence of smaller, globular mitochondria, actively undergoing fission when compared to the larger mitochondria with well-defined cristae seen in the control GFP HEK-293 cells (Fig. 3.14).
Figure 3.12 Immunofluorescence of JEG3 cells stained with Mitotracker® and probed for BOK.

IF analysis of BOK (green) in JEG3 cells treated with CER 16:0 or EtOH vehicle and live stained with Mitotracker® Red . Nucleus was stained with DAPI (blue). CER16 treatment increases colocalization of BOK to the mitochondria (white arrow) and results in punctate mitochondria indicative of fission (white star).
Figure 3.13 DRP1 and BOK expression in HEK-293 cells stably transfected with BOK and induced with doxycycline.

Representative western blot of BOK and DRP1 protein expression in HEK-293 cells stably transfected with GFP-BOK and induced with Dox. Densitometric analysis normalized to TUBA and expressed as a fold change relative to Dox 0.00, dH2O vehicle. DRP1 expression was significantly increased by BOK. Stable transfection was validated by WB of BOK protein expression and was found to be statistically significant (n=3; one-way ANOVA, post-Tukey’s test *P<0.05, **P<0.01).
Figure 3.14 Transmission electron microscopy images of HEK-293 cells stably transfected with GFP-BOK and induced with doxycycline.

Representative transmission electron microscopy images of HEK-293 cells transfected with GFP-BOK and subjected to doxycycline treatment or dH2O vehicle. Healthy mitochondrial morphology indicated by white arrows, mitochondrial fission indicated by white stars (scale bar: 500 nm; Dox 0.00 ng/mL, n=3; Dox 2.5 ng/mL, n=3).
Figure 3.15 DRP1 protein expression in HEK-293 cells silenced with BOK siRNA.

Representative western blot of DRP1 and BOK protein expression in HEK-293 cells transfected with 30nM BOK siRNA, or scramble sequence (SS) as a control. DRP1 expression was significantly decreased when BOK was silenced. Densitometric analysis normalized to TUBA and expressed as a fold change relative to scramble sequence (n=3; unpaired Student’s t-test *P<0.05).
3.3.1 BH3 and Transmembrane Domains of BOK are Important in Mitochondrial Fission

BOK contains four Bcl-2 homology domains (BH1-BH4), where the BH3 domain is associated with apoptotic function in both multi-domain (BOK, BAX, BAK) and BH3-only (BIM, BID) Bcl-2 family members [127]. To establish the relative contributions of the BH3 domain in mediating BOK’s effects on DRP1, we transiently transfected HEK-293 cells with a plasmid overexpressing BOK with a 17 base-pair deletion of the BH3 domain (∆BH3). The experimental strategy was to compare the effect of ∆BH3 to WT BOK, and empty vector (EV) control, in the presence or absence of CER 16:0 treatment. In line with our inducible model, we found a significant increase in DRP1 expression in JEG3 cells following transient overexpression of WT BOK relative to EV control. Furthermore, CER 16:0 treatment augmented the increased DRP1 expression observed in cells overexpressed with WT BOK. Notably, transient transfection of BOK ∆BH3 resulted in a rescue of DRP1 expression relative to WT BOK in cells treated with CER 16:0 (Fig. 3.16).

BOK also contains a C-terminus transmembrane domain (TMD) that is critical for its mitochondrial translocation [136] and in BNIP3, the TMD is responsible for mitochondrial depolarization and fragmentation [188]. To ascertain the relevance of the TMD of BOK upon regulating mitochondrial fission, we generated a stably transfected HEK-293 cell line that overexpressed BOK with a deleted TMD upon Dox induction. We found a significant decrease in DRP1 expression in cells induced with Dox that lacked the BOK TMD relative to controls, as measured by WB and densitometric analysis (Fig. 3.17 A).

The three-dimensional protein structures of Bcl-2 family members contain a number of alpha-helices [189]. The αH5, and αH6 of placental BOK are located between the BH2 and BH1 domains and were reported to be required for mitochondrial cell death [140]. Thus, we
generated stably transfected HEK-293 cell lines containing deletions of three BOK alpha-helices (αH4, αH5, and αH6) upon induction with Dox. WB analysis demonstrated that deletion of ΔαH4 and ΔαH5 did not abrogate the positive effect of BOK on DRP1 expression, while ΔαH6 deletion resulted in no changes in DRP1 expression relative to cells not induced with Dox (Fig. 3.17 B,C,D).
Figure 3.16 DRP1 protein expression in HEK-293 cells transfected with EV, WT BOK or BOK-ΔBH3, in the presence of CER16 or EtOH vehicle.

