The cloning of hBok, Bcl2-L12 and ADAMTS-16 and the functional research into their regulation in physiology of the ovary and other reproductive tissues

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To My Family

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Summary

Ovarian folliculogenesis is a complex process involving the development of a considerable number of small primordial follicles entering a predefined growth pattern finally resulting in the selection of a single, large preovulatory follicle. This complex process encompasses either the death or the successful ovulation of ovarian follicles, involving a multitude of hormonal signalling, proliferation of tissue and cellular apoptosis. Female mammals are endowed at birth with a finite number of primordial follicles, each consisting of an oocyte and a single layer of process of follicular degeneration is called atresia, which has been recognized at the cellular level as apoptosis. It is the ultimate fate of 99.9 % of all 266.000 to 2.000.000 originally available follicles in the ovary to undergo apoptosis. During the years between menarche and menopause only 400 of this entire population of ovarian follicles will achieve ovulation.

During the menstrual cycle until ovulation are well characterized and predominantly regulated by hormones, most notably those secreted by the pituitary. Conversely, a large proportion of the local, intraovarians mechanisms involved in this selection process are largely unknown. It is thought that some of the regulatory mechanisms within the ovarian follicle are modulated by oocyte-centered signalling.

The present series of studies aimed at characterizing the regulation of some novel factors involved in human ovarian folliculogenesis, most notably the Bcl-2 family member proteins, together with specific proteases and endocrine mediators thought to be important regulators of ECM modelling both during ovarian development and ovulation.

The mechanisms involved in atresia in the ovary are still largely unknown, although they are based on cellular apoptosis and are often hormonally regulated. Dysregulation of apoptosis in the ovarian follicle may be responsible for female infertility or associated endocrine disorders. Furthermore, the study of ovarian follicular apoptosis may give clues to the pathogenesis of related diseases such as ovarian or breast cancer.

From EST of NCBI GenBank derived from non-normalized human ovarian cDNA libraries, such as the Stanford microarray database, with the TBLASTN program by searching the GenBank and EST database, we have cloned and identified two novel human Bcl-2-related genes, the hBok gene (Bcl2-related ovarian killer or Bcl2-L9 gene) and the Bcl2-L12 gene. We were able to define the function of their gene products and to elucidate their roles both in folliculogenesis and in cancer. To further explore the functions of these two genes we cloned 7 Hbok deletion mutants and 5 Bcl2-L12 deletion mutants according to their structural domains.

In addition, during search of the ovary-specific gene expression database and trying to find out novel markers of granulosa cell function, we identified a new member of the family of proteases ADAMTS-16 (a disintegrin-like and metalloproteinase with thrombospondin type I motifs): This group of proteases is thought to be important in ECM remodelling during ovarian development and ovulation. We also identified a splicing variant of ADAMTS-16, which was termed ADAMTS-16s.

The following three conclusions can be drawn from our research work:

a) The hBok protein contains all four Bcl-2 like domains (BH1, 2, 3 and 4) and is a pro-apoptotic Bcl-2 protein identified in the ovary. By fluorescence *in situ* hybridization (FISH) and *in silico* analysis, hBok was found to be located on chromosome 2q37.3. Its expression was detected in various organs and in several hormonally regulated cancer cells. Expression of hBok was shown to be upregulated in estrogen-dependent breast cancer by estrogen deprivation and in myocardial cells during hypoxy. Confocal laser scanning microscopy examinations and subcellular fractionation studies showed that hBok was distributed both in cytosol and in membrane in healthy cells. Upon overexpression of hBok or stimulation of apoptosis, hBok became mainly associated with the intracellular membrane. Furthermore oligomerization were promoted by BH3-only proteins, such as Bid, Bnip3 and p53 but prevented by BFL-1. hBok was found to interact with Bnip3. Our findings suggest that functional BH3-only proteins facilite the oligomerization and insertion of the hBok into the membrane.

b) We cloned another apoptotic-related gene which is found highly expressed in breast, placenta, and known as Bcl-2 related proline-rich protein - Bcl2-L12. We found that is localized in the nucleus and that it posses an anti-apoptotic function. As evidenced by its structure. It can increase in the S/G2 phase of the cell cycle and enhance DNA replication. We analysed the Bcl2-L12 gene by cloning 5 different deletion mutations according to its structure domain, including the full length form (828bp), the deleted N-terminal (BH2-only), the deleted one coiled-coil domain (12cc) which contained 5 special proline-rich motifs (PXXP) without the BH2 and (PPPP) domains. The transfection of different deleted mutants into cells showed that the Bcl2-L12 deleted

BH2 domain possesses signalling activity outside the nucleus, indicating that believed the BH2 domain has a functional role for the nuclear localization of Bcl2-L12. Cell cycle analysis from FACS for Bcl2-L12 and its deletion mutants further confirmed that Bcl2-L12 has an anti-apoptotic effect in the MG 132 induced cell apoptosis but not in the STS induced apoptotic pathway. Each of the 5 mutants has their own function in modifying the cell cycle. Further results showed that the nuclear signalling would migrate out off the nucleus and and become fixed in the nuclear membrane or in mitochondria or in other organelles. We confirmed that Bcl2-L12 is regulated by many other Bcl2 apoptotic family members and that the cell cycle also changed after co-transfection with these Bcl2 family genes, such as with Bax, Bid, tbid, hBok, Bcl2, $Bcl2_{XL}$, Furthermore, we found that Bcl2-L12 is regulated by some circadian rhythm genes such as clock, Bmall and Rev-erb.

c) We further characterized full length ADAMTS-16, a novel member of the disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family together with its splicing variant, ADAMTS-16s, the latter containing the metallopeptidase domain only. ADAMTS-16 is highly expressed in the kidney and in the ovary, where it is predominantly expressed in luteinizing granulosa cells but only little in cumulus oophorus. Expression in several cancer tissues was also detected. In fully differentiated luteinizing granulosa cells, FSH and forskolin induced expression of ADAMTS-16, suggesting that it is regulated via cAMP pathway. LH did not have an effect on the expression of ADAMTS-16. ADAMTS-16 is capable of cleaving α_2-macroglobulin, a widely used substrate for proteases. These studies provide the first evidence that ADAMTS-16 is an active protease of α_2-macroglobulin, which is

present in high concentrations in mature ovarian follicles and which is known to participate both in estradiol production and in the formation of the perifollicular vascularization. The FSH dependency of ADAMTS-16 expression in granulosa cells and its protease activity on α_2-macroglobulin suggest a role of ADAMTS-16 in modulating the maturation of ovarian follicles during the late stages of their development.

1. Introduction

1. 1. The development of the oocyte

Throughout the fertile life span, primordial follicles continuously grow into primary follicles, preantral and antral follicles. Only antral follicles are capable of releasing oocytes at a stage when the oocyte can be fertilized (Tomic et al., 2004). Thus, the continuous growth of primordial follicles to the antral stage is essential for female fertility. When primordial follicles enter their growth phase, granulosa cells begin to divide, the oocyte enlarges and becomes surrounded by a zona pellucida. Gradually the follicles become secondary follicles and, when fibroblast cells in the inner thecal layer differentiate, the secondary follicle is defined as a preantral follicle (Nussey et al., 2001). The early growth phase is considered to be independent of gonadotropin stimulation despite some evidence about the presence of FSH receptors in these immature follicles. It is not known, why a few primordial follicles begin to grow, nor how they are selected, but paracrine factors within the ovary such as cytokines and epidermal growth factor may be involved. In the early luteal phase of each menstrual cycle, cohorts of preantral follicles undergo further growth into antral follicles (Nussey et al., 2001). At this time, the follicles enlarge, the thecal cells become richly supplied with blood vessels and a fluid-filled cavity (the antrum) forms. The oocyte itself becomes surrounded by several layers of granulosa cells known as the cumulus oophorus. During the basal growth phase follicle diameter increases from about 0.2 mm to 2 mm. By the late luteal phase of the third cycle those follicles that have not degenerated have reached the selectable phase (alternatively denominated recruitment phase). Over a period of about 5

days (termed the selection window) the follicles continue to grow but only one is selected to undergo final maturation. The mechanisms responsible for the selection of the dominant follicle are not well understood but may be determined by the number of FSH receptors or the concentrations of steroids or growth factors within the follicle. Over 15 days, the dominant follicle increases in diameter from 5 mm to around 20 mm, secretes increasing concentrations of estradiol and becomes a fully mature Graafian follicle (Nussey et al., 2001). Ovulation occurs in the third cycle (Figure1).

