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A connection in life and death: The BCL-2 family coordinates mitochondrial network dynamics and stem cell fate

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Abstract

The B cell CLL/lymphoma-2 (BCL-2) family of proteins control the mitochondrial pathway of apoptosis, also known as intrinsic apoptosis. Direct binding between members of the BCL-2 family regulates mitochondrial outer membrane permeabilization (MOMP) after an apoptotic insult. The ability of the cell to sense stress and translate it into a death signal has been a major theme of research for nearly three decades; however, other mechanisms by which the BCL-2 family coordinates cellular homeostasis beyond its role in initiating apoptosis are emerging. One developing area of research is understanding how the BCL-2 family of proteins regulate development using pluripotent stem cells as a model system. Understanding BCL-2 family-mediated regulation of mitochondrial homeostasis in cell death and beyond would uncover new facets of stem cell maintenance and differentiation potential.

Keywords

apoptosis; stem cells; pluripotency; BCL-2 family; mitochondria; mitochondrial dynamics

1. Introduction

Stem cells can simulate the earliest stages of development, since they give rise to the three main tissue lineages (Thomson et al., 1998). This capacity to differentiate into ectoderm, mesoderm, and endoderm lineages is known as pluripotency. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), collectively referred to as PSCs, are the two stem cell types that harbor this ability. Adult stem cells, also known as somatic stem cells, are multipotent and can replenish dying cells in case of tissue damage, and include hematopoietic stem cells, mesenchymal stem cells, and hair follicle stem cells (reviewed in (Goodell et al., 2015)). Stem cells also have the capacity of self-renewal, which is the process by which the stem cell pool is maintained indefinitely. These capabilities to regenerate and to give rise to the three germ layers have propelled an entire field of research

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dedicated to modeling embryonic development in culture by manipulating key signaling pathways and growth factors. The first human ESC (hESC) line was derived in 1998 from the inner cell mass (ICM) of human blastocysts (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998), while the discoveries of reprogramming mouse and human somatic cells into iPSCs were published in 2006 and 2007, respectively (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Reprogramming was initially achieved by inducing the expression of master pluripotency transcription factors OCT4 (Octamer-binding transcription factor 4), SOX2 (SRY (sex-determining region Y)-box 2), KLF4 (Kruppel-like factor 4) and c-MYC, collectively known as OSKM), but other methods of attaining iPSCs have been reported (reviewed in (Takahashi and Yamanaka, 2015)). The ability of PSCs to self-renew and differentiate has become an efficient tool to study basic processes of human development and various aspects of human diseases, such as diabetes, cardiomyopathy, and cancer (Assady et al., 2001; Hinson et al., 2015; Smith and Tabar, 2019). During embryonic development, genomic instability is especially dangerous for the integrity of rapidly dividing cells of the ICM. Thus, not surprisingly, stem cells are capable of executing intricate programs to quickly respond to apoptotic stress and prevent the propagation of deleterious mutations. Along with the primed cell death program, a growing number of studies on the BCL-2 family have shown changes in mitochondrial dynamics and metabolic function and regulation as stem cells differentiate and as somatic cells reprogram into iPSCs (Rinkenberger et al., 2000; Madden et al., 2011; Prigione et al., 2011; Dumitru et al., 2012; Gama and Deshmukh, 2012; Rasmussen et al., 2018). In the following chapter, we will discuss the known fundamental mechanisms involved in these changes, centering on the BCL-2 family, as well as describe areas that are open to more detailed exploration (Figure 1). In addition, many aspects of mitochondrial biology are beginning to emerge as hallmarks of pluripotency and self-renewal (Wanet et al., 2015; Rastogi et al., 2019). The increased sensitivity to apoptosis, the changes in mitochondrial morphology and localization, and the shifting of the metabolic program all accompany reprogramming. Furthermore, cellular events such as mitochondrial biogenesis, mitochondrial trafficking and motility, and mitochondrial DNA (mtDNA) transcription could also be important for reprogramming and generation of specialized tissues. Thus, the unique properties of ESCs and iPSCs make them a valuable model system to illuminate the effects of these processes on self-renewal and differentiation.

2. The BCL-2 family in stem cell death

2.1 Mitochondrial pathway of apoptosis

Caspase-dependent apoptosis occurs through both extrinsic and intrinsic pathways, which are mediated by external death ligands and mitochondrial-localized proteins, respectively (Elmore, 2007). The focus of this chapter will be on the intrinsic or mitochondrial pathway of apoptosis; the extrinsic apoptotic pathway is another form of regulated cell death that depends on detection and propagation of extracellular signals, which has been comprehensively reviewed here (Ashkenazi and Dixit, 1998; Mehlen and Bredesen, 2011; Galluzzi et al., 2018). The intrinsic pathway of apoptosis is crucial for embryonic development, tissue homeostasis, and in cellular response to irreversible perturbations. When the proteins that regulate apoptosis are dysregulated, cells can become cancerous and