Representative western blot of DRP1 and BOK expression in HEK-293 cells transfected with pcDNA BOK-L (WT BOK), pcDNA BOK-ΔBH3 and pcDNA3.1 (empty vector) and treated with CER 16:0 or EtOH vehicle. WT BOK transfection significantly increased the protein expression of DRP1 both with and without CER 16:0 hindrance. There is a significant decrease of DRP1 expression in cells transfected with BOK-ΔBH3 and treated with CER 16:0 relative to WT BOK. Densitometric analysis normalized to TUBA and expressed as a fold change relative to empty vector (n=3; one-way ANOVA, post-Tukey’s test *P<0.05  **P<0.01).
Figure 3.17 Protein expression of DRP1 in HEK-293 cells with deletions of the transmembrane domain, and alpha-helices 4, 5 and 6 of the protein structure of BOK

(A) Representative western blot of DRP1 and BOK in HEK-293 cells transfected with BOK-ΔTMD and induced with Dox. DRP1 expression is significantly decreased when the TMD is deleted. Densitometric analysis normalized to TUBA and expressed as a fold change relative to dH₂O vehicle (n=3; unpaired Student’s t-test P<0.05). (B)(C)(D) Representative western blots of DRP1 and BOK protein expression in HEK-293 cells transfected with BOK deletions of the following helices: ΔH4, ΔH5, and ΔH6 and induced with Dox (n=2)
3.4 Mitochondrial Fission Occurs in the Mitochondria-Associated ER Membranes

The mitochondrial-associated endoplasmic reticulum membranes (MAM’s) are enriched in glycosphingolipids [190], and represent the microenvironments that enable mitochondrial fission [191]. Tethering of the mitochondria to the ER is essential for MAM formation, and it requires MFN2 [192]. BOK induction using Dox in HEK-293 cells stably transfected with GFP-BOK resulted in a significant increase in MFN2 expression (Fig. 3.18 A). In addition, exposure of JEG3 cells to CER 16:0 resulted in a trend towards increased MFN2 expression relative to EtOH vehicle as assessed by WB analysis (Fig. 3.18 B). To examine the spatial subcellular localization of the key players/regulators of mitochondrial fission to the MAM following CER 16:0 hindrance, we employed IF analysis. Calreticulin is a protein localized to the ER lumen which participates in calcium signalling [193]. Treatment of primary trophoblast cells with CER 16:0 and revealed a striking redistribution of BOK and p-DRP1 to the ER relative to control vehicle (Fig. 3.19). It is established that the ER compartment is linked to the mitochondria by MFN2 to form the MAM [194]. Exposure of primary trophoblast cells to CER 16:0 resulted in association of both BOK and p-DRP1 to MFN2 (Fig. 3.20). These data provide evidence that CER 16:0 signals BOK and p-DRP1 recruitment to the MAM. Notably TEM analysis of section form PE placentae showed the presence of small, globular mitochondria, and mitochondrial fission events in close proximity to the ER (Fig. 3.21). The MAM is enriched in a number of proteins including DRP1, calreticulin, and binding immunoglobulin protein (BiP), which is important for protein translocation into the ER [63]. Hence, to evaluate the purity of our MAM isolation, we determined the presence of DRP1, calreticulin and BiP expression, but not TOM20, an OMM protein marker, in MAMs isolated from PE and PTC placentae (Fig. 3.21). To determine the
levels of CERs in the MAM we employed LC-MS/MS and found no significant differences between PE and PTC (Fig. 3.21), indicating that CER accumulation in PE that is responsible for mitochondrial fission is localized to the mitochondria, but not the MAM.
Figure 3.18 Mitofusin 2 Forms the MAM and is increased by BOK.

(A) Representative western blot of MFN2 expression in HEK-293 cells stably transfected with *GFP-BOK* and induced Dox or dH2O vehicle. Densitometric analysis normalized to TUBA and expressed as a fold change relative to dH2O vehicle (n=4, unpaired Student’s t-test *P<0.01). (B) Representative western blot of MFN2 expression in JEG3 cells treated with CER 16:0 or EtOH vehicle. Densitometric analysis normalized to ACTB and expressed as a fold change relative to EtOH vehicle (n=3).
Figure 3.19. Immunofluorescence of BOK or DRP1 and Calreticulin in primary cytотrophoblast cells treated with CER16

i/ii) Primary isolated cytотrophoblast cells treated with CER16 or EtOH vehicle were stained for BOK and calreticulin. There is increased colocalization of BOK to the ER (calreticulin) under CER16 treatment (white arrow) (n=3); iii/iv) Primary isolated cytотrophoblast cells treated with CER16 or EtOH vehicle were stained for p-DRP1 and calreticulin. There is increased colocalization of p-DRP1 to the ER (calreticulin) under CER16 treatment (white arrow) (n=3). Nuclei were stained with DAPI (blue).
Figure 3.20 Immunofluorescence of BOK or DRP1 and Mitofusin 2 in primary cytотrophoblast cells treated with CER16.

i/ii) Primary isolated cytотrophoblast cells treated with CER16 or EtOH vehicle were stained for BOK and MFN2. There is increased colocalization of BOK to the MAM (MFN2) under CER16 treatment (white arrow) (n=3); iii/iv) Primary isolated cytотrophoblast cells treated with CER16 or EtOH vehicle were stained for p-DRP1 and MFN2. There is increased colocalization of p-DRP1 to the MAM (MFN2) under CER16 treatment (white arrow) (n=3). Nuclei were stained with DAPI (blue).
Figure 3.21 MAMs isolated from PE placentae are enriched in DRP1, calreticulin, and BiP, but not TOM20 or ceramides.