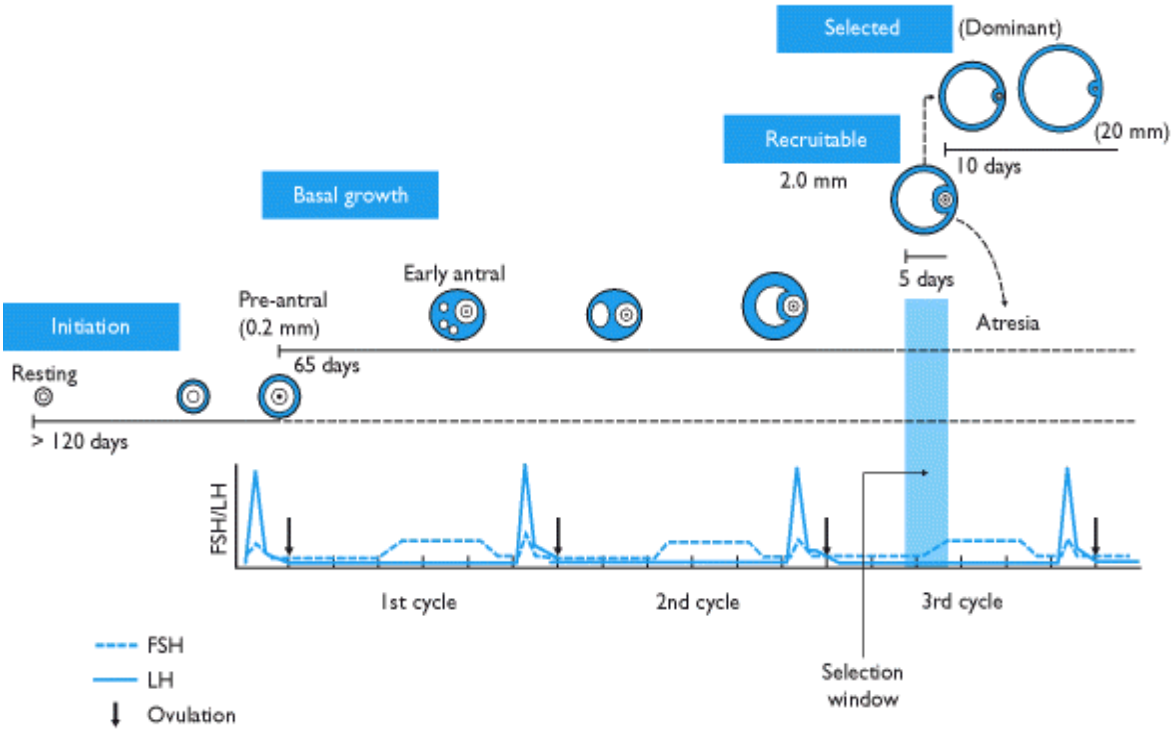


Figure 1: Ovarian control and the menstrual cycle: The development of a primordial follicle to a preovulatory follicle lasts approximately 120 days. After it has become a preantral follicle with a diameter of 0.2 mm, approximately 65 days are needed for its development to a preovulatory follicle. Cohorts of follicles continually develop but ultimately only one is 'selected' and becomes the dominant follicle. All the others undergo atresia (Nussey et al., 2001)

The LH surge, which immediately precedes ovulation, induces the oocyte to complete its first meiotic division producing two haploid daughter cells containing 23 chromosomes each. One cell, however, retains most of the cytoplasm and the smaller one (the first polar body) sits cramped in a small space, the vitelline space, between the secondary oocyte and the zona pellucida. The process of follicular growth is controlled by both extraovarian and intraovarian factors, and the importance of each of these factors depends on the developmental stage of the follicle (Tomic et al., 2004). Extra-ovarian factors regulate antral and preovulatory follicle growth, whereas intraovarian factors regulate preantral and early antral growth (Tomic et al., 2004). During follicular development, gonadotropins, together with local ovarian growth factors (IGF-I, EGF/TGF-α, basic FGF) and cytokine (interleukin-1β), as well as estrogens, activate different intracellular pathways to rescue follicles from apoptotic demise. In contrast, TNF-α, Fas ligand, presumably acting through receptors with a death domain, and androgens are atretogenic factors. These diverse hormonal signals probably converge on selective intracellular pathways (including genes of the bcl-2 and ICE families) to regulate apoptosis (Kaipia et al., 1997).

1. 2. Apoptosis and follicular development

Apoptosis, also known as programmed cell death, is a form of cellular demise, in which the cells activate an intracellular death programme and kill themselves in a controlled fashion. Apoptosis is intrinsic component of embryonic development, metamorphosis, tissue renewal, hormone-induced tissue atrophy and many pathological conditions, not the least of

them also cancer. In multicellular organisms, apoptosis ensures the elimination of superfluous cells, cells that have already completed their specific function or that are harmful to the organism. In reproductive tissues that are characterized by cyclic functional changes, such as the ovarian follicle of the endometrium, massive cell death occurs under the control of hormonal signals. A growing body of evidence suggests that the intracellular "death program" activated during apoptosis is similar in different cell types and has been conserved during evolution (Thompson et al., 1995; Steller et al., 1995).

During apoptosis, the cells shrink and exhibit several typical features, including cell membrane disruption, cytoskeletal rearrangement, nuclear condensation, and internucleosomal DNA fragmentation (Kaufmann et al., 2000) and form apoptotic bodies. Although the final step (i.e. DNA degradation and cellular fragmentation) is common to all apoptotic pathways, the activation of this death process is initiated either by changes of endogenous signals (e.g., hormones and growth/survival factors) or through damage to the cell induced by various exogenous agents (e.g. radiation, chemicals, viruses). Growth factors may actively suppress a default death pathway (Gross et al., 1999).

The **mitochondrial pathway** of caspase activation involves mitochondrial events such as membrane permeability transition (PT), with results in the release of mitochondrial proteins such as cytochrome *c* into the cytoplasm (Figure 2) (Kaufmann et al., 2001; Reed et al., 1997). When released into the cytoplasm, cytochrome *c* binds to Apaf-1 (apoptotic protease activating factor-1), resulting in the assembly of a high molecular-mass complexes called apoptosomes, which contain cytochrome *c*, Apaf-1, and procaspase-9 (Kaufmann et al., 2001). The interaction between Apaf-1 and procaspase-9 leads to the formation of an active

caspase-9 which, in turn, proteolytically activates caspase-3. In the death receptor-initiated pathway of the type II cells, the release of cytochrome *c* from the mitochondria results from caspase-8-mediated cleavage of the cytoplasmic Bcl-2-family member Bid. Bid can also be activated proteolytically by the cytotoxic lymphocyte protease granzyme B and by certain lysosomal cathepsins (Kaufmann et al., 2001). Importantly, various stimuli can induce PT and thus the release of cytochrome *c* independently of any caspase-8 activation (Kroemer et al., 2000).

The mitochondrial death pathway *(right*), which is triggered by many apoptotic stimuli and proapoptotic Bcl-2 members, involves the release of cytochrome *c* into the cytosol. This event can be triggered by anticancer drug or γ-irradiation induced, environmental stresses, such as heat, UV-light, or dedicated death receptors (e.g., FAS/ APO-1 and tumour necrosis factor TNF receptors), may inhibit cell growth and trigger apoptotic cell death (Johnson et al., 1996).

The **death receptor pathway** is initiated upon receptor ligation, resulting in the recruitment of the adapter protein FADD through interaction between the death domains of both proteins. The death effector domain of FADD in turn recruits procaspase-8 which undergoes autoproteolytic activation at the receptor complex.

Female mammals are endowed at birth with a finite number of primordial follicles, each consisting of an oocyte and a single layer of somatic (granulosa) cells (Hodgen et al., 1986). These follicles remain in a resting stage (prophase I) until their final development to an ovulating follicle after puberty or their degeneration. The process of follicular degeneration is called atresia, which has been recognized at cellular level as apoptosis (Tilly et al., 1992).

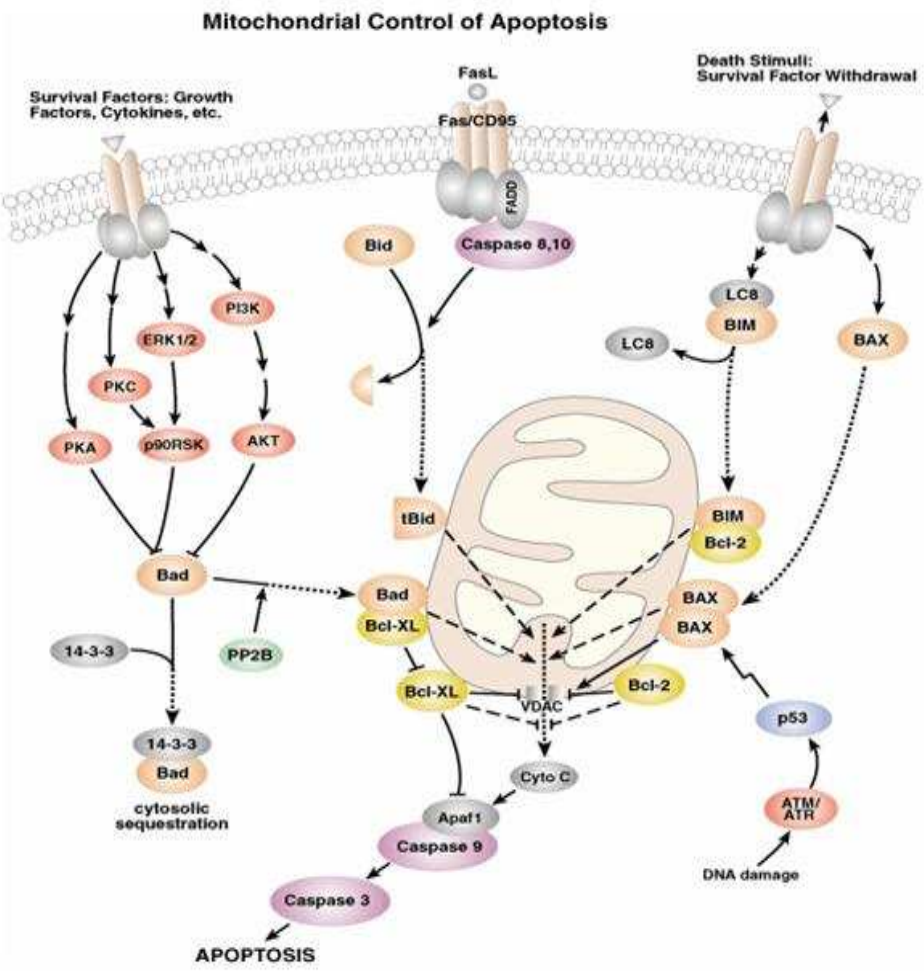


Figure 2: The mitochondrial pathway of apoptosis (Sigma)

It is the ultimate fate of 99.9% of all 266.000 to 2.000.000 originally pooled follicles to undergo atresia. In the years between the menarche and menopause only approximately 400 follicles reach ovulation (Tilly et al., 1998). The relationship between the apoptosis of granulosa cells and follicle atresia in the human was first demonstrated by Zeleznik AJ (Zeleznik et al., 1986) and by Hughes & Gorospe (Hughes et al., 1991). The factors regulating the recruitment and the development of a dominant ovarian follicle during the

menstrual cycle until ovulation are well characterized. These processes are predominantly regulated by hormons, most notably those secreted by the pituitary, but how the follicle atresia happened and which factors is involved in this process is still a puzzle.

