The role of BH3-only proteins in apoptosis within the ovary

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Abstract

BH3-only proteins are pro-apoptotic members of the BCL2 family that play pivotal roles in embryonic development, tissue homeostasis and immunity by triggering cell death through the intrinsic apoptosis pathway. Recent *in vitro* and *in vivo* studies have demonstrated that BH3-only proteins are also essential mediators of apoptosis within the ovary and are responsible for the initiation of the cell death signalling cascade in a cell type and stimulus-specific fashion. This review gives a brief overview of the intrinsic apoptosis pathway and summarise the roles of individual BH3-only proteins in the promotion of apoptosis in embryonic germ cells, oocytes, follicular granulosa cells and luteal cells. The role of these proteins in activating apoptosis in response to developmental cues and cell stressors, such as exposure to chemotherapy, radiation and environmental toxicants, is described. Studies on the function of BH3-only proteins in the ovary are providing valuable insights into the regulation of oocyte number and quality, as well as ovarian endocrine function, which collectively influence the female reproductive lifespan and health.

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Introduction

Programmed cell death, or apoptosis morphology (hereafter referred to as ‘apoptosis’), is a major feature of normal ovarian development, with more than two-thirds of the germ cells produced during female embryonic development undergoing cell death shortly after they are formed (Pepling & Spradling 2001, Myers *et al.* 2014). The apoptotic elimination of germ cells ultimately results in a reduced number of oocytes stored as primordial follicles within the ovary at birth (Myers *et al.* 2014). Once formed, primordial follicles may be lost from the ovarian reserve as a consequence of exposure to endogenous and exogenous stressors, such as chemotherapy, radiation and environmental toxicants (Kerr *et al.* 2012a, Sobinoff *et al.* 2013, Sivakumar *et al.* 2014). These agents are known to activate apoptotic responses within the primordial follicle oocyte itself, as well as in the granulosa cells of growing follicles (Suh *et al.* 2006) (reviewed in Morgan *et al.* (2012)). Furthermore, the majority of follicles that initiate growth and development throughout reproductive life do not progress to ovulatory status, but instead undergo selective atresia, a process underpinned by the apoptosis of granulosa and theca cells, followed by death of the enclosed oocyte (reviewed in Matsuda *et al.* (2012)). Thus, apoptosis plays a critical role in regulating the number of oocytes available for ovulation, which

influences the length of a women’s fertile lifespan and the timing of menopause. Apoptosis is also intimately involved in the constant and cyclical remodelling of the ovary that occurs after ovulation, and is particularly important for the later phase of corpus luteum regression involving structural involution (Juengel *et al.* 1993, Shikone *et al.* 1996, Bowen *et al.* 1999, Goyeneche *et al.* 2006), which occurs after the initial decline of progesterone production. Given the clear importance of apoptosis for germ cell development, folliculogenesis and overall female fertility, much research effort has focussed on identifying and characterising the proteins that regulate this process. In this regard, the pro-apoptotic BH3-only proteins are emerging as the key regulators of apoptosis within the ovary. BH3-only proteins are members of the BCL2 family that trigger apoptosis through the intrinsic apoptosis pathway, which will be described briefly below.

Pathways to apoptosis

Apoptosis is a genetically programmed, evolutionarily conserved process essential for the elimination of dangerous, damaged or supernumerary cells (reviewed in Golstein (1998)). There are two apoptotic pathways in mammalian cells: the intrinsic pathway, which is triggered by activation of pro-apoptotic BH3-only proteins in response to a diverse range of developmental

Figure 1 The extrinsic (death receptor-mediated) and intrinsic (mitochondrial) pathways to apoptosis. The intrinsic apoptosis pathway is activated by developmental cues or cytotoxic stimuli, such as cytokine deprivation or DNA damage caused by u.v., γ-irradiation (γ-IR) and chemotherapy. These stimuli trigger the BH3-only proteins, which then inhibit the BCL2-like pro-survival proteins. Inhibition of the BCL2-like pro-survival proteins leads to the activation of BAX and BAK, which induce mitochondrial outer membrane permeabilisation and release of cytochrome c from the mitochondria. Some BH3-only proteins may directly activate BAX and BAK. The release of cytochrome c from the mitochondria promotes the activation of caspase-9, which then activates the effector caspases (caspase-3, caspase-7, and caspase-6) leading to cell demolition. The extrinsic apoptosis pathway is activated when the ligands of the tumour necrosis factor family, such as FAS ligand (FASL) and tumour necrosis factor (TNF), bind to their respective death receptors on the cell membrane, for example FAS and TNFR1. This results in cleavage of caspase-8, leading to the activation of effector caspases (caspase-3, caspase-7, and caspase-6) and cellular destruction. Death receptor signalling can also result in BID cleavage by caspase-8, leading to the generation of active tBID, which then engages the intrinsic apoptosis pathway.