(A) Representative transmission electron microscopy image of PE cytotrophoblast showing close proximity of the ER to the mitochondria (MAMs) as indicated by white arrows. Mitochondrial fragments and fission events indicated by white stars (scale bar: 500nm). (B) WB analysis of MAM protein markers DRP1, BiP, and calreticulin from MAMs isolated from PE ant PTC placentae. WB demonstrating absence of TOM20 protein expression in the MAMs. (C) Sphingolipidomic analysis of ceramide content in MAMs isolated from PE and PTC as assessed by LC-MS/MS. There is are no significant differences in CER content in MAMs isolated from PE versus PTC placentae (PE, n=3; PTC, n=3).
3.5 Mitochondrial Fragments are Removed by Mitophagy

Mitophagy is selective autophagic process that degrades non-functional mitochondrial fragments produced by fission [86]. TEM analysis of PE placentae identified mitophagy in cytotrophoblast cells (Fig. 3.22). PINK1 is 63 kDa mitochondrial protein that is physiologically cleaved by PARL to an inactive 53 kDa form in the IMM. However, in damaged mitochondrial fragments, PINK1 cleavage is inhibited, and its full form accumulates on the OMM where it phosphorylates cytoplasmic Parkin and ubiquitin resulting in the recruitment of lethal mitophagic machinery to carry out degradation [88]. Hence, we evaluated PINK1 \( \frac{63\text{kDa}}{53\text{kDa}} \) ratio in mitochondrial isolates from PE and PTC placentae and found a significant increase in the ratio of pro-mitophagy PINK1 \( 63\text{kDa} \) to its non-active form, in PE relative to PTC by WB analysis (Fig. 8E, upper). Furthermore, we assessed the expression of mitochondrial Parkin, and found it to be significantly increased in PE relative to PTC (Fig. 3.23). PINK1 and Parkin were normalized to Ponceau as the mitochondrial isolate is devoid of cytokeletal housekeeping proteins.
Figure 3.22 TEM image of mitophagy in PE cytotrophoblast cell.

Representative transmission electron microscopy image of mitophagolysosome in cytotrophoblast from PE placenta collected at 29 weeks. Mitophagolysosome indicated by white arrow (scale bar: 500 nm; PE, n=8).
Figure 3.23 PINK1 and Parkin are increased in PE mitochondria

Representative western blot of PINK1 and Parkin expression in the mitochondrial isolate of PE vs. PTC placentae. There is a statistically significant increase in PINK1 (63kDa)/cleaved-PINK1 (53kDa) ratio, and Parkin (52kDa) in PE mitochondria relative to PTC. Densitometric analysis normalized to ponceau and expressed as a fold change relative to PTC (PE, n=4; PTC, n=4 *P<0.05).
Chapter 4
Discussion

4 General Discussion

Studies conducted in placentae from preeclamptic pregnancies have focussed on affirming the role of the mitochondria as ‘powerhouses’ of trophoblast cells [195], in a balance between oxidative stress and antioxidant defenses [196], and as regulators of intrinsic apoptosis [197]. However, to date, the involvement of mitochondrial dynamics to placental cell homeostasis remains to be established. In the present study we provide evidence that mitochondrial fission is an event that occurs in the human placenta, and is augmented in PE. Furthermore, we report for the first time that ceramide, a sphingolipid found in excess in preeclampsia, plays a critical role in regulating mitochondrial fission, via a mechanism that involves BOK, a pro-apoptotic member of the Bcl-2 family. We have also identified the mitochondria-associated endoplasmic reticulum membrane (MAM) as the microenvironment in which the interplay between ceramide, BOK, and the key players of mitochondrial fission interact, and that mitophagy is a cellular defence that removes excess mitochondrial fragments in PE.

Excess mitochondrial fission leads to the accumulation of non-functional fragments with impaired mitochondria membrane potentials, and importantly, increased generation of reactive oxygen species (ROS) which overcome insufficient antioxidant defenses [198]. Excessive DRP1-driven mitochondrial fission has been implicated in the pathophysiology of many human diseases, though we are the first to report that mitochondrial fission is increased in placentae from pregnancies complicated by PE. Fission can participate in pathways leading to cell death, as seen in ischemia-reperfusion injury and heritable juvenile Parkinsonism [7]. Post myocardial infarction, ischemia-reperfusion injury remains a critical cause of cardiac arrest leading to increased myocardial cell death, with release of Ca^{2+} and subsequent activation of calcineurin.
resulting in mitochondrial fission by means of DRP1 dephosphorylation at S637 [80, 199].

Early-onset Parkinson’s disease is characterized by a loss of glutathione antioxidant defence and increased ROS causing neuronal degeneration and impaired striatal dopamine production; which, in turn, leads to mitochondrial fission and mitophagy [81]. In mice, supranigral injections of a recombinant adeno-associated virus to deliver a Drp1-K38A mutant, identified a new therapeutic target for Parkinson’s disease as this effectively blocked mitochondrial fission, attenuated neurotoxicity and restored striatal dopamine release [82, 83].