1.3. Ovarian follicle apoptotic cell death (follicle atresia) and Bcl2 Family members

The majority of ovarian follicles undergo atresia is mediated by apoptosis. Bcl-2-related proteins act as regulators of apoptosis via the formation of dimers with proteins inside and outside the Bcl-2 family. Members of the Bcl-2 family serve as a central checkpoint for cell death regulation in the ovary. Overexpression of Bcl-2 in transgenic mice led to the suppression of follicle cell apoptosis and subsequent formation of teratoma of germ cell origin (Hsu et al., 1998), whereas deletion of the pro-apoptotic Bax gene resulted in the accumulation of apoptotic follicular cells (Knudson et al., 1995). These data suggest that the Bcl-2 family proteins have important roles in the regulation of follicular atresia. It is likely that selective pairs of Bcl-2 agonists/antagonists may play tissue-specific roles in the regulation of apoptosis. Indeed, mutant mice deficient in Bcl-2 or Bax showed abnormality in apoptosis regulation only in distinct cell lineage (Hsu et al., 1997; Fulton et al., 2005). Although Bax-deficient mice showed an accumulation of granulosa cells in atretic follicles, these cells were still apoptotic (Hsu et al., 1997), suggesting the involvement of additional pro-apoptotic factors during ovarian follicle atresia. Because the pro-apoptotic protein Bax has been suggested to function as a tumor suppressor gene in colon adenocarcinomas (Hsu et al., 1997; Rampino et al., 1997) and because inactivation of Bax in transgenic mice leads

To enhanced tumorigenesis (Yin et al., 1997), further study of the pathways of apoptosis in the gonads has the potential to provide clues for both gonadal and uterine tumorigenesis. Therefore, regulation of Bcl-2-related genes expression is important step in mediating apoptosis. The expression of the Bcl-2, bcl-x and bax genes in the rodent ovary relative to the absence or presence of follicular atresia, documented a strong correlation between elevated levels of RNA encoding the Bax death-promoting protein and apoptosis in granulosa cells both in vivo and in vitro (Knudson. et al., 1995; Johnson et al., 1996) any changes in Bcl-2 and Bcl-x mRNA levels were observed, suggesting that a shift in the balance of Bcl-2-related death-repressors to death-inducers was accomplished primarily via alterations in bax expression. The increased expression of Bax in granulosa cells, at both the mRNA levels and protein levels, correlates well with the induction of atresia, and that the protein encoded by bax gene may actually be required for normal death of granulosa cells in follicles committed to this degenerative pathway (Eskes et al., 2000). Although in this same study expression of both the long (death-repressing) and short (death-inducing) forms of bcl-x mRNA were also detected in human granulosa-lutein cells (Kugu et al., 1998; Johnson et al., 1996), the role(s) played by these and other members of the Bcl-2 gene family in human follicular atresia awaits more in-depth analysis. The increased expression of Bcl-x-long has been linked to the apoptosis resistant phenotype observed in granulosa cells of avian follicles recruited into the rapidly growing hierarchy that are no longer prone to atresia (Johnson et al.,1996). A growing number of reports have implicated this anti-death factor and other related members of this gene family. BOD (Bcl-2-related ovarian death agonist) is isolated from an ovarian fusion cDNA library. BOD mRNA is found ubiquitously expressed in the ovary and in multiple other tissues (Hsu et al., 1998). The BOD gene is also

conserved in diverse mammalian species. It is also known that BAD overexpression in granulosa cells leads to apoptosis (Kaipia et al., 1997). It is known that phosphorylation of BAD induced by survival factors leads to its preferential binding to 14-3-3 and suppression of the death-inducing function of BAD. P11, a member of the S100 family of calcium-binding proteins, has been shown to interact with BAD (Hsueh et al., 2001), another member of the Bcl-2-family of proteins. Bok and Bcl2-L12 are likely to be major players in the regulation of apoptosis both in the gonads and in the uterus, as suggested by its restricted expression in these organs.

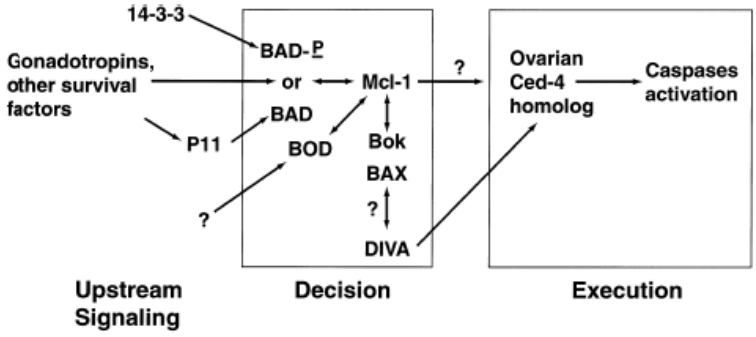


Figure 3: Three stages of some Bcl2 genes involved in ovarian cell apoptosis are depicted. Taking advantage of the dimerization property of Bcl-2-related proteins, Bcl-2 was used as bait to search for endogenous Bcl-2-interacting preys in the yeast 2-hybrid system. After characterization of each prey, new bait was chosen and, in turn, used for a subsequent round of screens. Repeating this procedure multiple times allowed the identification of BAD as an important Bcl-2 member in the ovary that bridges the upstream signalling proteins, 14–3-3 and P11, to the Bcl-2 family of proteins. In addition, the antiapoptotic Mcl-1 was found to be the likely mammalian ced-9 homolog in the ovary and Mcl-1 dimerized with Bax and two novel ovarian proapoptotic Bcl-2 members, Bok and BOD, in the decision step of apoptosis. The antiapoptotic protein, Mcl-1 or the recently discovered DIVA/Boo, may interact with downstream ovarian ced-4/Apaf-1 homolog which, in turn, activates caspases in the execution step of apoptosis (Hsu et al., 2000).

1.4. The potential role of hBok and Bcl2-L12 in reproductive tissues and in the treatment of cancer

The Bcl-2 family proteins are crucial regulators of apoptosis, in which they exert direct control through the release of apoptogenic factors, such as cytochrome *c*, to activate the proteolytic caspase cascade (Green et al., 2004). In mammalian cells at least 20 different Bcl-2-related proteins have already been identified (Cory et al., 2002; Gross et al., 1999). Based on functional studies and the retention of BH (e.g. "Bcl-2 homology") domains, the Bcl-2 family are divided into three subgroups: (Fig. 4):

1. The Bcl-2 subgroup including all anti-apoptotic proteins, such as Bcl-2, Bcl-xL, A1/Bfl-1 and Mcl-1.
2. The Bax subgroup consisting of multi-BH domain pro-apoptosis members, such as Bax, Bak, Bok and Bcl-rambo (Hsu et al.,1997; Zhang et al.,2000; Kataoka et al., 2001). Both groups contain more than one BH domain.
3. The third subgroup containing BH3-only proteins, such Bid and Bim, which can interact with either anti-apoptotic proteins or pro-apoptosis members and promotes apoptosis (Hsu et al., 1997).

Full complement of BH domains may not be necessary to confer apoptotic or anti-apoptotic activity. And hBok presence of all of BH1, BH2, BH3 and BH4 domains and a TM domain.

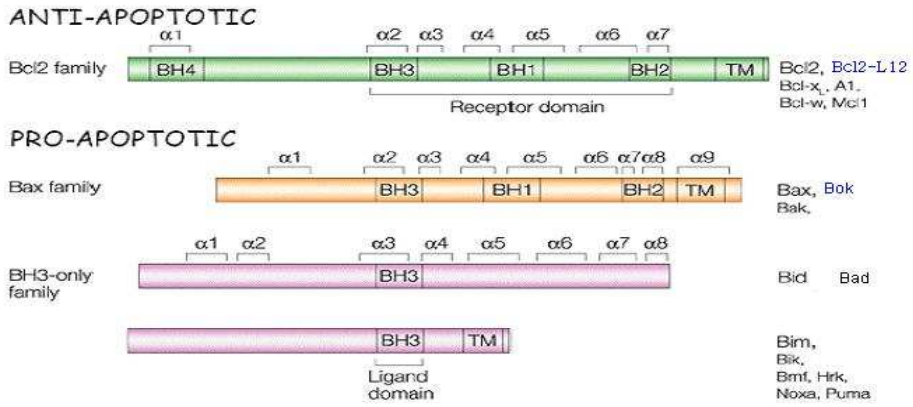


Figure 4: Bcl-2 homology (e.g. BH) domains

It has been demonstrated that BH3-only proteins are crucial in facilitating oligomerization of Bax and Bak and pore-formation in the mitochondrial membrane allowing the release of cytochrome *c* (Kuwana et al., 2002; Antonsson et al., 2001; Roucou et al., 2002). It has also been reported that Bax kills cells by a caspase-independent mechanism (Knudson et al., 1997). Recent studies have also shown that in some tissues the activation of Bak requires Bax (Lindsten et al., 2000). Knockout studies in mice revealed that inactivation of either Bax or Bak alone has little consequence, but that elimination of both genes dramatically impairs developmental apoptosis in many tissues (Mikhailov et al., 2003). These results suggest that the regulation of Bax and Bak interaction may not be simply redundant (Gillissen et al., 2003). Modulating the activities of Bax subgroups are of considerable importance for the control of apoptosis, which may be induced by more than one mechanism

(Xiang et al., 1996). Biochemical studies revealed that oligomerization of both Bax and Bak could be stimulated by p53, although the latter can only interact with Bak but not with Bax. Therefore, additional pathways and interactions must be at work too.The mechanisms of action of other multi-domain proteins, such as Bok, and their contribution to developmental susceptibility of apoptosis have not yet been established.