contribute to tumor formation, or die uncontrollably resulting in neurodegeneration. The BCL-2 family are the main regulatory proteins that control the intrinsic pathway of cell death (Figure 2). The direct binding interactions that occur between pro-apoptotic and anti-apoptotic family members govern their activities, ultimately resulting in MOMP after receiving a death signal (Oltval et al., 1993; Kale et al., 2018). Roughly 30 family members have been identified to belong to the BCL-2 family, which is made up of structurally similar globular proteins that are defined by multiple domains, known as BCL-2 homology (BH) domains (BH1, BH2, BH3, and BH4) (Tsujimoto et al., 1984; Wang et al., 1996; Yang et al., 1996; Lindsten et al., 2000; Chipuk et al., 2010). Family members with multiple BH domains are either pro-apoptotic “effectors” (BCL-2 associated X (BAX), BCL-2 antagonist killer 1 (BAK1; known as BAK), BCL-2 ovarian killer (BOK)) or anti-apoptotic/pro-survival (BCL-2 apoptosis regulator (BCL-2), BCL-2-like 1 (known as BCL-xL), BCL-2-like 2 (BCL-w), Myeloid cell leukemia (MCL-1), and BCL-2-related protein A1 (BFL-1/A1)). Another group of family members have only one BH domain, known as the BH3-only proteins. The “activator” pro-apoptotic proteins (BCL-2 interacting mediator of cell death (BIM), BCL-2 interacting domain death agonist (BID), P53-upregulated modulator of apoptosis (PUMA)) and “sensitizer” pro-apoptotic proteins (phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1, best known as NOXA), BCL-2-associated agonist of cell death (BAD), BCL-2 interacting killer (BIK), BCL-2 modifying factor (BMF), and Harakiri (HRK)) work to either activate the effector proteins directly or sequester the anti-apoptotic family members from binding the effectors. After activation of BAK, BAX and/or BOK by the BH3-only proteins, they oligomerize to form pores in the outer mitochondrial membrane (OMM), initiating MOMP, reducing mitochondrial membrane potential and releasing cytochrome c from the inner membrane space (IMS) (Liu et al., 1996; Lindsten et al., 2000; Goldstein et al., 2000; Eskes et al., 2000; Kalkavan and Green, 2018; Moldoveanu and Czabotar, 2019). Mechanistically, each of the effector proteins are regulated through slightly different means. While BAK is usually found anchored to the OMM, BAX resides in the cytosol in an inactive state and must undergo a conformational change to insert into the OMM through its C-terminal domain (Hsu and Youle, 1997; Wolter et al., 1997; Goping et al., 1998; Gavathiotis et al., 2008; Green and Chipuk, 2008; Todt et al., 2015). Less is known about BOK activity and regulation, but it is proposed to have the ability to initiate MOMP independent of BAK or BAX, and it is modulated by the endoplasmic-reticulum-associated protein degradation (ERAD) pathway (Llambi et al., 2016; Schulman et al., 2016; Zheng et al., 2018; Schulman et al., 2019). Cytochrome c release results in Apoptotic protease activating factor-1 (APAF-1)/Caspase-9 assembly, known as the apoptosome (Zou et al., 1997; Kim et al., 2005; Taylor et al., 2008; Inoue et al., 2009), which activates the executioner caspases, leading to the extensive cleavage and destruction of critical cellular components (Li et al., 1997; Dix et al., 2008; Mahrus et al., 2008; Fuchs and Steller, 2011; Ramirez and Salvesen, 2018). Morphological changes occur, including cell constriction, organelle fragmentation, chromatin condensation, plasma membrane “blebbing” as the cortex ruptures, and finally the break-up of the cell into apoptotic bodies, which are the defining features of apoptosis (Kerr et al., 1972).

There are several proposed models of how the BCL-2 family execute cell death upstream of MOMP (reviewed in (Shamas-Din et al., 2013)): the direct activation model, the

displacement model, the embedded together model, and the unified model. The latter two models, which built upon the direction activation and displacement models, highlight the complexity of the mitochondrial cell death pathway. The embedded together model proposes that the specific interactions of the BCL-2 family members are dependent on cellular equilibria, with binding events occurring based on concentration and binding affinities of each protein (Leber et al., 2007). These affinities are also dependent on proximity to the mitochondrial membrane as well as post-translational modifications. The unified model of apoptosis differs in that it proposes that the anti-apoptotic proteins have two “modes”: one in binding and sequestering the activator BH3-only proteins, which can be overcome by the sensitizer BH3-only proteins, and the other binding and sequestering BAK/BAX, which results in a more robust inhibition of MOMP (Llambi et al., 2011; Kalkavan and Green, 2018). The unified model also includes the effects that the BCL-2 family proteins have on mitochondrial dynamics, thus expanding on the embedded together model.