PE placentae exhibit shallow cytotrophoblast invasion and impaired vascular transformation which render the placenta vulnerable to hypoxia/oxidative stress [181, 200]. Hypoxia in PE has been attributed to impaired perfusion and oxidative stress and results from hypoxia-reoxygenation injury, with ensuing trophoblast apoptosis [172, 201]. Our group has identified a number of targets of hypoxia-induced injury in placental models. Of note, oxidative stress induced with sodium nitroprusside treatment in JEG3 cells was found to increase the expression of pro-apoptotic Bcl-2 family member BOK leading to autophagy, which was prevented by overexpression of pro-survival Mcl-1 [144]. Also, hypoxia-inducible factor 1-alpha (HIF1α) and ceramide have both been identified as regulators of increased expression of BOK, which typifies PE and leads to aberrant trophoblast cell death [119, 202]. One study has found that ETC respiratory enzymes and ATP production are diminished in PE, and this is associated with increased oxidative and nitrative stress resulting in a compensatory increase in heat shock protein 70 which functions to maintain oxidative phosphorylation [203]. Using a placental perfusion model, another group demonstrated impaired endothelial responsiveness to superoxide and nitric oxide-induced peroxynitrate accumulation in PE placentae [204, 205]. Interestingly, we have found that JEG3 cells subjected to hypoxia increase DRP1 expression relative to normoxia (data not shown) underscoring that hypoxia-induced oxidative stress favors mitochondrial fission.
It should be noted that mitochondrial dynamics involve a balance between mitochondrial fission and fusion. In pulmonary arterial hypertension, increased HIF1α promotes DRP1-driven mitochondrial fission, and simultaneously downregulates MFN2 activity via decreased peroxisome proliferator-activated receptor γ coactivator 1α in human lung and pulmonary arterial smooth muscle cells, exaggerating mitochondrial fragmentation [206]. Loss of IMM fusion regulator OPA1 in HeLa cells has been found to alter mitochondrial membrane integrity and cristae remodelling, leading to increased fragmentation and apoptosis [207]. In line with a mitochondrial dynamics balance model, we have demonstrated that increased DRP1-dependent mitochondrial fission inversely correlate to fusion as identified by decreased OPA1 in PE placentae, and in JEG3 and primary isolated trophoblast cells following CER 16:0 hindrances. Yu et al. recently reported that mitochondrial fusion is downregulated in PE placentae and human first-trimester extravillous trophoblast TEV-1 cells subjected to hypoxia, as evident by decreased MFN2 mRNA expression, and this associated with impaired mitochondrial membrane potentials and ATP production - common prerequisites for mitochondrial fission [37]. Interestingly, transfection of mouse cardiac HL-1 cells with mutant Drp1(K38A) abrogates mitochondrial fragmentation in a similar fashion as MFN1 and MFN2 overexpression [208].

Ceramides are powerful inducers of cell death via a mechanism that involves the formation of protein-permeable channels that facilitate the cytoplasmic extrusion of pro-apoptotic molecules, irreversibly initiating the apoptotic cascade [94]. We have identified that p-DRP1 is significantly expressed in the mitochondrial compartment of PE placentae, which are enriched in CER 16:0 and CER 18:0. In whole-placenta lysates and serum collected from PE women, CERs 16:0, 18:0, 20:0 and 24:0 are increased, and this is dependent on the effect of the oxidative stress status upon ceramide regulatory enzymes [119]. Parra et al. found increased DRP1 expression and recruitment to the OMM in the mitochondria of neonatal rat cardiomyocytes treated with
synthetic CER 2:0, and this was accompanied by a more spherical mitochondrial conformation, and initiation of apoptosis [121]. In line with this observation, we show that exposure of primary isolated cytotrophoblast cells and human choriocarcinoma JEG3 to synthetic CER 16:0, increased p-DRP1/DRP1 expression and recruitment to the mitochondria. Furthermore, our findings are in agreement with a study using C2C12 murine myoblasts, where CER 2:0 hindrance resulted in increased Drp1 mRNA expression after 4 hours of treatment, with apparent fission events at 16 hours [120]. Myoblasts exhibited diminished mitochondrial respiration localized to impaired complex II function of the electron transport chain [120]. The placenta already functions in a physiologically hypoxic environment despite its requirement of oxygen for aerobic respiration, and studies have shown that even in healthy term placentae, mitochondrial ATP stores are rapidly depleted when the maternal blood supply is disrupted at delivery [158, 160]. Using isobaric tag for relative and absolute quantitation (iTRAQ) analysis linked to LC-MS/MS, Shi et al. identified a number of downregulated mitochondrial proteins vital to ATP production and as well as decreased placental fatty acid oxidation in PE placentae [209]. In the present study, we observed fragmented, globular, and unhealthy mitochondria in PE placentae and primary cytotrophoblasts treated with CER 16:0. We propose that the function of these perturbed mitochondria with respect to membrane potentia, and ATP production is detrimentally impaired.

Important to the formation of permeable channels at the mitochondria are the Bcl-2 family members, a group of proteins that act as either pro-apoptotic (BAK, BAX, BOK) or pro-survival (Bcl-2, Bcl-XL, Mcl-1) inducers [13, 15, 71]. Pro-death Bcl-2 family members are critical for mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release into the cytoplasm [72]. BOK is an important pro-death Bcl-2 family member, contains a homologous BH3 domain, and is highly expressed in reproductive tissues including the human placenta [135].
Importantly, oxidative stress-induced CER triggers BOK leading to trophoblast autophagy [119]. The OMM produces ceramides which has been referred to as the ‘mitochondrial ceramide rich macrodomain’ (MCRM), a platform by which BAX inserts, oligomerizes and forms channels responsible for apoptosis in irradiated cells [110]. Interestingly, one group has shown BOK to induce intrinsic apoptosis through a BAX/BAK-dependent mechanism [136], whereas we have previously reported that mitochondrial membrane destabilization and apoptosis are directly regulated by the αH5, αH6 and TMD of BOK [140]. Mitochondrial fission is attenuated in primary neurons isolated from Bak-deficient mice brains, underscoring the importance of BAK as a positive regulator of fission [210]. In addition, studies conducted in HeLa cells, demonstrated that DRP1 membrane association to the mitochondria is BAX/BAK dependent [146]. One study in fibroblasts has shown that DRP1 is not required for BAX/BAK regulated apoptosis, and once cells are primed for cell death, this is independent of mitochondrial fission [145]. However, Montessuit et al. have indicated that BAX oligomerization, a critical step in MOMP is dependent on DRP1-induced membrane hemifusion, thus resulting in increased apoptosis [148]. Our data on BOK regulation of DRP1 and fission events further underscore the importance of pro-apoptotic Bcl-2 proteins in fission.