From a rat ovarian fusion cDNA library, a new pro-apoptotic Bcl-2 gene, Bcl-2-related ovarian killer (Bok) was first cloned by yeast two-hybrid screen (Hsu et al., 1997). The mouse homolog (*Mtd*) and was identified bioinformatically later (Inohara et al., 1998). Unlike other channel-forming proapoptotic Bcl-2 proteins including Bax and Bak, Bok preferentially dimerizes with antiapoptotic proteins Mcl-1, Bfl-1, and BHRF1 but does not interact with Bcl-2, Bcl-w, and Bcl-xL. Although low levels of mRNA for Bok, also named as Mtd, are present in diverse tissues (Inohara, N. et al., 1998), it is expressed at high levels in several reproductive organs, including ovary, testis, and uterus (Hsu et al., 1997). The distinct heterodimerization property and tissue distribution pattern of Bok suggested that it is a tissue-specific mediator of cell death and could promote apoptosis by interacting with selective antiapoptotic protein. This notion is consistent with the hypothesis that apoptosis is regulated through the dimerization of Bcl-2 family proteins in a tissue-specific manner. The human Bok gene (hBok) was identified in human granulosa cells in our research laboratory (Gynecological Endocrinology, Department of Research) from a human infant brain library (GenBank accession number AF174487) (Zhang et al., 2000). We found that it is more widely expressed in other tissues than the rat Bok (Zhang et al., 2000). However, the function and regulation of this gene was not yet researched in detail.

Members of the Bcl-2 family of apoptosis-regulating proteins contain at least one of the four evolutionarily conserved domains, termed BH1, BH2, BH3, or BH4. The BH1 and BH2 domains are present in all antiapoptotic proteins, while the BH3 domain is present in the pro-apoptotic members of the family. The BH4 domain appears to be present in the N-terminal domain of the anti-apoptotic Bcl-2, Bcl2-XL, and Bcl2-w proteins, and its function is not clear as yet, Bcl2-L12 is a Bcl-2 related proline-rich protein. This new gene has been reported previously to be mapped on the chromosome 19q13.3 and is located between the IRF3 and the PRMT1/HRMT1L2 genes, close to the RRAS gene. Bcl2-L12 is composed of seven coding exons and six intervening introns, spanning a genomic area of 8.8 kb. The Bcl2-L12 protein (Scorilas et al., 2000) is composed of 334 amino acids and has a calculated molecular mass of 36.8 kDa. The Bcl2-L12 protein contains one BH2 homology domain, one proline-rich region similar to the TC21 protein and five consensus PXXP tetra-peptide sequences (Scorilas et al., 2000). Bcl2-L12 is reported to be expressed mainly in the breast, thymus, prostate, fetal liver, colon, placenta, pancreas, small intestine, spinal cord, kidney, and bone marrow (Scorilas et al., 2000). It was found that Bcl2-L12 is overactive in a majority of glioblastoma multiform (GBM) tumors (Chin, unpublished data). Analysis of the Bcl2-L12 gene expression in breast cancer confirmed an association of Bcl2-L12 with a more favorable prognosis of breast cancer (Mathioudaki et al., 2004). It has also been reported that Bcl2-L12 is overexpressed in the breast cancer (Mathioudaki et al., 2004), but also in benign breast tissue (Scorilas et al., 2001). Overexpression of various anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, has been implicated in cancer chemoresistance, whereas high levels of pro-apoptotic proteins such as Bax promote apoptosis and sensitize tumor cells to various anticancer therapies.

1.5. Human ADAMTS-16 and ECM

The ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are a group of proteases that are found both in mammals and invertebrates. Since the prototype ADAMTS-1 was first described in 1997, there has been a rapidly expanding body of literature describing this gene family and the proteins they encode. It is known that ADAMTS-1 together with ADAMTS-4 and ADAMTS-5 has been shown to degrade members of the lectican family of proteoglycans. ADAMTS-1 mRNA is induced in granulosa cells of periovulatory follicles by the luteinizing hormone surge through a progesterone receptor-dependent mechanism. Female progesterone receptor knockout (PRKO) mice are infertile primarily due to ovulatory failure and they lack the normal periovulatory induction of ADAMTS-1 mRNA (Russell et al., 2003).

The complete human family has 20 ADAMTS genes, together with three members of a newly identified subgroup, the ADAMTSL (ADAMTS-like) proteins, which share several domains with the ADAMTSs. The ADAMTSs are extracellular, multidomain enzymes whose known functions include:

1) collagen processing as procollagen N-proteinase
2) cleavage of the matrix proteoglycans aggrecan, versican and brevican;
3) inhibition of angiogenesis
4) blood coagulation homoeostasis (protease of the von Willebrand factor)
5) organogenesis, inflammation and fertility are also apparent.

Recently, some ADAMTS genes have been found to show altered expression in arthritis and various cancers, All of them show a domain organization similar to that of previously characterized family members, consisting of a signal sequence, a propeptide, a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich region, and a variable number of TS-1 repeats.

ADAMTS proteases are modular, consisting of a protease domain and an ancillary domain (Apte et al., 2004). The protease domain of these enzymes, like that of ADAMs, but not MMPs, is of the reprolysin (snake venom) type. The hallmark of the ADAMTS proteases is the presence of at least one thrombospondin type 1 repeat (TSR). Other highly conserved modules are arranged around this central TSR in a specific organization, and there are additional TSRs near the carboxyl terminus in all members of the ADAMTS family with the exception of ADAMTS4 (Apte et al., 2004, Figure 1). ADAMTS proteases are synthesized as zymogens that are targeted to the secretory pathway and activated by protein convertases. Zymogen processing leads to removal of a 200–220-amino acid-long prodomain in the secretory pathway or at the cell surface.

Phylogenetic analysis of the ADAMTS suggests that the family members can be categorised into seven subgroups based on sequence similarities and, where known, their targeted substrates (Sarah P. et al., 2005). These seven subgroups are: a) ADAMTS-1, -4, -5, -8 and -15; b) ADAMTS-2, -3 and -14; c) ADAMTS-9 and -20; d) ADAMTS-7 and -12, plus ADAMTS-6 and -10; e) ADAMTS-16 and -18; f) ADAMTS-17 and -19; and g) ADAMTS-13, which is least like any other ADAMTS (Figure 5).

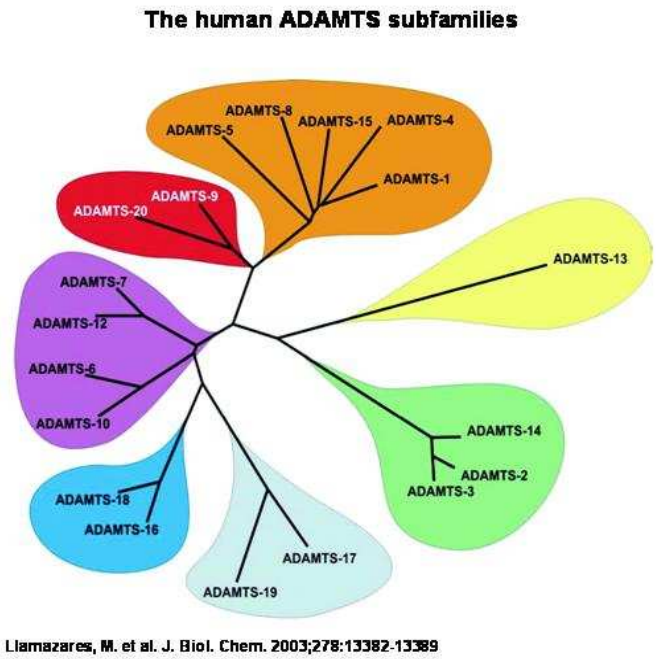


Figure 5: The seven subgroups of various members of the ADAMTS family

These dual proteins, with both protease and cell adhesion domains, were initially associated with reproductive processes like spermatogenesis and sperm-egg binding and fusion (Wolfsberg et al., 1996). However, in the last few years, these proteins have been implicated in other biological processes such as modulation of cell migration (Alfandari et al., 2001), differentiation of osteoblastic cells (Inoue et al., 1998), stimulation of adhesive properties of tumor cells (Cal et al., 2000) or activation of signalling pathways by shedding of membrane-bound cytokines and growth factors (Black et al., 1997). Thus, ADAM-17 or TACE is involved in the release of the cytokine tumor necrosis factor-α (TNF-α) from the cell membrane. To date, at least 11 different ADAMTSs have been identified in human tissues (Cal et al., 2001) and only in few of them a functional role in normal or pathological

processes has been described. Thus, ADAMTS-1 (also called METH-1) and ADAMTS-8 (METH-2) have angio-inhibitory activities (Vazquez et al., 1999). ADAMTS-2 and ADAMTS-3 are procollagen N-propeptidases (Colige et al., 1995; Fernandes et al., 2001), and deficiency of ADAMTS-2 causes the Ehlers–Danlos syndrome VIIC in humans (Colige et al., 1999).