cues or cellular stressors, and the extrinsic apoptotic pathway, which is triggered when cell surface death receptors are activated by their respective ligands (Fig. 1) (reviewed in Happo *et al.* (2012)). These pathways converge with the activation of the caspases (aspartate-specific cysteine proteases), which are responsible for cleaving essential proteins, ultimately leading to cellular destruction (Fig. 1).

The intrinsic apoptosis pathway, also known as the mitochondrial or BCL2 regulated apoptosis pathway, is regulated by the relative levels and activities of both pro- and anti-apoptotic members of the BCL2 superfamily (Figs 1 and 2; reviewed in Happo *et al.* (2012) and Czabotar *et al.* (2014)). The members of the BCL2 superfamily share one or more of the four BCL2 homology domains (BH1–BH4) and can be divided into three subgroups according to their structure and function: i) the multi-BH domain pro-survival proteins,

BCL2, BCL extra-large (BCL-X_L), myeloid cell leukaemia sequence 1 (MCL1), A1 and BCL-W (also known as BCL2L2), which inhibit apoptosis and are essential for cell survival; ii) the multi-BH domain pro-apoptotic members BCL2-associated X protein (BAX), BCL2 antagonist/killer (BAK) and possibly also BCL2-related ovarian killer protein (BOK), which are critical for activation of the downstream events in cell destruction; and iii) the pro-apoptotic BH3-only proteins, including BCL2 interacting mediator of death (BIM, also known as BCL2L11), p53 upregulated modulator of apoptosis (PUMA, also known as BBC3), BH3 interacting domain death agonist (BID), BCL2 antagonist of cell death (BAD), BCL2 modifying factor (BMF), BCL2 interacting killer (BIK, also known as BLK/NBK), HRK (also known as DP5) and NOXA (also known as PMAIP1) (Fig. 2; reviewed in Czabotar *et al.* (2014)).

BH3-only proteins are the critical sensors of cell stress and initiators of the intrinsic apoptosis signalling cascade (reviewed in Tsujimoto & Shimizu (2000)). They promote apoptosis by binding, via the BH3 domain, the pro-survival BCL2 family members and neutralising their activity by stabilising and inhibiting their function (Chen *et al.* 2005; reviewed in Willis *et al.* (2007), Happo *et al.* (2012) and Czabotar *et al.* (2014)). Inhibition of the pro-survival BCL2 proteins then leads to the release from inhibition and activation of apoptotic effectors BAX and BAK (reviewed in Hsu & Hsueh (2000), Willis & Adams (2005) and Adhikari & Liu (2009)). Some BH3-only proteins, such as tBID (activated form of BID, see below), BIM and possibly PUMA, may also directly bind and activate BAX and BAK and in this instance a key role of the pro-survival BCL2 proteins may be to sequester these BH3-only proteins

BCL2 family proteins	Members
Pro-apoptotic BH3-only proteins <div><div>BH3</div><div>TM</div></div>	PUMA, NOXA, BMF, tBID, BIK, BAD, HRK, BIM
Pro-survival BCL2 like proteins <div><div>BH4</div><div>BH3</div><div>BH1</div><div>BH2</div><div>TM</div></div>	BCL2, BCL-X _L , BCL-W, MCL1, A1
Pro-apoptotic effector proteins <div><div>BH4</div><div>BH3</div><div>BH1</div><div>BH2</div><div>TM</div></div>	BAX, BAK, BOK

Figure 2 The BCL2 protein family. The BCL2 family of apoptotic regulators can be divided into three broad groups based on their general structure and function. The BH3-only proteins contain only a BH3 domain and comprise family members PUMA, NOXA, BMF, tBID, BIK, BAD, HRK and BIM. The pro-survival BCL2-like proteins have all four BCL2 homology domains (BH1–4) and comprise family members BCL2, BCL-X_L, BCL-W, MCL1 and A1. Finally, the proapoptotic effectors BAX, BAK, and possibly BOK all contain four BH domains. Most of the BCL2 family members also have a transmembrane domain (TM), which enables them to anchor to the mitochondria.