Pro-apoptotic members of the Bcl-2 family can contain up to four Bcl-2 homology domains (BH1-4), however, the BH3 domain remains homologous amongst all, and crucial to apoptosis. BH3-only proteins, such as BAD, BID and BIM ascribe their pro-apoptotic activity to a number of mechanisms including direct BAX/BAK activation and the neutralization of pro-survival Bcl-2 proteins [16]. Interestingly, deletion of the BH3 domain of the Bcl-2/adenovirus E1B 19-kDa interacting protein 1 (BNIP1) results in the abrogation of mitochondrial fission in HeLa cells [147] highlighting the importance of this domain for fission. Our data show the abrogation of BOK-induced fission following transient overexpression of BOK- ΔBH3 in JEG3 cells with and
without CER 16:0 hindrance, further underscoring the significance of this domain in regulating DRP1 expression. Most Bcl-2 family members, including BOK, contain a hydrophobic C-terminal α-helical transmembrane domain (TMD) which functions to anchor the protein to the mitochondrial membranes so that they may exert their apoptotic function [211]. Interestingly, using nuclear magnetic resonance spectroscopy (NMR), the TMD of BAX has been identified and critically liked to its ability to translocate from the cytosol to the OMM [189]. One study deleted the TMD and found that its recruitment to the ER and golgi was detrimentally impaired [136], and herein we show that the TMD of BOK may be vital for its mitochondrial recruitment and regulation of fission.

Mitochondrial fission may alternatively aid to proliferation, as seen in lung adenocarcinoma. Indeed, mitochondrial fission is enhanced during malignant cell proliferation in a HeLa cell model to produce more mitochondria for daughter cells, and this is regulated by cyclin B1-cyclin dependent kinase-1 which initiates mitosis and activates DRP1 through phosphorylation at S616 [53]. Interestingly, Redline et al. propose that PE is characterized by a hyperproliferative intermediate phenotype of trophoblasts [212]. We previously reported that nuclear BOK is increased in placentae from early development and PE, where it contributed to the trophoblast proliferative phenotype by increasing the expression of Ki67 and cyclin E1; however, apoptotic stimuli increase BOK recruitment to the mitochondria, favoring cell death [141]. In the present study we observe a striking redistribution of BOK to the mitochondria of JEG3 cells following CER 16:0 treatment, indicating that following this stimuli, cell death prevails, whereas other conditions may trigger proliferation. A dual role of mitochondrial fission in PE may exist, such that mitochondrial fragments with impeded membrane potentials may contribute to trophoblast cell death, whereas fragments with intact membrane potentials favor mitotic fission and trophoblast proliferation.
ER-mitochondria crosstalk is crucial for intracellular calcium signaling as it functions as a critical membrane contact site for lipid exchange and conversion [63]. Mitofusin 2 (MFN2) is the GTPase responsible for tethering the ER and mitochondria together [39], though its most notable function is in mitochondrial fusion [36]. We found a significant increase in MFN2 protein expression when BOK is induced by Dox in stably transfected HEK-293 cells. We propose that BOK increases MFN2 bridging between the ER and mitochondria to facilitate the process of mitochondrial fission. Ganesan et al. have reported that ceramide produced in the ER is trafficked to the mitochondria via the MAM [71], where together with Bcl-2 family members carry out apoptosis [72]. However, we found no significant differences in the CER content of MAMs from PE relative to PTC placentae, indicating that CERs may transport from the de novo synthesis site in the ER via the MAM, but accumulate in the mitochondria where they exert their effect on mitochondrial fission. In HeLa cells, the MAM is enriched in DRP1 bound to Rab32 [75], and we also find the MAM’s of PE and PTC placentae to express DRP1, in addition to other MAM markers BiP and calreticulin, but not TOM20, an OMM marker.