2.2 The BCL-2 family in stem cells

The intrinsic apoptosis has been shown to be uniquely regulated in stem cells (Madden et al., 2011; Dumitru et al., 2012; Gama and Deshmukh, 2012; Liu et al., 2013; Rasmussen et al., 2018). Human PSCs (hPSCs) are extremely sensitive to DNA perturbations in a P53-dependent manner. P53 protein expression, however, was not significantly different between hPSCs and differentiated cells, pointing to another mode of action for this rapid apoptotic response. It was found that expression of the BCL-2 family in stem cells is shifted so that pro-apoptotic members are upregulated, while pro-survival members are downregulated, which serves to position them closer to the threshold of apoptosis, a property known as mitochondrial priming (Certo et al., 2006; Liu et al., 2013). BAX aids in this high mitochondrial priming state of hPSCs (Dumitru et al., 2012). BAX was found in an activated state in homeostatic conditions but localized at the Golgi until an apoptosis-initiating event occurred (e.g. etoposide-mediated DNA damage), when it would translocate to the mitochondria (Dumitru et al., 2012). How BAX is maintained at the Golgi in an active state remains elusive, as up to this point active BAX, which is identified by its N-terminal domain (Upton et al., 2007), had only been known to localize at the OMM, and furthermore only in dying cells. When hPSCs were differentiated into stem cell aggregates known as embryoid bodies, BAX was no longer active during homeostasis and retained its mitochondrial localization during apoptotic stress, indicating a stem cell-specific mechanism of BCL-2 family regulation (Dumitru et al., 2012). Maintaining BAX in an active conformation and in a high mitochondrial priming state likely allows for hPSCs to respond rapidly to cell death, thereby preventing potential mutations induced by DNA damage from persisting in the stem cell pool or in the differentiating cell lineage. Conversely, sustaining BAX in an active state would also pose significant risk for spontaneous apoptosis. Thus, isolating BAX at the Golgi could serve to safeguard hPSCs from unnecessary cell death while still allowing for its rapid induction if the cell is antagonized. At the organismal level, this sensitive response may stop the development of abnormal cells in the growing embryo. If constitutive BAX activation is also maintained by other highly proliferative cells in adult tissue, then perhaps it prevents aberrant growth of cancer cells and tumors. Dissection of the mechanisms underpinning BAX activation and localization, both at the Golgi and at the mitochondria, will be necessary to fully elucidate this apoptotic sensitivity as a defining component of stem cell homeostasis.

As PSCs begin to differentiate, they quickly become resistant to DNA damage-mediated apoptosis; this was shown to be facilitated in part by a decrease in mitochondrial priming (Madden et al., 2011; Liu et al., 2013). Mitochondrial priming and apoptotic sensitivity have been exploited in recent years to identify dependencies of cancer cells on specific BCL-2 family proteins. This technique is known as BH3 profiling (Certo et al., 2006; Montero et al., 2015; Montero and Letai, 2016), and it is useful to measure the different binding affinities between the BCL-2 family members across cell types and patient-derived tumors.

The pro-apoptotic members of the BCL-2 family become activated through several upstream sensing mechanisms. As previously mentioned, PSCs are highly sensitive to DNA damage, which in turn initiates the transcription factor P53 and triggers a repair response and cell cycle arrest (Kruse and Gu, 2009). If the cell cannot resolve the damage, the BCL-2 family facilitates MOMP and the cell undergoes apoptosis in a caspase-dependent manner. Fluctuations in P53 activation have been shown to determine cell fate (Purvis et al., 2012). During reprogramming of somatic cells back to a pluripotent state, inhibition of P53 greatly improves cell survival due to the bypass of BCL-2 family-mediated cell death (Kawamura et al., 2009; Li et al., 2013). Excellent reviews covering the details of P53 function in stem cells can be found here (Bonizzi et al., 2012; Levine et al., 2016; Jain and Barton, 2018). Downstream of BCL-2 family facilitation of MOMP, activity of caspases has been shown to play dual roles in both promoting differentiation and maintaining proper distribution of differentiated cell types, as well as driving reprogramming of somatic cells (Fujita et al., 2008; Li et al., 2010; Fu et al., 2019).

3. The BCL-2 family in mitochondrial dynamics

The main players of intrinsic apoptosis, the BCL-2 family of proteins, execute their functions at the mitochondria. Beyond being the energy-producing powerhouses of the cell, mitochondria serve as major signaling organelles and constantly fuse and divide in a process known as mitochondrial dynamics (Chan, 2007; Berman et al., 2008; Friedman and Nunnari, 2014) (Figure 3). These continuous, energy-consuming events are proposed to occur both in response to various stimuli and to relay signals throughout the cell to other organelles. Dynamic movement and fragmentation of the mitochondrial network was first documented in 1915, with various morphological changes described in detailed, hand-drawn illustrations (Lewis and Lewis, 1914). Yet, the notion that mitochondria are static, bean-like organelles persevered over the decades. The development of more advanced imaging technologies has allowed for improved observation and tracking of mitochondrial dynamics and motility, which has propelled the field and revealed the remarkable changes that mitochondria undergo within cells (Johnson et al., 1981; Bereiter-Hahn and Vöth, 1994; Rizzuto et al., 1996). Emerging studies have also highlighted new links between mitochondrial dynamics machinery, apoptosis and mitochondrial autophagy (mitophagy) (Kageyama et al., 2014; Morciano et al., 2016b; Rasmussen et al., 2018; Fu et al., 2019). Proper equilibrium of mitochondrial dynamics is essential for cellular and organismal homeostasis, and diseases often manifest when the proteins that regulate fission and fusion are mutated (Chan, 2007; Westermann, 2010; Dorn, 2013; Chan, 2019).