to prevent or curtail direct activation of BAX and BAK in the absence of sufficient apoptotic stimuli (Letai *et al.* 2002, Kuwana *et al.* 2005, Certo *et al.* 2006, Gavathiotis *et al.* 2008). Activation of BAX/BAK proteins results in permeabilisation of the mitochondrial outer membrane, followed by release of cytochrome c and other apoptogenic factors from the mitochondria. Formation of the apoptosome then promotes the activation of initiator caspase-9, with subsequent activation of the effector caspases (e.g. caspase-3, -7, -6; reviewed in Happon *et al.* (2012)). The fate of the cell is thus determined by the balance or ratio of these three groups of apoptotic regulators.

Stress factors known to result in activation of the intrinsic apoptosis signalling cascade include reactive oxygen species, detachment from the extracellular matrix, heat, hypoxia, γ -irradiation, chemotherapy or cytokine deprivation (reviewed in Youle & Strasser (2008)). Importantly, individual BH3-only proteins are activated by different apoptotic stimuli, with some overlap and functional compensation. They also differ in their affinity for individual pro-survival BCL2 proteins (reviewed in Czabotar *et al.* (2014)). This difference defines the apoptotic potency of each BH3-only protein because it is likely that most of the pro-survival proteins expressed by a given cell must be neutralised for apoptosis to occur efficiently (Chen *et al.* 2005, Willis *et al.* 2007). In this regard, PUMA, BIM and tBID are particularly potent cell killers because they can heterodimerise with and inhibit all pro-survival BCL2 family members (BCL2, BCL-X_L, BCL-W, MCL1 and A1) (Tsujimoto & Shimizu 2000; reviewed in Kuwana & Newmeyer (2003) and Kelly & Strasser (2011)). By contrast, the killing capacity of NOXA is considerably reduced because it can only bind and inhibit MCL1 and A1 (reviewed in Yu & Zhang (2008)).

The extrinsic apoptotic pathway is initiated by the activation of cell surface death receptors belonging to the tumour necrosis factor receptor family (e.g. FAS and TNFR1) by binding of their respective pro-apoptotic ligands (e.g. FASL and TNF α) (Fig. 1; reviewed in Strasser *et al.* (2000)). The activation of death receptor is followed by cleavage and activation of caspase-8, which then directly activates effector caspase-3 (Fig. 1; reviewed in Kaufmann *et al.* (2012)). While the intrinsic and extrinsic pathways to apoptosis are largely independent, some cells require simultaneous activation of both pathways for death to occur (Kaufmann *et al.* 2009). In these cells, death receptor activation also leads to the cleavage of the BH3-only protein BID by caspase-8 (Fig. 1; Kaufmann *et al.* 2009). BID cleavage results in the generation of a 15 kDa truncated active protein called tBID, which translocates to the mitochondria, where it forms a heterodimer with the pro-survival BCL2 proteins, BCL2 itself, BCL-X_L, BCL-W, MCL1 and A1 (Fig. 1; reviewed in Happon *et al.* (2012)). This interaction results in the inactivation of the pro-survival BCL2 proteins, followed

by activation of pro-apoptotic proteins BAX/BAK. Activation of BAX/BAK results in destabilisation of the mitochondrial membrane, cytochrome c release, formation of the apoptosome, caspase activation and apoptosis. Thus, BID is the essential bridge between the intrinsic and extrinsic apoptosis pathways and cleavage of BID results in the simultaneous activation of the cell death machinery of both the intrinsic and extrinsic pathways.

Unique and overlapping roles for individual BH3-only proteins in triggering apoptosis have been extensively studied in many non-ovarian cell types and organ systems, with much progress made though the generation and study of gene knockout mouse models. Knowledge regarding the function and regulation of BH3-only proteins gained from studies in other cells is now being applied to the ovary, and important roles for BH3-only proteins in triggering apoptosis in germ cells, oocytes, granulosa cells and luteal cells are now emerging.