Mitophagy is a highly specialized autophagic degradation pathway for mitochondrial fragments, that is classically dependent on P-TEN-induced putative kinase 1 (PINK1) and Parkin regulation [213]; mitochondrial fragments with impaired inner mitochondrial membrane potentials fail to import PINK1 to the IMM for cleavage by presenilin-associated rhomboid-like serine protease (PARL), allowing PINK1 accumulation on the OMM where it can recruit Parkin [214]. Mitophagy is physiologically required to remove excess, non-functional mitochondrial mass to protect the cell from ROS and oxidative insult [86]. We have observed mitophagy in CER 16:0 treated primary cytotrophoblasts (data not shown), and similar to Kalkat et al. we find evidence of mitophagy in PE placentae [144]. Furthermore, we found increased PINK1 relative to its cleaved form, and increased Parkin protein expression in mitochondria isolated from PE women
relative to PTC. Both FUNDC1 and BH3-only pro-apoptotic BNIP3 contain LC3-interacting regions that can also trigger mitophagy [215]. In cardiac HL-1 cells, Zhu et al. demonstrated that phosphorylation of BNIP3 at serine residues 17 and 24 targets unhealthy mitochondria for mitophagy via increased binding to LC3 [216]. Interestingly, in a breast cancer mouse model, Bnip3 null tumors have deficient mitophagic defenses and exhibit a more severe phenotype due to the inability to compensate for elevated ROS and subsequent hypoxia inducible factor activation [217]; however, previous work from our group has reported that BNIP3 expression is not altered in PE [144]. The present study has identified that PINK1/Parkin regulated mitophagy is primed in PE as a compensatory mechanism against oxidative stress.

In accordance with our putative model of PE (Fig. 4.1), our data show that elevated ceramide in PE mitochondria, favour pro-apoptotic BOK recruitment to the OMM, and increased p-DRP1 dependent mitochondrial fission. Furthermore, mitochondrial fragments produced by fission are targeted for mitophagy. We have also demonstrated that the crosstalk between mitochondrial ceramide, BOK and p-DRP1 occur at the MAM. Increased mitochondrial fission in PE supports the notion that preeclampsia is characterized by cell death and autophagy, both downstream events of mitochondrial fission.
**Figure 4.1 Putative model of mechanisms underlying mitochondrial fission in PE.**

Putative model of the mechanisms underlying increased mitochondrial fission in PE. i) DRP1 and BOK are cytosolic proteins, whereas, MFF is resident at the OMM. ii) Mitochondrial fission is primed by the cross-talk between ceramide, BOK and p-DRP1 in the mitochondria-associated ER membranes (MAMs), which are tethered together by MFN2. The MAMs are the conduits for ceramide transfer from the site of *de novo* synthesis in the ER to the mitochondria. Elevated mitochondrial ceramide content in PE phosphorylates DRP1 and increases recruitment to MFF at the OMM. BOK is also recruited to the OMM under the influence of ceramide and augments DRP1 expression and activation. Excess, non-functional mitochondrial fragments are degraded by PINK1/Parkin regulated mitophagy.
4.1 Conclusions

Both mitochondrial fusion and fission have physiological roles that are vital to maintain homeostasis. Mitochondrial fission is required to partition non-functional components of the organelle to preserve cellular energy, and protect the cell from toxic reactive oxygen species through subsequent mitophagic degradation. Excess mitochondrial fission is mechanistically intertwined with apoptotic machinery, and has been identified as crucial mediators of a number of human pathologies including neurodegenerative diseases like Parkinson’s and Alzheimer’s diseases, cardiometabolic diseases like post-myocardial infarction reperfusion injury and pulmonary arterial hypertension, as well as malignancies like lung cancer [7]. The precise mechanisms that regulate mitochondrial fission are an intense focus of recent research in a number of different systems and models. Preeclampsia is a serious placental pathology characterized by increased cell death and has adverse maternal and fetal outcomes. Our lab has identified ceramide, a pro-death bioactive sphingolipid as a contributor to cell death in preeclampsia. My hypothesis is that ceramide increases mitochondrial fission in preeclampsia.

The first objective of my project was to determine the presence of mitochondrial fission in preeclampsia. I addressed this by evaluating the protein expression of fission-regulator DRP1 and its receptor MFF in PE and PTC placentae. Furthermore, I isolated the mitochondria from PE and PTC placentae and evaluated the protein expression of activated p-DRP1 which is recruited to the OMM during fission. The mitochondrial isolate further underwent sphingolipidomic analysis for ceramides. Herein, I report for the first time that DRP1-dependent mitochondrial fission is increased in PE compared to PTC, and the mitochondria of PE placentae are enriched in active p-DRP1 and CER 16:0 and CER18:0. Using TEM, I observed significant
ultrastructural morphological changes such that PE placentae have smaller mitochondria, and are more numerous – both outcomes of increased mitochondrial fission.

My second objective was to determine whether the CER enrichment observed in PE mitochondria had any effect on the fission machinery. Using cultured JEG3 and primary isolated cytotrophoblast cells, I determined that synthetic CER 16:0 hindrance increased DRP1 expression and p-DRP1 recruitment to the mitochondria, and this was associated with increased fission observed by TEM. To understand how ceramide was exerting its effect on DRP1, we turned to BOK, a pro-apoptotic member of the Bcl-2 family that our group has reported to be an inducer of trophoblast cell death and autophagy in PE [144]. We found that CER 16:0 treatment in JEG3 cells recruited BOK to the mitochondria, and stable transfection of BOK in HEK-293 cells increased DRP1 protein expression, whereas silencing BOK decreased DRP1 protein expression. Furthermore, we identified that the BH3 and TM domains of BOK are important for DRP1 regulation. Under CER 16:0 treatment, BOK and p-DRP1 are both transported to the ER (calreticulin) and MAM (MFN2), highlighting their presence in this important subcellular region that facilitates fission. Moreover, mitochondria isolates from PE placentae display increased expression of PINK1 and Parkin, and we observe mitophagolysosomes in PE tissue, indicating that mitophagy is the degradation pathway for excess mitochondrial fragments.