3.1 Mitochondrial fission

Mitochondrial shape changes drastically depending on the cell type, cell cycle stage, and metabolic state (Chen and Chan, 2017). Differentiated cell types, such as fibroblasts, myocytes, and endothelial cells, generally have elongated, cristae-rich mitochondria that make up complex networks, which promote mtDNA homogenization and energy generation (Kuznetsov et al., 2009). Mitochondrial fission is necessary for distribution of mitochondria to daughter cells during cell division, mitochondrial transport within cells, and isolation of damaged mitochondria for degradation by mitophagy (Nunnari et al., 1997; Pon, 2013; Ganesan et al., 2019). As differentiated cells reprogram and transition into a stem-like state, mitochondria undergo higher levels of fission and arrange to be more perinuclear (Prieto et al., 2016; Chen and Chan, 2017). The mitochondria of hESCs and iPSCs are considered more functionally immature, with globular shapes and fewer cristae, contributing to a metabolic profile that is traditionally thought to be more dependent on glycolysis for energy requirements (Facucho-Oliveira and St. John, 2009; Chung et al., 2010). Constitutive activation of the dynamin-related guanosine triphosphates (GTPases) that control fission is likely a major participating factor in maintaining high levels of fragmentation in stem cells (Chung et al., 2010; Prieto et al., 2016; Rasmussen et al., 2018).

The large dynamin-related GTPases regulate mitochondrial dynamics by inducing mitochondrial fission (division) and fusion (reviewed in section 3.2) in a highly conserved manner (Praefcke and McMahon, 2004; Hoppins et al., 2007; Chan, 2012). Dynamin-related protein 1 (DRP-1) executes its function upon activation by several post-translational modifications, including phosphorylation, ubiquitination, sumoylation, and O-GlcNAcylation (Westermann, 2010). The enzymes that perform this activation of Dynamin-related proteins have not been completely identified, but ERK1/2 and CDK1 have been shown phosphorylate DRP-1 at Serine 616, providing insight into specific pathways that promote increased mitochondrial fragmentation (Taguchi et al., 2007; Prieto et al., 2016). Once DRP-1 is activated, it translocates from the cytosol to the OMM, where it hydrolyzes GTP and self-assembles around the mitochondria, constricting both membranes until the organelle is divided in two (Hoppins et al., 2007; Pon, 2013; Antonny et al., 2016; Francy et al., 2017). The structural domains and mechanistic details of action for DRP-1 have been described in several studies and reviews (Hoppins et al., 2007; Meglei and McQuibban, 2009; Chappie et al., 2010; Gao et al., 2010; van der Bliek and Payne, 2010; Mears et al., 2011; Francy et al., 2017); however, whether or not DRP-1 activation is differentially regulated in PSCs, which have a more fragmented mitochondrial network, has not been elucidated. DRP-1 function in mitochondrial fission is required for proper achievement of pluripotency early on in the reprogramming process (Prieto et al., 2016). As DRP-1 is also involved in the mitochondrial fragmentation observed during apoptosis (Frank et al., 2001), and iPSCs are highly sensitive to apoptotic stimuli, these cells are likely highly dependent on DRP-1 beyond its role in homeostatic conditions. It is important to note that some studies suggest the possibility that mitochondrial fission can occur in the absence of DRP-1 (Onoue et al., 2013; Stavru et al., 2013), and/or that DRP-1 requires additional proteins for severance of the mitochondria (i.e. dynamin-2) (Lee et al., 2016), a phenomenon that remains controversial (Kamerkar et al., 2018; Fonseca et al., 2019). Moving forward, additional studies into the mechanisms by which mitochondrial fission is regulated across different cell

types, cell states and model systems are needed for appropriately interpreting conflicting results, and to better elucidate the intricate events coordinating mitochondrial fragmentation.