PUMA and NOXA

Studies carried out in somatic cells have shown that PUMA and NOXA are direct transcriptional targets of p53, with important roles for these proteins in the activation of apoptosis in response to DNA damage induced by γ -irradiation and chemotherapy drugs (Jeffers *et al.* 2003, Villunger *et al.* 2003). By contrast, transcriptional induction of PUMA and NOXA in the oocytes of primordial follicles following γ -irradiation-induced DNA damage does not rely on p53, but is dependent on the p53 homologue Tap63 (Kerr *et al.* 2012b). Notably, while oocytes lacking NOXA alone are sensitive to γ -irradiation, oocytes deficient in PUMA are partially resistant and oocytes lacking both PUMA and NOXA are robustly resistant to this insult (Kerr *et al.* 2012b). Thus, PUMA, alone and together with NOXA, plays a critical role in the elimination of primordial follicles from the ovarian reserve that have sustained DNA damage.

PUMA has also been implicated in the developmentally regulated germ cell loss that occurs during the embryonic migration of germ cells to the gonad and in the newly forming ovary (Myers *et al.* 2014). Interestingly, mice genetically deficient in PUMA are endowed with an almost twofold increase in the normal number of primordial follicles, highlighting the key role PUMA-mediated apoptosis plays in determining how many primordial follicles are initially established in the ovarian reserve (Myers *et al.* 2014). However, it is not yet clear whether PUMA directly mediates germ cell apoptosis during this time, or whether PUMA initiates somatic cell apoptosis, which subsequently limits germ cell survival.

BCL2 modifying factor

BMF initiates apoptosis by binding pro-survival BCL2 proteins BCL2, BCL-X_L and BCL-W, and MCL1, but unlike BID, BIM and PUMA, does not interact with the

pro-apoptotic family members BAX and BAK. There are two major apoptosis inducing isoforms of BMF that arise from different translation start sites on a single transcript (Grespi *et al.* 2010). Some studies have suggested that the pro-apoptotic activity of BMF is negatively regulated by binding to dynein light chain 2 (DLC2), a component of the myosin V actin motor complex, which sequesters BMF to the cellular cytoskeleton (Puthalakath *et al.* 2001, Grespi *et al.* 2010). DLC2 binding is facilitated by a highly conserved motif (K/RXTQT) found near the N-termini of BMF (Puthalakath *et al.* 2001). Upon activation, BMF is released from the myosin V motor complex, allowing it to translocate to the mitochondria and inhibit pro-survival BCL2 family members, thereby initiating the apoptotic program. The release of BMF from the myosin V motor complex may be regulated through phosphorylation by JNK on the serine residue adjacent to the BMF actin/myosin domain (Lei & Davis 2003, Labi *et al.* 2008). Interestingly, the pro-apoptotic BH3-only protein BIM is also negatively regulated by cytoskeletal sequestration (Puthalakath *et al.* 2001).

Bmf^{-/-} mice are viable and fertile, but have disrupted B-cell lymphocyte homeostasis, with excess B-cells observed (Labi *et al.* 2008, Hubner *et al.* 2010). Furthermore, embryonic fibroblasts and lymphocytes from mice lacking BMF are resistant to apoptosis induced by the inhibition of phosphatidylinositol 3-kinase, glucocorticoids and histone-deacetylase inhibitors (Labi *et al.* 2008, Grespi *et al.* 2010). Some studies using primary mammary epithelial cells, intestinal cells and transformed cell lines have suggested that BMF initiates cell death resulting from loss of cellular attachment from the basal lamina (anoikis) (Puthalakath *et al.* 2001, Schmelzle *et al.* 2007, Hausmann *et al.* 2011), whereas other studies using embryonic fibroblasts and gastrointestinal epithelial cells from *Bmf*^{-/-} mice have suggested that BMF may be dispensable for anoikis of certain cell types (Labi *et al.* 2008).

While male *Bmf*^{-/-} mice do not exhibit impaired or altered fertility (Labi *et al.* 2008), two *in vitro* studies have suggested that BMF may be important for mediating the death of germ cells when they lose Sertoli cell attachment and thus may act as a germ cell quality control mechanism (Show *et al.* 2004a,b). It is proposed that separation of germ cells from the Sertoli cells activates JNK1, which leads to the phosphorylation of BMF and its relocation from the sub-acrosomal space to the cytoplasm of round spermatids, triggering their apoptosis (Show *et al.* 2008).