ADAMTS-4 and ADAMTS-5/11 are aggrecanases involved in cartilage aggrecan degradation in arthritic diseases (Tortorella et al., 1999; Abbaszade et al., 1999). ADAMTS-4 was also found to be responsible for brevican degradation in glioma cells, which is a critical step in the invasive properties of these tumors (Nakamura et al., 2000). It has been also shown that ADAMTS-1 and ADAMTS-4 are able to catalyze the hydrolysis of versican in the human aorta (Sandy et al., 2001). Finally, ADAMTS-6, ADAMTS-7 (Hurskainen et al., 1999), ADAMTS-9 (Clark et al., 1999), ADAMTS-10 (accession number AF163762), and ADAMTS-12 (Cal et al., 2001) have been only characterized at the structural level and their putative roles remain unknown, although some of them are overexpressed in various tumors.

Additionally, several members of the ADAMTS family play important roles in ovarian physiology, which is the main focus of our research group. It has been demonstrated that ADAMTS-1 mRNA and protein were induced in granulosa cells of periovulatory follicles by the luteinizing hormone in wild-type but not in the progesterone-receptor knockout mice (PRKO), indicating that ADAMTS-1 is a transcriptional target of the progesterone receptor around ovulation (Robker et al., 2000; Doyle et al., 2004). Studies in ADAMTS-1 (-/-) knockout mice demonstrated that the number of ovulated oocytes is significantly reduced as

compared with heterozygous controls (Shindo et al., 2000; Shozu et al., 2005). The presence of ovulations in ADAMTS-1 null mice, albeit significantly reduced, and the involvement of other members of the ADAMTS family of proteases in the ovulatory process demonstrate a large degree of redundancy among the various members of this family (Richards et al., 2005). Two proteases both closely structurally and functionally related to ADAMTS-1, ADAMTS-4 and ADAMTS-5, are present in the granulosa cells of small ovarian follicles, indicating that various members of the ADAMTS family also have functions beyond ovulation and the formation of the luteal body (Richards et al., 2005).

It is reported that ADAMTS-16 gene expression in cartilage and synovium and upregulation in osteoarthritis (OA) but the function of these enzymes is as yet unknown (Kevorkian et al., 2004). Searching for novel markers of granulosa cell function, based on its high expression level in the ovary we identified ADAMTS-16 during the search of our ovary-specific gene expression database. In this report, we carried out the first investigation on regulation and function of ADAMTS-16 in the ovary.

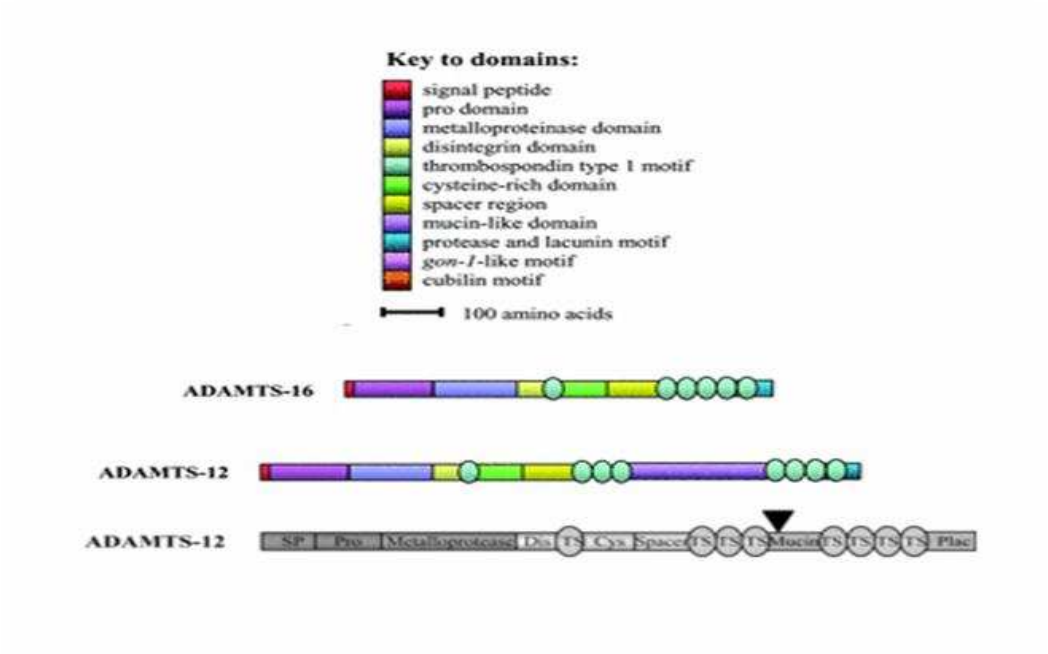


Fig. 6: Domain structure of the ADAMTS-16 protein (TS: thrombospondin-like)

2. The aims of these studies

For decades, the mechanisms responsible for germ cell depletion from the ovary, either directly during the prenatal period or indirectly via follicular atresia during postnatal life, have remained relatively obscure. It has been shown that these proces involve the intrinsic apoptosis pathway known from other organ systems. Conversely, the ovary physiology may also serve as a valuable model for studying the regulation of cell death through the action of diverse extracellular and intracellular signaling mechanisms. One of the projects in our research laboratory is focused on functional studies of the Bcl2 family proteins which are the most prominent regulators of apoptosis. Understanding the regulation of apoptosis in the ovary may provide an additional therapeutic approach in infertile women.

My current studies were designed to characterize Bcl2-L9 (hBok) and Bcl2-L12. In addition, I have also studied the function of a novel member of ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like repeats), namely ADAMTS- 16, which is highly expressed in ovary.

3. Materials and methods

3.1. Cloning of hBok and Bcl2-L12 and their mutants´ plasmid constructions

Using the human Bcl-2 sequence to perform TBLASTN searches, a fragment of HBok was found in the human infant brain library (GenBank accession number AF174487). By search of the expressed sequence tags (EST) database/Drosophila genome sequence database using the amino acid sequences of HBcl-xL and HBok, DNA was prepared from a cDNA library, (EST 30920). An EST clone (IMAGE 32090) was also obtained from the collection of the IMAGE Consortium and amplified by PCR using HBok-specific primers. Wild-type Bcl2-L12 was cloned with PCR using the following primers:

5’CCGCTCGAGGAGTTCAGTGGAGGAGACCGCAAG-3’

5’-CCCAAGCTTGCTCAGTCCAATGGCAAGTTC-3’.

cDNA IRALp96200217 was provided by rzpd GmbH (Germany). The full length Bcl2-L12 contains 828 bp. Using pLEGFP-828bp, we further subcloned 4 deletion mutants: (1) the deleted coil–coil domain (121 bp), (2) the deleted BH2, (3) the BH2-only domain and (4) the mutated PPPP domain, which is a Src binding domain.

PCR was performed for 35 cycles at 94 °C for 45 sec, at 55 °C for 1 min and at 72 °C for 1 min. Amplified fragments were run on a 1 % agarose gel. The expected band was cut under the UV-light, subsequently purified with the QIAquick Gel Extraction kit (QIAGEN) and digested with Enzyme equal to vector. We ligated the vector and its fragment with Rapid DNA ligation kit for 5-10 min (Roche), transformed to DH5a or top 10 or HB101 competent cells. The cells were grown on an Ampicillin or a Kanamycin plate for 12-16 hours at 37 °C.

10 to 16 colonies were picked up for Plasmid amplification with corresponding Ampecilin or Kanemycin-enriched LM medium. The amplified plasmid was then purified by using QIAprep spin minprep kit (QIAGEN) and then cut with the original enzyme to digest the vector and its fragment. In order to select positive clones the material was again run on a gel for plasmid amplification in order to obtain about 100-200 µg of DNA, which was then confirmed by sequencing.

Pblue-script-k-Kiaa2029 (ADAMTS-16) was kindly provided by Kazusa DNA Research Institute (Kikuno et al., 2004). Full-length was cloned in-frame into pGEX-4T-2 Vector with Sal I /Not I site. ADAMTS-16s was generated by PCR using were cloned by using the primer 5‘-CGTGGATCCCATTTCTGTGGAAGACGC-3‘ and 5‘-CGGGAATTCGCCACTTGCACT GTG TGT TT –3‘ and the digested the PCR product with BamHI and EcoRI, cloned in-frame into the EcoRI/BamH I sites of pGEX-4T-1 and pcDNA3-HisC (Invitrogen, Groningen, The Netherlands). All of these constructs were confirmed by sequencing.

3.2. Chromosomal localization of hBok as demonstrated by PCR amplification and partial sequencing

The molecular cloning of hBok was described previously with the GenBank accession numbers AF174487 (Zhang et al. 2000). A BAC was obtained by PCR screening of P1 Human Libraries with primers 5’-CCTCGCGGGTCTGAATGGAAGG-3’ and 5’-CGAGCGGTCAAAGGCGTCCAT-3’ and sequenced partially. On one hand, fluorescence

in situ hybridization (FISH) was performed on human metaphase chromosomes. The hBok BAC DNA probe was labelled with biotin-16-dUTP using a nick translation kit (Roche) and ethanol-precipitated with salmon sperm DNA and *E. coli* tRNA. Hybridization and detection of fluorescence signals were performed according to (Matsuda et al., 1992; Matsuda & Chapman., 1995). Fluorescence images were observed by an Olympus BX-60 epifluorescence microscope with Olympus filter (Olympus Optical Co. Ltd., Tokyo, Japan) set U-MWIB (excitation at 460-490 nm), U-MSWG (480-550 nm) and U-MWU (330-385 nm), and photographed with Kodak Ektachrome ISO 100 films (Eastman Kodak Co., Rochester, NY, U.S.A.). On the other hand, this gene is located on human chromosome 2q37.2 by using the Human Genome Browser programme created by Jim Kent at UCSC, U.S.A. Polymerase chain reaction was used to determine the size of the introns.