3.2 Mitochondrial fusion

As stem cells differentiate and become more mature, the mitochondrial network transforms to support the changing needs of the differentiating cell. Mitochondrial fusion is required for the proper maturation of several tissue types, and it is necessary for mtDNA homogenization and assembly of the electron transport chain (ETC) (Chan, 2012). The specific mechanisms by which proteins control mitochondrial fusion are not well understood. In vitro studies have shown that OMM fusion is a mechanistically distinct process from fusion of the inner mitochondrial membrane (IMM) (Legros et al., 2002; Meeusen et al., 2004). The large GTPases that govern the process of mitochondrial fusion are Mitofusin 1 (MFN1), Mitofusin 2 (MFN2), and Optic Atrophy 1 (OPA1) (Alexander et al., 2000; Delettre et al., 2000; Chen et al., 2003). MFN1 and MFN2 are localized to the OMM, where they facilitate outer membrane fusion by homo- or hetero-dimerizing with MFNs on adjacent mitochondria and actively fusing the outer membranes. While the MFN paralogs are highly similar, they are not completely redundant and are both required for mitochondrial fusion (Escobar-Henriques and Joaquim, 2019). Depletion of MFN1 in mouse embryonic fibroblasts (MEFs) leads to increased fragmentation of individual mitochondria, resulting in small fragments, while MFN2 depletion results in larger fragments of aggregated mitochondria (Chen et al., 2003). Interestingly, double knockout embryos die earlier than single mutants (Chen et al., 2003) and both MFN1 and 2 levels vary in expression among different tissues (Santel et al., 2003), pointing to a non-redundant requirement for the Mitofusins during early development (Schrepfer and Scorrano, 2016).

Once OMM fusion occurs, the IMM is fused through the activity of OPA1. OPA1 regulation is complex, as it has eight different splice isoforms that lead to the formation of two proteolytically cleaved proteins, designated as long OPA1 (OPA1-L) and short OPA1 (OPA1-S), which serve several distinct and overlapping purposes (Mishra et al., 2014; Del Dotto et al., 2017). OPA1-L is anchored in the IMM with its GTPase domain exposed to IMS. It coordinates the active process of IMM fusion by forming homo-dimers with OPA1-L proteins on the opposite target membrane. OPA1-S is proposed to provide a more passive, structural role at the mitochondrial matrix in cristae formation and maintenance, while combinations of both long and short forms work together to fine-tune mitochondrial morphology and function (Del Dotto et al., 2018). The OPA1-S form has also been implicated in fission of the IMM (Anand et al., 2014). These studies were performed in MEFs, but it would be interesting to probe if the homeostatic balance of OPA1 isoforms is differentially regulated in stem cells and over the course of differentiation. Disproportionate levels of either mitochondrial fusion or fission results in profound pathological abnormalities. These include embryonic lethality at mid-gestation in mice deficient in any of the dynamin-related proteins (i.e. MFN1, MFN2, OPA1, and DRP1) (Chen et al., 2003; Davies et al., 2007; Alavi et al., 2007; Ishihara et al., 2009; MacVicar and Langer, 2016), as well as neurodegenerative diseases such as Charcot-Marie-Tooth syndrome and dominant optic atrophy (Züchner et al., 2004; Alavi et al., 2007; Davies et al., 2007; Waterham et al., 2007) caused by mutations in MFN2 and OPA1, respectively.

3.3 Mitochondrial dynamics and apoptosis

Mitochondrial dynamics also change during apoptosis; mitochondrial fusion is thought to protect against mitochondrial pore formation (Estaquier and Arnoult, 2007; Jahani-Asl et al., 2010), while mitochondrial fission has been shown occur concurrently with BAK/BAX-mediated cell death (Frank et al., 2001; Youle and van der Bliek, 2012). Parallel to this, studies have shown that pro-apoptotic BAK and BAX must be inactivated for mitochondrial fusion to occur, and BAX has been shown to modulate fusion through interactions with the Mitofusins (Karbowski et al., 2006; Brooks et al., 2007; Hoppins et al., 2007, 2011). Additionally, other BCL-2 family members have recently been associated with the maintenance of mitochondrial dynamics in various cell types (Chen et al., 2011; Hardwick and Soane, 2013). In adult neurons, the anti-apoptotic protein BCL-xL is enriched in the mitochondria, where it promotes proper mitochondrial length and size, as well as localization of the mitochondria to the highly energetic synapses (Berman et al., 2008; Li et al., 2008). BCL-xL has been reported to be involved in neuronal connectivity and communication, which are required for normal brain development (Nakamura et al., 2016). Another anti-apoptotic family member, MCL-1, has been shown to also function in the regulation of mitochondrial dynamics in cancer cells and hPSCs (Morciano et al., 2016a; Rasmussen et al., 2018). MCL-1 is expressed at high levels in hPSCs, and its depletion causes mitochondrial elongation in stem cells that coincides with the loss of expression of pluripotency markers OCT4 and NANOG (Rasmussen et al., 2018). The changes in mitochondrial morphology are likely due to MCL-1's interaction with DRP-1, but the details of the state of DRP-1 activation are still unknown. Interestingly, MCL-1 was also shown to interact with OPA1, an association that likely occurs through MCL-1's matrix-localized form (Rasmussen et al., 2018). These non-apoptotic interactions further implicate MCL-1, and perhaps other BH domain proteins, in the modulation of mitochondrial dynamics and homeostasis beyond their roles in cell death. A recent study showed that BID, a pro-apoptotic BH3-only protein, is also imported into the mitochondrial matrix where it regulates cristae morphology and organization (Salisbury-Ruf et al., 2018). As BID is a known binding protein of MCL-1, this study showed these two family members interact at the matrix in cancer cells. It would be interesting to elucidate the dynamics of this interaction in stem cells. For instance, MCL-1 deficiency causes significant changes in the mitochondrial network of iPSCs where BID is highly expressed (Rasmussen et al., 2018). Whether MCL-1's remodeling of the mitochondria is dependent on BID or whether MCL-1 and BID work in concert at the matrix to maintain pluripotency/self-renewal properties in stem cells is not known. Understanding the regulation of cell fate from the perspective of mitochondrial dynamics and the BCL-2 family of proteins could shed light on the intricacies of cell signaling and organelle biology.