Taken together I propose that CER accumulation in PE increases BOK expression, which in turn activates DRP1 resulting in their recruitment to the MAM, with ensuing mitochondrial fission. I further propose that the mitochondria are equipped with mitophagic machinery to compensate for increased fission; however, when cellular stresses overcome this defence mechanism, trophoblast cell death ensues.
Chapter 5
Future Directions

5 Future Directions

The data contained within this thesis are the first to identify that mitochondrial fission is increased in preeclampsia, and that the mitochondria of PE placentae are enriched in CERs. Furthermore, I identified the novel role of BOK as a ceramide-induced regulator of DRP1 expression using in vitro JEG3 and HEK-293 models. My work represents the initial explorations into excess mitochondrial fission in preeclampsia. During the process of data collection for this thesis, a number of important questions were raised that have not yet been addressed. Herein, I would like to highlight four avenues for future exploration which may provide further insight into the complex pathophysiology of PE, with the possibility to identify early diagnostic biomarkers and potential therapeutic targets for this serious disease.

5.1 Is mitochondrial fission altered in placental development?

Early first trimester placental development prior to gestational week 10 is characterized by physiological low oxygen tension which rapidly increases by the 10-12th gestational week when the maternal vasculature remolds [200]. Hypoxic environments can induce oxidative stress by blocking complex III-IV of the ETC resulting in the build-up of ROS [175], and rapid increases in oxygenation as seen in the 10-12th gestational week can similarly overwhelm the ETC leading to hyperoxic oxidative damage [218]. Maternal physiology has evolved to a perfect balance, such that oxygen tensions are fine tuned to embryonic demand in early gestation when there is an absence of antioxidant defenses. This is achieved by the formation of ‘capillary plugs’ by the
extravillous trophoblast that regulate appropriate blood flow to the site of the embryo until the 11-13\textsuperscript{th} weeks [219].

Hence, I mapped the expression of DRP1 in placentae obtained from elective abortions between the 6\textsuperscript{th} and 13\textsuperscript{th} weeks of gestation and found that as oxygen tensions increase in the developing embryo there is a statistically significant decrease in DRP1 expression (\textbf{Figure 5.1}). There are a few possible explanations for these preliminary findings. First, the developing embryo is constantly dividing, and the increase in DRP1 in early development may represent ‘mitotic fission’ to produce healthy organelles for daughter cells. Also, these samples were obtained from elective abortions, and as such, it is impossible to determine whether these subjects would go on to have preeclampsia. Mitochondrial fission may in fact be the physiological mechanism by which mitochondria in the early first trimester developing placenta are able to function without antioxidant defenses by dividing off non-functional fragments for degradation by mitophagy. Therefore, future experiments should address the expression of p-DRP1 in the mitochondria of placentae from freshly collected aborted embryos along a variety of gestational ages. Next, the mitophagy markers PINK1 and Parkin should be probed in similar samples to ascertain whether the mitochondrial fission found in early development is producing mitochondria capable of further fusion with intact $\Delta\psi_m$, or is it producing fragments with impaired $\Delta\psi_m$ for mitophagy. Furthermore, morphological characterization by the gold standard TEM would be valuable.
Figure 5.1 DRP1 and MFF are increased in early placental development.

Representative western blot of DRP1 and MFF expression in placental development. Densitometric analysis normalized to ACTB and expressed as a fold change relative to 6-9 weeks gestation group. Both DRP1 and MFF are significantly increased in early first trimester placental development (6-9 wks, n=7, 10-13 wks, n=7; unpaired Student’s t-test *P<0.05).
5.2 Does placental hypoxia in PE contribute to increased mitochondrial fission?

Severe early-onset preeclampsia is characterized by utero-placental hypoxia which can lead to fetal intra-uterine growth restriction and prematurity [220]. This thesis has used normoxic in vitro techniques to establish the role of ceramide in regulating mitochondrial fission. These experiments were designed to remove oxygen tension as a variable. One study has reported that DRP1 gene expression is upregulated by hypoxia-inducible factor 1-alpha (HIF1α) in human glioblastoma cell lines which resulted in increased tumor migration. In the same study, U251 cells stably transfected with DRP1-K38A, a mutation that renders DRP1 inactive, found that this mutation diminished hypoxia-induced cell migration [221]. As a preliminary experiment, I performed a mitochondrial isolation of JEG3 cells subjected to hypoxic (3% pO2) and normoxic (20% pO2) conditions for 24 hours. Interestingly, in both the mitochondrial isolate, and the post-mitochondrial supernatant, there was an apparent increase of DRP1 expression in hypoxic JEG3 cells (Figure 5.2). To determine the effect of oxygen on DRP1 activation/regulation, JEG3 cells should be subjected to hypoxic, normoxic and hyperoxic conditions, and pDRP1 protein expression should be assessed by WB and compared to HIF1α expression levels. As HIF1α regulates DRP1 gene expression, the mRNA expression of DRP1 should also be assessed by qPCR in JEG3 cells subjected to hypoxia, normoxia, and hyperoxia. As ceramide is central to the data I have shown, the above experiments should be conducted in the presence of CER compared to EtOH vehicle. As a model of HIF1α activation in mice, our lab has used the pharmacological agent 2-(4-hydroxy-1-methyl-7-phenoxyisoquinoline-3-carboxamido) acetic acid (FG-4592), which inhibits HIF1α degradation. It would be valuable to assess the expression of DRP1 and pDRP1 in the placental lysates of mice treated with FG-4592.
Figure 5.2 Mitochondrial Isolation in JEG3 cells in hypoxic and normoxic conditions.