OR0021 was identified based on its high level of expression in the ovary in our ovarian-rich gene expression database and OR0021 was found to be identical to ADAMTS-16 (Cal et al., 2002). Searching the GenBank database of human expressed sequence tags (ESTs) with the ADAMTS-16 cDNA sequence, an another EST sequence (GenBank accession no. AK122980) was detected and sequenced. This gene was named ADAMTS-16s. The search of the Genbank database for STSs using ADAMTS-16 as a template revealed that a human STS WI-6822 (accession no. G06277) matches 100 % to the nucleotides +941 to +1229 of the ADAMTS-16 sequence. ADAMTS-16 was found to be located on chromosome 5 by using the NCBI Unigen programme. The GenBank accession numbers for ADAMTS-16s is DQ266047.

3.3. Tissue expression pattern analysis by RT-PCR

In order to determine the expression patterns of hBok mRNA in different human tissues, PCR was performed, using the primers 5'-GCAAATGGCTCTTCCTTGAG-3' and 5'-AGCAGCACATGAAGTTGTGG-3' on a panel of first-strand cDNAs from various human tissues (Origene, Maryland, U.S.A.). Bcl2-L12 and ACTIN gene sequences, two pairs of gene-specific primers were designed: GGA GAC CGC AAG TTG AGT GG forward and GTC ATC CCG GCT ACA GAA CA reverse for Bcl2-L12; ATC TGG CAC CAC ACC TTC TA forward and CGT CAT ACT CCT GCT TGC TG reverse for ACTIN. PCR was carried out in a reaction mixture containing 1 μl of cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 mM dNTPs, 150 ng of primers and 2.5 units of DNA polymerase (Roche) in an Eppendorf thermocycler. The cycling conditions were as follows: a denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, at 62 °C for 30 s, at 72 °C for 1 min and a final extension step at 72 °C for 10 min. Equal amounts of PCR products were electrophoresed on 1.5 % agarose gels and visualized by ethidium bromide staining. Gels were photographed under UV light and images were analyzed for presence (positive) or absence (negative) of Bcl2 and Bcl2-L12 gene expression by the NIH Image program, Actin was used as internal control for the integrity of the mRNA.

In order to determine the expression patterns of ADAMTS-16 mRNA in different human tissues, PCR was performed, using the primers 5'- AACTCAGCCTGCACGATTCAC -3' and 5'- CAGTGCCCATTCAGGTAGTAC -3' on a panel of first-strand cDNAs from various human tissues (Origene, Maryland/USA). The PCR conditions were as described in the manufacturer's protocol with 30 cycles performed on the β-actin control employing *Taq* polymerase.

Microarray data of mouse testis, generated by the GNF Mouse Atlas v2 project (Su et al., 2004), were obtained from the hgFixed database of the UCSC Genome Browser (Karolchik et al., 2003).

3.4. Cell culture, cell treatment with estradiol or STS and MG132

The MCF-7, HEK293 or Hela cells were grown in full medium (RPMI 1640 or DMDM, Gibco, Basel) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco) in 5 % CO_2 at 37 °C. For estrogen deprivation, MCF-7 cells were washed 3 times with PBS then further cultured in DMEM with 10% charcoal/dextran treated FBS (Hyclone, USA) for 48 hours. This time point served as a 0 hour time point control. Cells were either further cultured for 10 hours or add 50 nM 17β-estradiol for various time before harvest. To mimic hypoxia condition, 500 μM of $CoCl_2$ was applied to $2x10^5$ MCF-7 or HEK293 for indicated time points. To induce apoptosis, HEK293 cell or the transfectants were incubated with 100 nM Staurosporine(STS) or MG132 for 12 hours.

3.5. Cell transfection

The MCF-7, HEK293 or Hela cells cells were grown in full medium (RPMI 1640 (Gibco) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco) in 5 % CO_2 at 37 °C. Briefly, 5 x 10^6 cells/ml resuspended in 400 μl of ice-cold PBS buffer (Gibco, Basel, Switzerland) were co-electroporated with 15 μg of DNA at 400 V, 1000 mF in a Bio-Rad electroporation cuvette, followed by 2 days recovery. The clones were selected by G418.

For transient transfection, Effectene Transfection Reagent kit was used for cell transfection following the protocol recommended by the manufacture. Cells was seeded on 24 well plate, 24 hour later cells reach about 60 % confluent ($2x10^5$), transfected with Effectene® Transfection Regent (QIAGEN)and follow its 24 well plate transfection protocol. Simply dilute 0.3 µg DNA with 75 µl buffer EC, add 2.4 µl enhancer and mix by vortexing 1 s, incubate the mix at room temperature for 5 min, add 6 µl Effctene and vortex 10 s. Incubate 10 min at room temperature. add 400 µl DMEM (contain10% FBS and 1% P/S) to transfection complexes , mixed the medium with pipette, added to the in the 24 well plate containing the cells, Incubate the cells with transfction complexes at 37 °C and 5 % CO_2 for 24 to 48 h.

3.6. Cellular fractionation and Western blotting

Cells were pelleted and suspended in 100 µl of an enzymatic reaction buffer (Lutter et al., 2000) supplemented with protease inhibitors, lysed by sonication for 15 seconds in an ice bath. Subsequently, the lysate was centrifuged for 10 min at 1000 g for removal of unlysed cells and nuclei. The supernatant was then centrifuged for 1 hour at 100`000 g (Kontron Instruments). The final supernatant served as a cytosolic fraction, the pellet as a crude membrane fraction which was resuspended in one volume of the lysis buffer with 10 % Triton X-100. The protein concentration of both cytosolic and membrane fractions was determined by a BioRad protein assay (BioRad, CA, U.S.A.). Fractionated cell lysates were boiled for 10 min. 20 µg of samples were separated on 15 % SDS-PAGE, electrotransferred to an Immobilon-P membrane (Millipore, Switzerland), immunoblotted with a rabbit

polyclonal anti-His antibody (Milan Analytica AG/CH) or anti-cytochrome *c* antibody (Pharmingen, CA, U.S.A.), and developed with NBT/BCIP (Sigma, St. Louis, U.S.A.). For Bcl2-L12 protein extracts were incubated with the (Abgent, San Diego, CA) or EGFP (Santa Cruz Biotechnology) antibodies and subjected to SDS-PAGE.

3.7. Cell viability assay

At various time points at least 100 cells from each individual culture were analyzed by trypan blue exclusion staining. Viability was determined at various time points by trypan blue exclusion, counting at least 100 cells from each individual culture. The percentage of cell survival was calculated as the number of surviving cells per total cell count (Zhang et al., 1998).

3.8. Immunofluorescence of Mitochondrial, ER and Golgi Staining

HEK 293 or Hela cells were seeded on 24 well plate containing 12 mm glass coverslips. 24 hour later cells reached approximately 60 % to 70 % confluency (about $2x10^5$). Cells were transfected with Effectene® Transfection Regent (QIAGEN). 24 hours after transfection, the cells were washed with PBS three times, incubated with fresh medium containing 100 nM MitoTracker red (ER or Golgi) for 30 min at 37 °C in a 5 % CO_2 incubator, washed 3 times with PBS for additional 30 min and fixed with 3.7 % formaldehyde at room temperature. The cells were than mounted on slides. The cells were visualized through Leica confocal laser scanning microscopy.

3.9. In vitro transcription, translation and co-immunoprecipitation

Expression plasmids encoding BNIP3 and its mutants cloned into pcDNA3 were used as templates for *in vitro* transcription/translation in the presence of methionine (Amersham Pharmacia Biotech) by the TnT-coupled Reticulocyte Lysate System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. For co-IP, equivalent amounts of *in vitro* protein products were incubated with 3 µg of purified Bcl-2 protein and hamster monoclonal anti-Bcl-2 antibody in 250 µl of 0.2 % Nonidet-P40 IP buffer (100 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, and 0.2 % Nonidet-P40) for 2 h at 4 °C. Immune complexes were captured with protein A-Sepharose 4B for an additional hour, washed four times in excess IP buffer, and resuspended in SDS sample buffer. *In vitro* protein products or solubilized immune complexes were separated by Laemlli 12 % SDS-PAGE. Gels were fixed with 25 % 2-propanol and 10 % acetic acid for 20 min, followed by an additional 20 min in Amplify solution (Amersham Pharmacia Biotech) and then analyzed by autoradiography. To detect Bcl-2 protein in each co-IP reaction, gels were stained with Coomassie Blue.

3.10. Subcellular Localization of hBok and Bcl2-L12 Proteins

To examine subcellular localization of endogenous hBok, 10^5 of MCF-7 cells were seeded on a sterile glass coverslips. After 12 hours culture, cells were washed with PBS and fixed with 4% paraformaldehyde (in 10 mM PIPES, 2 mM $MgCl_2$, 2 mM KCl, 300 mM sucrose, and 2 mM EGTA). Cells were further quenched with 20 mM NH_4Cl and permeabilized in

0.25 % Triton X-100. A rabbit anti-Bok antibody (Sc-11424, Santa Cruz, USA) was used as primary antibody in a 1:200 dilution. The secondary antibody was Alexa-488 conjugated goat anti-rabbit IgG in a 1:1000 dilution. To examine subcellular localization of transfected hBok, HEK293 cells or Hela cells were transfected with 0.3 µg of expression plasmids encoding GFP-hBok or GFP-Bcl2-L12 using Effectene reagent (Qiagen). Cell were incubated with 100 nM Mitotracker Red 580 (Molecular Probes, Oregon USA) and then fixed in 3.7 % formaldehyde 24 hours post-transfection. Images were acquired with a confocal microscope (Leica NT SP1, Germany) with oil immersion using a 63× lens on a Leica DM RxE. The images were acquired with Leica confocal software with the image format of 1024 x 1024 pixels x 8 bit, and transferred to graphics program for printing.