As previously mentioned, extensive remodeling of the mitochondrial network occurs during apoptosis. These changes result in the release of cytochrome c and other caspase-inducing proteins from the IMS (Goldstein et al., 2000; Garrido et al., 2006; Galluzzi et al., 2018). Mitochondrial fragmentation during apoptosis takes place through two synchronized, but independent, events: dissociation of cristae junctions, where pools of cytochrome c are located, and BAK/BAX oligomerization and pore formation at the outer membrane (Gao et al., 2001; Lee et al., 2004; Ow et al., 2008; Sheridan et al., 2008; Montessuit et al., 2010;

Sinibaldi et al., 2013). A growing number of studies show that DRP-1 acts in response to apoptotic signals, consistent with the idea that mitochondrial fission occurs along with MOMP. DRP-1 has been shown promote BAX translocation to the mitochondria (Wang et al., 2015), and it also localizes to BAX/BAK pores (Frank et al., 2001; Tanaka and Youle, 2008) where it promotes extensive mitochondrial network fission. DRP-1 depletion prevents mitochondrial fission during apoptosis, and overexpression of the DRP-1 mutant K38A, which inhibits GTP binding by DRP-1, prevents apoptosis-induced mitochondrial fission (Smirnova et al., 2001; Sugioka et al., 2004; Estaquier and Arnoult, 2007). BAK and BAX have also been reported to localize at fission sites along with DRP-1 and MFN2, providing further evidence that MOMP is associated with the fission regulators (Karbowski et al., 2002; Hoppins et al., 2011).

OPA1 has been shown to be involved during MOMP where its disassembly is needed to facilitate cytochrome c release (Frezza et al., 2006; Yamaguchi et al., 2008; Jiang et al., 2014). A number of studies demonstrated that apoptosis requires OPA1-dependent cristae remodeling initiated by metalloprotease OMA1-mediated cleavage of OPA1. Furthermore, activation of OMA1 is mediated by pro-apoptotic members of the BCL-2 family. How these functions of OPA1 are regulated during apoptosis in stem cells is not clear, however, these studies point to distinct functions of OPA1 in mitochondrial fusion and in cristae remodeling during apoptosis (Arnoult et al., 2005; Frezza et al., 2006; Yamaguchi et al., 2008; Jiang et al., 2014).

3.4 Mitophagy

Concomitant with increased mitochondrial fission is the clearance of damaged mitochondria by mitophagy (Zhang, 2013; Burman et al., 2017) (Figure 3). Mitophagy is proposed to be an opposing process to apoptosis, and initiation of either pathway is dependent on signaling crosstalk and cell state (Pickles et al., 2018). Activation of PTEN-induced putative kinase 1 (PINK1) aids in the selective clearance of mitochondria through recruitment of the E3 ubiquitin ligase Parkin, which translocates to the mitochondria from the cytosol and targets mitochondrial membrane proteins for degradation by the autophagosome (Narendra et al., 2008). MFN1 and MFN2 are substrates for Parkin-mediated ubiquitination (Gegg et al., 2010). However, this modification targets them for proteasomal degradation rather than acting as a mitophagy signal, suggesting that elimination of mitochondrial fusion and a shift to fission occurs prior to mitophagy. BCL-2 and BCL-xL have been shown to negatively regulate mitophagy through inhibition of Beclin-1 (also known as ATG6), a BH3 domain-containing protein that regulates formation of the phagophore along with adaptor proteins such as BNIP3 (BCL2 and adenovirus E1B 19 kDa-interacting protein 3) and NIX (BNIP3-like) (Pattingre et al., 2005; Maiuri et al., 2007; Zhang and Ney, 2009; Dhingra et al., 2014; Vásquez-Trincado et al., 2016; Fernández et al., 2018). Another study in HeLa cells showed that overexpression of any of the anti-apoptotic family members (BCL-2, BCL-xL, BCL-w, MCL-1, A1) prevented PINK1/Parkin-mediated mitophagy, but this block was independent of Beclin-1 (Hollville et al., 2014).