Recently, the role of BMF within the adult female reproductive system has been studied in detail using *Bmf*^{-/-} mice (Liew *et al.* 2014). BMF was shown to regulate the number of primordial follicles maintained in the adult mouse ovary, and loss of BMF was associated with increased numbers of primordial follicles between post natal days 100 and 545 and conferred prolonged fertility (Liew *et al.* 2014). While primordial follicle

depletion during female reproductive ageing is known to occur as a result of their activation and entry into the growing follicle pool, this study suggests that it is also possible that primordial follicles are directly lost from the pool through apoptotic processes involving BMF. Notably, the follicle stages and ovarian cells in which BMF is expressed were not identified, and it remains unknown whether BMF directly initiates death within oocytes or if BMF mediates death of somatic cells (e.g. granulosa cells), indirectly leading to oocyte death (Liew *et al.* 2014). Clearly, further work must be done before this issue can be resolved.

BH3 interacting domain death agonist

Studies in non-ovarian somatic cells show that BID is a potent cell killer, but it is significantly different to all other BH3-only proteins because it induces death only after the extrinsic apoptotic pathway is triggered, which leads to its cleavage by caspase-8 to form truncated tBID (Fig. 1; reviewed in Kantari & Walczak (2011) and Happo *et al.* (2012)). Consequently, BID has a unique role in the connection of proximal signals from the death receptors of the extrinsic apoptotic pathway to the common intrinsic apoptotic pathway, thereby providing a mechanism for amplification of the apoptotic cascade.

Though a comprehensive study of the role of BID in cell death within the ovary using knockout mice is yet to be published, several studies have hinted at important roles in follicular atresia and corpora lutea regression (Yadav *et al.* 2005, Goyeneche *et al.* 2006, Sai *et al.* 2011, 2012, Paulose *et al.* 2012, Craig *et al.* 2013). More than 99% of the growing follicles in the ovary are destined for atresia, which is usually first evident by apoptosis of the granulosa cells. Notably, BID expression increases during follicular atresia in the porcine ovary and knockdown of BID expression in human granulosa cell-derived KGN cells can suppress apoptosis (Sai *et al.* 2011, 2012), suggesting a role for BID in regulating the normal process of follicular atresia. In addition to this natural process, BID may also be responsible for triggering follicular atresia following exposure to exogenous environmental toxicants such as di-*n*-butyl phthalate and the organochlorine pesticide methoxychlor, which are known to increase follicular atresia (Paulose *et al.* 2012, Craig *et al.* 2013).

The apoptotic mechanisms involved in structural luteolysis and the eventual disappearance of the corpus luteum from the ovary in normally cycling mammals are not well characterised. Inter-species differences in the factors that induce luteal cell apoptosis and the low number of apoptotic cells observed *in situ* at any given time has made clarification of the precise mechanisms involved challenging (reviewed in Sugino & Okuda (2007)). In an attempt to overcome this latter problem, Goyeneche *et al.* (2006) used an *in vitro* model of rat luteal cell apoptosis to investigate the cellular machinery involved. While high levels of BID were detected in

protein extracts from healthy luteal cells cultured in the presence of serum, when luteal cell apoptosis was induced under conditions of serum starvation, they unexpectedly found reduction in BID expression (Goyeneche *et al.* 2006). The authors implied that this reduction in BID expression might have been due to its cleavage to the active tBID form, but tBID expression itself was not evaluated and active caspase-8 could not be detected (Goyeneche *et al.* 2006). Another study clearly showed increased expression of FAS and FASL increased caspase-8 activity, BID cleavage to tBID and increased caspase-3 and -9 cleavage during PGF2 α -induced bovine luteal tissue apoptosis (Yadav *et al.* 2005). Indeed, a potential role for BID in triggering luteal cell apoptosis during normal cycling is supported by many studies carried out in rodents, cattle and humans showing FAS signalling and caspase-8 activation during luteolysis (Quirk *et al.* 1995, Sakamaki *et al.* 1997, Kuranaga *et al.* 2000a,b, Taniguchi *et al.* 2002, Carambula *et al.* 2003).