3.11. His or GST pull-down experiment

TOP10 *E. co*li (Invitrogen) transformed by an expression vector were grown in 2 ml of LB medium containing 50 µg/ml ampicillin at 37 °C to an O.D. of 575=0.3-0.5. Following the addition of 10 µl of 0.1 M IPTG (final concentration: 0.5 mM) to the culture medium, and the induced cells were grown for an additional 3-4 h at 37 °C, pelleted, washed once with PBS, and resuspended in 400 µl PBS containing 1 % Triton-X100 and the protease inhibitor cocktail Complete Mini EDTA free (Roche). The cells were then subjected to freeze-thaw cycles (3×) using liquid nitrogen. The cell lysates were clarified by centrifugation at 17000 g for 30 minutes and were stored at –80 °C until needed for further use. The concentration of bait protein among the lysates was assessed by SDS-PAGE. For the analysis of affinity precipitations, cell lysates were prepared using a Hela cells. 1-10^8 cells were harvested and

lysed in 1 ml of lysis buffer (75 mM HEPES1 pH7.5, 100 mM NaCl, 100 mM $(NH_4)2SO_4$, 1 mM $CaCl_2$ or EGTA, 0.5% CHAPS1, and 0.1 mM DTT) containing the protease inhibitor cocktail Complete Mini EDTA free (Roche) for 1 h at 4 °C. Transformed *E. co*li lysates containing approximately 10 µg of bait proteins were added to the lysates. The mixture was incubated for 24 h at 4 °C. Then, a 5 µl bed volume of GS4B beads equilibrated by applying lysis buffer with or without calcium was added to the mixture, and the mixture was incubated for 1 h at 4 °C. Finally, the beads were washed extensively in the appropriate lysis buffer, dried for a few minutes using a Speed Vac, and stored at –30 °C until use.

3.12. Immunofluorescence Microscopy Assessments

MCF7 or Hela cells were transiently transfected with plasmids pLEGFP-hBok or pLEGFP-Bcl2-L12(828) grown on 24 well plate with sterile glass coverslips. The cells were then washed with PBS before fixation with 4 % paraformaldehyde (in 10 mM PIPES, 2 mM MgCl, 2 mM KCl, 300 mM sucrose, and 2 mM EGTA) for 20 min . Thereupon, the well was quenched with 20 mM NH_4Cl in PBS and incubated at room temperature for 5 min. The cells were then washed 3 times with PBS for 10 min in the shaker and permeabilized in 0.25 % Triton X-100/PBS for 5 min, then washed 3 times with PBS for 10 Min. Primary rabbit antibody anti-Bok Sc-11424 were diluted 1:200 with PBS and incubated for 1 hour at room temperature. As a secondary antibody Alexa-488 conjugated goat anti-rabbit IgG (1:1000 dilutions) was used (dark). The cells were mounted with mowiol. Images were acquired with confocal immunofluorescence microscopy was performed with a ×60 oil objective (Leica).

3.13. Flow cytometry (FACS) analysis.

HEK 293T cells were seeded in six-well plates at $1x10^5$ cells per well for one day and then transfected. $5x10^5$ cells with pLEGFP vector or pLEGFP-828 and its splice variants using transfection effected reagent protocol. After 24 h, adherent and floating cells were harvested by trypsinization, washed twice with PBS, resuspended in 5 ml of PBS, and then 1 ml of cold 70 % ethanol was added drop wise into each tube while the tubes were being vortexed at slow speed. The ethanol-fixed cells were stored at 20 °C until needed. Fixed cells were centrifuged between 1000 rpm for 5 min after which the pellet was resuspended in 2 ml PBS/RNAase (50 μg/ml) at 37°C for 30 min. The cells were incubated at 37 °C for 30 min, mixed with 1 ml PBS containing propidium iodide at a final concentration of 5 μl (stock 50 μg/ml), and analyzed by Flow Cytometer (CyAn^{TM} ADP, DakoCytomation). For apoptosis pLEGFP-bcl2L12 and its splicing variants were harvested after 24h treatment with 500 nM staurosporine or 100 μM MG132 and then analyzed by flow cytometry. Data for 50,000 to events were gathered by Cell Quest software and analyzed using Flowjo Macintosh (8.1.version) FACS Analysis software.

3.14. Purification of ADAMTS-16 GST-Fusion and His-tagged proteins

In brief, pGEX-4T-2_ADAMTS-16 and pGEX-4T-1_ADAMTS-16 pep were transformed to DE3(BL20). The positive clones were cultured in 250ml LB (100 ug/ml Ampicillin) for 3- 4 hours at 30 °C shaker untill O:D.600 was reached 0.6-0.8. Then IPTG was added to final concentration of 0.1 mM (stock 200 mg/ml and final concentration is 350 ng/ml) and

the bacteria were cultured for further 4 hours. Cells were collected by centrifugation and the pellets were stored at –70 °C. Subsequentially, the pellets were lysized in 3 ml cold lysis buffer (50mM Na_2HPO_4 pH 8.0, 0.3M NaCl, 1 mM PMSF with protease inhibitors) containing freshly added lysozyme (100 µg/ml) and sonicated 5 x 20 seconds until the sample cleared up and was no longer viscous. Upon centrifugation, samples were boiled at 95 °C for 5 min in loading samples and analysed on 10 % SDS-PAGE. His-tagged proteins were produced by transfection of pcDNA3-Hisc_ADAMTS-16 in HEK293 cells. Protein concentration was determined by BioRad mini kit.

3.15. Cleavage of Alpha 2-Macroglobulin

45 µl of 2X buffer (100 mMTris-HCl, 20 mM$CaCl_2$, and 200 mM NaCl, pH 7.5) were added to 45 µl 50 % GST beads (containing 5 mM Tris, 10 mM NaCl, pH 7.5, GE) and or His Bead (Roche). ADAMTS-16 and ADAMTS-16s at a concentration of 20ng/µl were incubated with 5 µl 10 mM $ZnCl_2$ and 5 µl of 100 ng α_2-macroglobulin at 37 °C for 2 hour in 1.5 ml eppendorf tubes. Subsequently, the products were analyzed for total protein by SDS-PAGE on 8-10 % gels followed by staining with Coomassie Brilliant Blue R-250 to analyze for cleavage of α_2-macroglobulin.

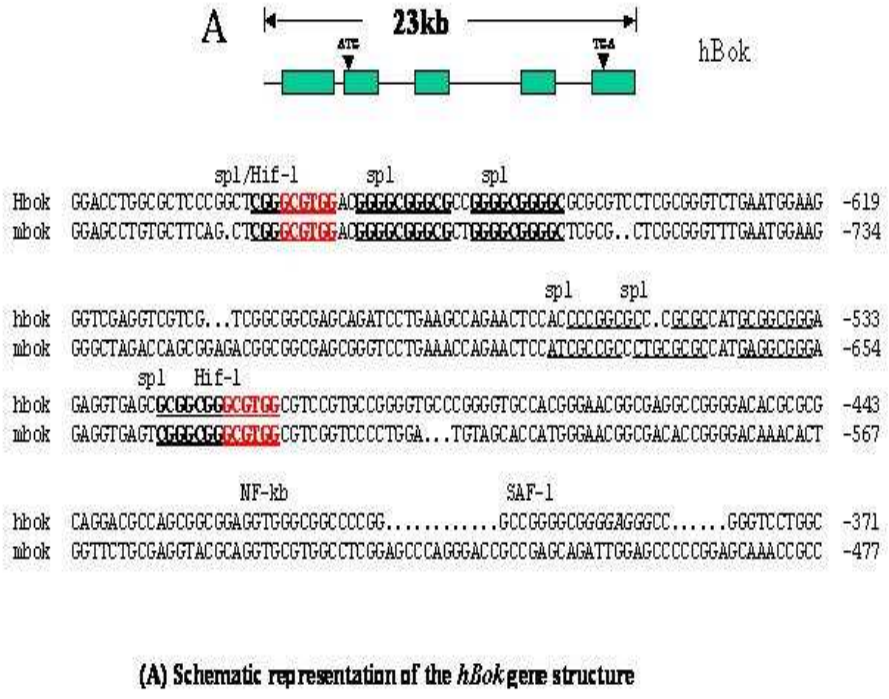
4. Results

4.1. The regulation of the expression of human Bcl-2-related ovarian killer gene (hBok), a pro-apoptotic member of the Bcl-2 family (Bcl2-L9), specific to reproductive organs

4.1.1. Isolation and characterization of the human Bok gene

To identify the chromosomal location of the hBok gene, we isolated and identified one positive BAC clone, which covered all coding regions of the human BOK gene as known from a human BAC genomic library (see Materials and Methods). Sequence analysis revealed that it contains 4 coding exons in which the exon-intron organization was perfectly conserved between human, mouse and rat genes, although the intron sizes were different among the genes of these species (Table 1, Fig.7A).

The complete hBok gene spanned about 23 kb of genomic DNA. We then used this BAC DNA as a probe for fluorescence *in situ* hybridization (FISH) studies. We examined chromosomes in metaphase from a normal male for fluorescent signal. Out of 40, 32 metaphases examined, possessed specific signal in the region 2q37 (Fig.7B). No background signals were observed. Localization to 2q37 is consistent with *in silico* analysis of the draft human DNA sequence at the National Center for Biotechnology Information, which have mapped the genomic locus of hBok to human chromosome 2 between the markers D2S125 and chr2_qTEL. Together with all data, the hbok gene was localized to human chromosome 2q37.3 (Fig.7B).