Mitophagy can maintain cellular homeostasis and is also triggered by nutrient deprivation and other stressors; however, mitophagy can also be developmentally initiated (Pickles et al.,

2018). In fact, decreased levels of mitophagy accompany increased mitochondrial fusion that occurs during stem cell differentiation, and increased mitophagy during somatic cell reprogramming promotes the switch in metabolism back to glycolytic dependence, as it reduces mitochondrial mass, mtDNA content, and ETC components that facilitate oxidative phosphorylation (Vazquez-Martin et al., 2012; Fu et al., 2019). Mitophagy is essential in post-mitotic cells, since they have lost their proliferative capacity and rely on maintaining a healthy pool of mitochondria (Zhang, 2013; Evans and Holzbaur, 2019). However, high levels of mitochondrial fission in stem cells would perhaps require high activation of mitophagy for quality control purposes (Naik et al., 2019). Supporting this, late-passage PINK1-deficient mouse iPSCs, while they expressed the pluripotency factors OCT4 and SOX2, were more prone to differentiation and showed accumulation of damaged mitochondria (Vazquez-Martin et al., 2016).

3.5 Inter-organelle contacts

Although it is known that mitochondrial fragmentation and the protein machinery that coordinate this process are essential, details of how mitochondrial membrane integrity is disrupted, yet in a controlled manner, are not well understood. Structural analysis of DRP-1 discovered that the rings formed by DRP-1 oligomers are smaller in diameter (30–50 nm) than the average diameter of mitochondria (Ingeman et al., 2005; Mears et al., 2011), suggesting there are likely other constriction mechanisms involved. Recent work has identified the endoplasmic reticulum (ER) as well as the actin cytoskeleton as key players in mitochondrial dynamics (Lewis et al., 2016; Rehklau et al., 2017; Steffen and Koehler, 2018). ER tubules can be found at sites of fission, where they form contacts with the mitochondria through the ER-mitochondria encounter structure (ERMES) complex and initiated fission even before DRP-1 constriction (Elgass et al., 2015; Lewis et al., 2016; Yang and Svitkina, 2019). Tethering of the ER to mitochondria might serve not only to facilitate mitochondrial fission, but also to regulate mitochondrial membrane biosynthesis, calcium signaling, and protein import (reviewed in (Kornmann and Walter, 2010)). Following ER tubule encirclement of the mitochondria, it is proposed that INF2-induced actin polymerization occurs, triggering an initial constriction mechanism to facilitate DRP-1 recruitment, assembly, and final scission of the membrane (Korobova et al., 2013; Pon, 2013). It will be interesting to determine if members of the BCL-2 family such as MCL-1 and BAK/BAX are involved in this mitochondrial fission “complex” in cells subjected to sublethal doses of apoptotic stimuli. It could also be informative to examine potential adaptations to these processes in hPSCs and cancer stem cells where the balance of mitochondrial dynamics is shifted to favor high fission activity.

Increased mitochondrial priming and mitochondrial fission are two vital components that allow for pluripotent maintenance, and these properties change upon cellular differentiation; however, the precise signaling events and protein interactions that govern these processes are yet to be elucidated. The BCL-2 family affects mitochondrial priming, but we also propose that BCL-2 proteins regulate fragmentation; reprogramming of differentiated cells into iPSCs will be a crucial tool to dissect this intriguing possibility in a temporal and cell-specific manner. Further investigation into the relationship between mitochondrial priming,

fragmentation and the acquisition of pluripotency or a particular stem cell fate opens an exciting opportunity for future mechanistic studies.

4. Concluding remarks and future perspectives

As discussed throughout this chapter, the BCL-2 family of proteins regulate the mitochondrial pathway of apoptosis and are thereby at the center of several essential signaling pathways. The mitochondrial network is responsible for the regulation of apoptosis, oxidative phosphorylation, coordination of neighboring organelles, and they provide the machinery needed to generate key metabolites that serve the bioenergetic and biosynthetic needs of the cell (Fu et al., 2019). The crosstalk between the pathways of BCL-2 family-mediated cell death, mitochondrial dynamics, and mitophagy must be explored further in PSCs, as it will likely provide important details into how cells navigate the differentiation and reprogramming processes. The role of reactive oxygen species on stem cell maintenance is also an intriguing area of study, since multiple redox-sensitive pathways also control apoptosis and mitochondrial dynamics (e.g. MAPK, ERK1/2, JNK) (Bigarella et al., 2014).

As discussed in this chapter, many questions remain; for example: 1) How do the signaling pathways that converge at the mitochondria intersect to regulate cell fate? 2) How are the changes in mitochondrial network, mitochondrial turnover, and mitochondrial connectivity to other organelles regulated as cells undergo differentiation and reprogramming? 3) How do changes in mitochondrial morphology contribute to adaptations in metabolic state? 4) How do metabolite levels modulate the activity of epigenetic enzymes ultimately affecting gene expression? Answering these questions would open the opportunity to advance the fields of stem cell biology and apoptosis as well as to rationalize new therapies, as shown by the emergence of pharmacological inhibitors of the BCL-2 family.