Representative western blot of DRP1 expression in the PMS, PNS, and MI of JEG3 cells subjected to 24 hrs in either hypoxic (3% pO2) or normoxic (20% pO2) conditions. DRP1 protein expression appears to be increased in hypoxic MI and PMS (n=1).
5.3 What is the role of autophagy in mitochondrial fission?

Though this thesis has addressed mitophagy, the selective autophagic degradation of mitochondrial fragments via the PINK1/Parkin pathway, the effect of excess BOK-induced autophagy observed in PE [144] upon mitochondrial fission is yet to be explored. In a short communication, Galluzzi et al. has described mitophagy as the cell’s physiological defense against ROS and subsequent apoptosis induction, and autophagy as a reaction to cellular nutrient deprevation. Interestingly, fission is required to produce the mitochondrial fragments with impaired $\Delta \psi_m$ for mitophagy, and autophagy stimuli promote mitochondrial elongation through fusion to sustain cell viability [222]. Also, Purnell et al. have demonstrated in HEK-293 cells that autophagy inhibition increases DRP1 expression, thereby postulating that autophagy may have a protective effect from the adverse effects of excess fission [223]. Hence, I performed one pilot experiment in JEG3 cells treated with bafilomycin, a pharmacological inhibitor of autophagy flux, for 4 and 24 hours. I found that when autophagy was inhibited, there was an apparent increase in DRP1 expression (Figure 5.3) which confirms Purnell’s findings, but does not align with our data indicating that pro-autophagic BOK increases DRP1 expression in PE. This may be explained by the fact that cytotrophoblast cells in PE are not simply starved, but are under extreme oxidative insult and may require mitophagic defenses to remove toxic ROS-producing fragments initially, and when these mechanisms are overwhelmed, may result in autophagy. To ascertain the relative contributions of both of these vital processes on mitochondrial fission, I would first inhibit autophagy machinery in JEG3 cells subjected to hypoxia, ceramide and BOK overexpression and compare DRP1/p-DRP1 protein expression to that of mitophagy markers (PINK1 and Parkin). Similarly, I would inhibit mitophagic machinery and compare DRP1/p-DRP1 to autophagy markers (LC3B-II) in under similar treatment conditions.
**Figure 5.3 Inhibition of autophagy flux increases DRP1 expression.**

Representative western blot of DRP1 expression in JEG3 cells treated with Bafilomycin to inhibit autophagy flux for 4 and 24 hrs. DRP1 protein expression appears to be increased in the presence of Bafilomycin (n=1).
5.4 Do other placental pathologies of pregnancy display alterations in mitochondrial dynamics?

Gestational diabetes mellitus (GDM) is characterized by new-onset hyperglycemia during pregnancy, usually manifesting in the late-second or early-third trimester [224]. Kuo et al. have used cytoplasmic hybrids from human osteosarcoma cells to study mtDNA, and in doing so have identified cells containing diabetes-susceptible (B4) and diabetes-protective (D4) haplotypes. Interestingly, this study found that cybrid B4 increased mitochondrial fragmentation, decreased ATP production, and impaired mitophagic defenses; whereas, insulin treatment increased cybrid D4 driven bioenergetics and network formation [225]. During the initial mapping of DRP1 expression in PE, I performed a WB analysis of placentae from term controls (TC), gestational diabetes mellitus (GDM) and GDM with superimposed PE. There appears to be an increase in DRP1 protein expression in GDM, and GDM+PE placentae relative to TC (Figure 5.4). To further assess mitochondrial dynamics in GDM, first tissues must be stratified based on diet-control (GDM-D) versus insulin-dependent (GDM-I), and to further, into well-controlled (WC) versus uncontrolled (UC). The first WB analysis should include TC, GDM-D WC, GDM-D UC, GDM-I WC, and GDM-I UC, probed for DRP1 and OPA1 expression. One could hypothesize that mitochondrial fission would be increased in GDM-D (more so in the uncontrolled group), and mitochondrial fusion would be increased in GDM-I as the mitochondrial stress is rescued. Thus, JEG3 cells should be treated with different glucose concentrations and with insulin and probed for DRP1 and OPA1 expression. I have found that BOK increases DRP1 expression in HEK-293 cells. BOK is opposed by anti-apoptotic Bcl-2 family member Mcl-1. Therefore, the mechanism by which insulin rescues cells from aberrant fission in GDM might be through upregulation of Mcl-1 or the downregulation of BOK. To assess this, BOK and Mcl-1 loss and gain of function studies should be done in the presence of glucose with and without insulin.
Figure 5.4 DRP1 expression is altered in GDM and GDM+PE.

Representative western blot of DRP1 expression in placentae from TC, GDM, and GDM with superimposed PE (GDM+PE). Expression of DRP1 appears to increase in GDM and GDM+PE relative to normotensive, normoglycemic term controls (TC, n=3; GDM, n=4; GDM+PE, n=3).
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