BCL2 interacting mediator of death

BIM binds all prosurvival BCL2 proteins with high affinity, and can also bind BAX and BAK (Merino *et al.* 2009, 2012). BIM exists as three major splice variants referred to as BIM_S, BIM_L and BIM_{EL} (O'Connor *et al.* 1998, Wang *et al.* 2012a). Studies in a number of somatic cell types have shown that BIM, both alone or together with other BH3-only proteins, promotes apoptosis in response to a variety of stimuli including cytokine deprivation, taxol, glucocorticoids, radiation, chemotherapy and endoplasmic reticulum (ER) stressors and has a well-characterised role in the apoptosis of autoreactive thymocytes and B-cells (Bouillet *et al.* 1999, 2002, Enders *et al.* 2003, Erlacher *et al.* 2006, Ekoff *et al.* 2007, Happo *et al.* 2010). Transcription of BIM is positively regulated by the forkhead transcription factor FOXO3a in response to cytokine deprivation (Dijkers *et al.* 2000) and by C/EBP homologous protein (CHOP) in response to endoplasmic reticulum stress (Puthalakath *et al.* 2007). Similar to BMF, BIM may be negatively regulated at the post-translational level by sequestration to the cellular cytoskeleton (Puthalakath *et al.* 2001). The activity of BIM can also be regulated by phosphorylation which, depending on the site of modification, can enhance (reviewed in Ewings *et al.* (2007)) or reduce (Lei & Davis 2003, Putcha *et al.* 2003) its activity.

Within the ovary, BIM protein has been detected in the granulosa cells of follicles at all stages of development (O'Reilly *et al.* 2000). Two studies hinting that BIM may be involved in the apoptosis of naked oocytes and oocytes from newly formed primordial follicles in the developing rat ovary have been published. One was an *in vitro* study showing that the survival cytokine, stem cell factor, reduces apoptosis and suppresses BIM expression in cultured naked rat oocytes (Liu *et al.* 2009). The second was a study of neonatal rat ovaries showing

that apoptotic (TUNEL positive) oocytes express FOXO3a and BIM (Sui *et al.* 2010). However, further work needs to be done before a physiological role for BIM in the developmental apoptosis of oocytes during follicle formation could be firmly established.

While the role of BIM in the regulation of apoptosis at the earliest stages of oocyte and follicle development remains unclear, recent work from Wang *et al.* (2012b) has demonstrated an important role for BIM in the atresia of antral follicles. Atretic porcine follicles have increased levels of BIM mRNA compared with healthy follicles and overexpression of BIM_{EL} triggers apoptosis in cultured granulosa cells (Wang *et al.* 2012b, Fu *et al.* 2013). Notably, the well-characterised ability of FSH to prevent atresia of antral follicles is, at least in part, achieved through its ability to suppress BIM_{EL} expression via the PI3K/AKT/FOXO3a pathway (Wang *et al.* 2012b). Work from this same group has also indicated that the low level of apoptosis observed in cumulus compared with mural granulosa cells is achieved through oocyte-secreted GDF9, which inhibits BIM_{EL} expression in cumulus cells (Hussein *et al.* 2005, Wang *et al.* 2013).

BCL2 antagonist of cell death

The proapoptotic activity of BAD is mediated through its ability to bind and neutralise BCL2, BCL-X_L and BCL-W, but not MCL1 or A1 (Chen *et al.* 2005). BAD has been implicated in the apoptosis of haematopoietic cells following withdrawal of cytokine: in the presence of cytokines, BAD can be inhibited by AKT-mediated phosphorylation, which leads to its sequestration by 14-3-3 scaffold proteins or by preventing its interaction with BCL-X_L (Zha *et al.* 1996, del Peso *et al.* 1997). By contrast, cytokine deprivation leads to dephosphorylation of BAD, enabling it to induce apoptosis. *Bad*^{-/-} mice are generally fertile and healthy, although have excess platelets and exhibit a tendency to develop lymphoma with ageing (Ranger *et al.* 2003, Kelly *et al.* 2010).

BAD was first identified in the ovary following a screen for BCL2 interacting proteins and was one of the first pro-apoptotic proteins shown to have a role in follicular atresia (Kaipia *et al.* 1997). Early studies have shown that BAD mRNA is expressed in the granulosa and theca cells of follicles of all sizes and overexpression of BAD in granulosa cells induces their apoptosis (Kaipia *et al.* 1997). However, BAD expression does not actually increase throughout the course of normal atresia or during atresia induced experimentally by estrogen withdrawal in immature rats (Kaipia *et al.* 1997). Instead, induction of granulosa cell apoptosis by loss of hormonal support is associated with the activation of BAD through its dephosphorylation, (Kaipia *et al.* 1997, Gebauer *et al.* 1999). More recent work has provided additional support for the involvement of BAD in granulosa cells apoptosis by showing that progesterone suppresses granulosa cell apoptosis by binding the progesterone receptor

membrane component-1 (PGRMC1) and inhibiting *BAD* gene expression (Peluso *et al.* 2010; reviewed in Peluso (2013)). In addition, one of the mechanisms by which VEGFA is thought to promote granulosa cell survival is through its ability to increase AKT-mediated phosphorylation of *BAD* (Abramovich *et al.* 2010).