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Box 1:**Abbreviations used in this chapter**

BCL-2	B cell chronic lymphocytic leukemia/lymphoma 2	BIK	BCL-2 interacting killer
MOMP	Mitochondrial outer membrane permeabilization	BMF	BCL-2 modifying factor
ESCs/hESCs	Embryonic stem cells/human embryonic stem cells	HRK	Harakiri
iPSCs	Induced pluripotent stem cells	OMM	Outer mitochondrial membrane
PSCs/hPSCs	Pluripotent stem cells (iPSCs & ESCs)/human pluripotent stem cells	IMS	Inner membrane space
ICM	Inner cell mass	IMM	Inner mitochondrial membrane
OCT4	Octamer-binding transcription factor 4	ERAD	Endoplasmic reticulum-associated protein degradation pathway
SOX2	Sex-determining region Y-box 2	APAF-1	Apoptotic protease activating factor 1
KLF4	Kruppel-like factor 4	mtDNA	Mitochondrial DNA
BAX	BCL-2 associated X	DRP-1	Dynamin-related protein 1
BAK1/BAK	BCL-2 antagonist killer 1	ERK1/2	Extracellular signal-regulated kinase 1/2
BOK	BCL-2 ovarian killer	CDK1	Cyclin-dependent kinase 1
BCL-xL	BCL-2-like 1	ETC	Electron transport chain
BCL-w	BCL-2-like 2	MFN1/2	Mitofusin 1/2
MCL-1	Myeloid cell leukemia 1	OPA1	Optic atrophy 1
BFL-1/A1	BCL-2-related protein A1	MEF	Mouse embryonic fibroblast
BIM	BCL-2 interacting mediator of cell death	PINK1	PTEN-induced putative kinase
BID	BCL-2 interacting domain death agonist	ERMES	Endoplasmic reticulum-mitochondria encounter structure
PUMA	P53-upregulated modulator of apoptosis	MAPK	Mitogen-activated protein kinase
PMAIP1/NOXA	Phorbol-12-myristate-13-acetate-induced protein 1	JNK	c-Jun N-terminal kinase
BAD	BCL-2-associated agonist of cell death	NIX	BNIP3-like (BNIP3L)
BNIP3	BCL2 and adenovirus E1B 19 kDa-interacting protein 3

Figure 1: The BCL-2 family regulates mitochondrial cell death and homeostasis in stem cells. This schematic depicts the canonical pathways of mitochondrial apoptosis and priming. Highlighted are the reported changes in PSC regulation of these pathways: 1) High levels of pro-apoptotic proteins. 2) BAX is maintained in an active state at the Golgi. 3) High levels of MCL-1, which is important for pluripotent maintenance and mitochondrial fission. 4) Increased fragmentation of the mitochondrial network and higher dependence on glycolytic metabolism.

Figure 2: The BCL-2 family regulates the mitochondrial pathway of apoptosis.

When cells receive an intrinsic death signal, BAX becomes activated by the BH3-only activators, undergoes a conformational change, and translocates to the outer mitochondrial membrane (OMM). BAX (in addition to BAK and BOK) then oligomerize and form pores in the OMM, causing MOMP and the release of cytochrome c into the cytosol. BAX activation can be prevented by sequestration of the activator BH3-only proteins through the pro-survival proteins BCL-2, MCL-1, BCL-xL, etc, which are inhibited by the sensitizer BH3-only family members. In human embryonic stem cells, BAX can be maintained in an active state at the Golgi, which confers quick translocation to the OMM in case of a death signal. Additionally, MCL-1 has been found to reside at the matrix in stem cells, where it is proposed to interact with OPA1.

Figure 3: The dynamic cycle of mitochondrial morphology and quality control.

In homeostasis, differentiation, reprogramming, and during stress, the mitochondrial network undergoes rounds of fusion and fission. During fission, DRP-1 is activated and recruited to pre-constricted regions of mitochondria (facilitated by actin and ER tubules) by its receptors (MFF, MID49/51, FIS1). Mitochondrial fusion is achieved by the activity of MFNs, which dimerize with MFNs on adjacent mitochondria to fuse the OMM. This is followed by OPA1-mediated fusion of the inner mitochondria membrane (IMM). If mitochondria are damaged (i.e. low membrane potential), PINK1 is stabilized at the mitochondria and phosphorylates proteins on the OMM. This phosphorylation triggers Parkin-mediated ubiquitination of OMM proteins, causing recruitment of various adapter proteins and receptors associated with the phagophore. The damaged mitochondrion is then engulfed by the mature mitophagosome, which fuses with the lysosome for degradation.