In addition to triggering follicular atresia in response to physiological stimuli, *BAD* may also be involved in follicle depletion caused by environmental pollutants. For example, 4-vinylcyclohexene diepoxide, a by-product in the manufacture of pesticides, flame retardants and plastics, is known to induce the atresia of small preantral follicles, which likely involves *BAD* (Hu *et al.* 2001). Similarly, hexavalent chromium (CrVI), a widely used industrial pollutant, induces apoptosis in cultured granulosa cells and is associated with decreased expression of BCL2, BCL-X_L, HSP70 and HSP90 and increased expression of BAX and BAD (Banu *et al.* 2011).

BCL2 interacting killer

There is little information available about the role of BIK in the ovary. However, studies of male mice deficient in both *BIK* and *BIM* suggest a role in the elimination of supernumerary germ cells. Whereas mice lacking BIK appear to be largely normal, likely due to functional redundancy with other BH3-only proteins (Coultas *et al.* 2004), testes from young males lacking both BIK and BIM have increased numbers of spermatogonia and spermatocytes. These males cannot produce mature sperm due to the inability of sertoli cells to support the excess germ cells (Coultas *et al.* 2005). Female *Bik*^{-/-}*Bim*^{-/-} mice are reported to be fertile (Coultas *et al.* 2005), but their ovarian phenotype has not yet been characterised and such a study may reveal a similar role for BIK, together with BIM, in female germ cell apoptosis.

HRK

HRK is best characterised for its role in neuronal cell death (Imaizumi *et al.* 1997, 1999, 2004, Coultas *et al.* 2007). Indeed, *in situ* hybridisation of mouse embryos and northern blottings of adult rodent tissues suggested that *Hrk* expression is limited to the central and peripheral nervous systems (Imaizumi *et al.* 1997, Coultas *et al.* 2007). However, these studies did not appear to screen for the expression of *HRK* during the latter stages of embryonic ovarian development when large numbers of germ cells are eliminated in response to unknown developmental cues (e.g. E15.5-PN1) or in the adult ovary, leaving open the possibility of a role for HRK in the mediation of apoptosis within the ovary. In this regard, Jurisicova *et al.* (2007) showed that polycyclic aromatic hydrocarbons (PAH), such as those found in cigarette smoke, can induce the expression of *HRK* mRNA and HRK protein in primordial and primary follicles. Furthermore, elimination of *HRK* in

mice conferred protection against PAH-induced primordial and primary follicle depletion (Jurisicova *et al.* 2007). Maternal exposure to cigarette smoke has also been recently shown to upregulate the expression of *HRK* mRNA in the human foetal ovary (Fowler *et al.* 2014). These studies implicate *HRK* in the apoptosis of oocytes and granulosa of very early follicles and should stimulate further investigation of *HRK* and ovarian cell apoptosis, particularly in response to PAH exposure.

Concluding remarks

BH3-only proteins are emerging as important regulators of apoptosis in the ovary during embryonic development and throughout reproductive life. In this regard, roles for individual BH3-only proteins in germ cell, oocyte, granulosa and luteal cell apoptosis have been described. Much of the information accumulated so far has been the result of expression studies and the analysis of gene-targeted mice, in response to both developmental cues and cellular stressors. However, further in-depth analysis of single-gene knockout mice will likely yield additional insight into the regulation of apoptosis in the ovary and targeted conditional knockouts will help to specifically identify roles in the different cellular compartments of the ovary. In addition, the analysis of compound mouse mutants, deficient in more than one BH3-only gene, will undoubtedly lead to the identification and characterisation of additional roles for BH3-only protein family members in the ovary, which may be obscured in single-gene knockouts by functional redundancy. It is also important to continue to verify findings in animal models using human tissue, where possible, in order to gain more understanding as to the involvement of apoptosis in the regulation of oocyte and follicle number and quality in the context of female reproductive longevity and health.

Declaration of interests

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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