(A) Schematic representation of the *hBok* gene structure

Figure7: Genomic organization and chromosomal localization of the human Bok gene (hBok). (A) Schematic representation of the hBok gene structure. The boxes represent the exons. The exon numbers are indicated below the boxes. The thin lines denote introns and flanking regions. The lower panel shows the alignment of the conserved 5 -UTR sequences of human (–371to –709) and mouse (–477 to –821) Bok genes. Underlines represent transcriptional factor-binding sites.

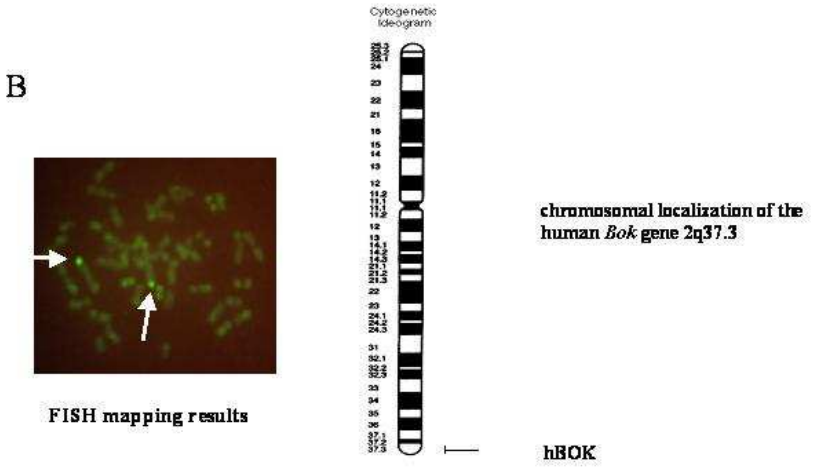


Figure 7 (B): Left panel shows that in situ hybridization of a biotin-labeled human Bok probe to human metaphase cells resulted in specific labelling on chromosome 2 (arrow). Right panel shows results of FISH mapping. The detailed position of the Bok gene, on human chromosome 2q37.3, was determined from careful analysis of 40 metaphase cells and compared to the NCBI STS database.

4.1.2. Differential expression of hBok in various human tissues

Semiquantitative RT-PCR analysis (semi-Q-RT-PCR) was performed by using a pair of primers corresponding to sequences flanking to the first intron (Materials and Methods). As shown in Fig. 8A, human Bok was expressed mainly in the colon, stomach, testis, placenta, pancreas, ovary and uterus.

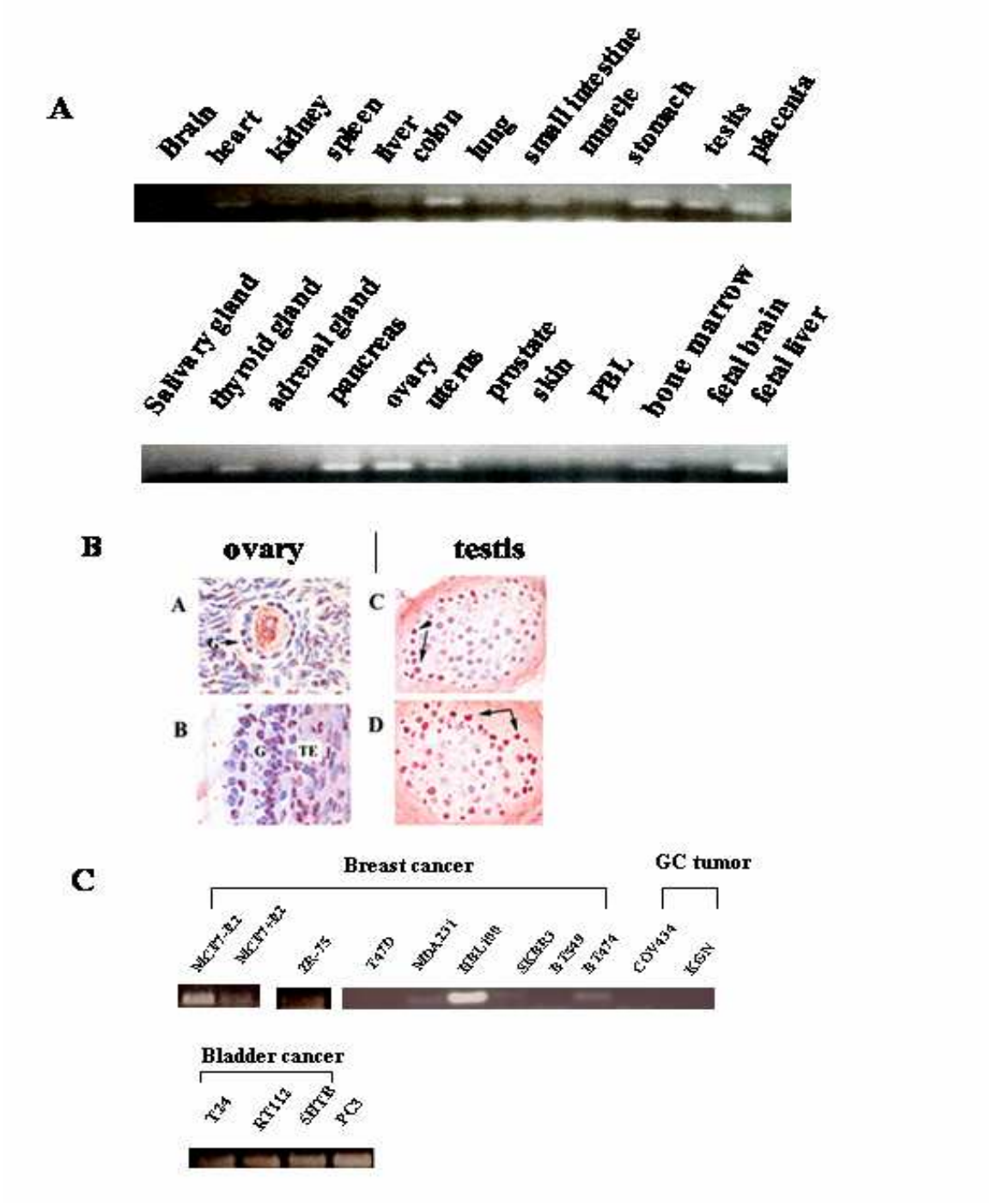


Figure 8: Expression of hBok mRNA species varies in different tissues. (A) PCR was performed on a panel of first-strand cDNAs prepared from different human tissues (Origene). The PCR products obtained from each reaction following 30 cycles were separated on a 1.2 % agarose gel and visualized by SYBR green I. (B) The localization of hBok protein was examined with immunohistochemistry in ovarian follicles (A, B) and testicular seminiferous tubules (C, D). G, granulosa cells; TE, theca cells; arrows, spermatocytes. (C). hBok expression was examined in various cancer cell lines using semi-Q-RT-PCR.

The expression in fetal liver exhibits higher level than that in adult tissue. The expression of the human Bok protein in various human tissues was also examined with immunohistochemistry. Figure 8B shows the localization of hBok protein in ovarian and in testicular tissue samples. Human Bok was found to be expressed predominately in the granulosa compartment of ovarian follicles (Figure 8A & 8B) and in the spermatocytes of testicular seminiferous tubules (Figure 8C & 8D).This result consistent with the observation for RBok (Hsu et al., 1997). The expression of hBok in various tissues was further examined in various hormonally regulated cancer cell lines using semi-Q-RT-PCR. All 7 breast cancer cell lines displayed much lower hBok expression than HBL100, which is considered a non-cancergenic cell line. In contrast, granulosa cell tumors lacked mRNA expression. However, a clearcut expression of hBok mRNA was detected in some other cancer cells, such as bladder and prostate cancer cells (Figure 8C).

4.1.3. Differential regulation of hBok expression

In our initial studies on influences of hypoxia to antiestrogen, ICI 182780 on apoptosis of breast cancer cells, we observed that hBok expression was enhanced in MCF-7 cells both by 17β-estradiol withdrawal and by hypoxia. MCF-7 cells showed low but detectable levels of Bok mRNA. Cells that were cultured in an estrogen-free, serum-free medium for 2 days or treated with the steroidal antiestrogen, ICI 182780, generated increased levels of hBok mRNA, whereas E2 treatment induced the downregulation of hBok mRNA (Figure 9A). In contrast, C-Myc expression was increased by addition of E2. Furthermore the expression in

MCF-7 treated with various concentration of $CoCl_2$, a condition mimicking hypoxy, resulted in an increased hBok expression after 24 h. However, combined treatment of ICI 182780 and hypoxia did not further enhance the expression of hBok and similar observations were made with HEK293 cells (data not shown). Although stauroporine induced apoptosis in both cell lines, expression of hBok was not enhanced, indicating that expression of hBok is regulated distinctively according to the apoptotic inducing signal.

It has been shown that hypoxia, a condition of ischemia, induced brain injury and cardiomyocyte apoptosis, which can be prevented by estrogens (Harm et al., 2001; Kim et al., 1996). We therefore further examined the expression of hBok in mouse cardiomyocytes. As demonstrated in Figure 9C, hBok is expressed at very level in primary cells but hypoxia caused a significant rose of hBok mRNA levels 24 h after treatment. The increased expression level was also observed for BNip3, which have been reported as hypoxia-response genes in hypoxia-induced apoptosis (Bruick et al., 2000). The expressions of three other BH3-only genes were also examined. Expression of Bad was decreased whereas Noxa (Oda et al., 2000) and Bmf (Puthalakath et al., 2001) remained unexpressed but the expression of Bcl2-L10 (Inohara et al., 1998; Zhang et al., 1999) was increased (Figure 9D). Thus, the observation that hypoxic regulation for hBok expression appears to be similar both in the human and in the mouse suggested that expression of hBok is hypoxia-specific regulated in certain organs.

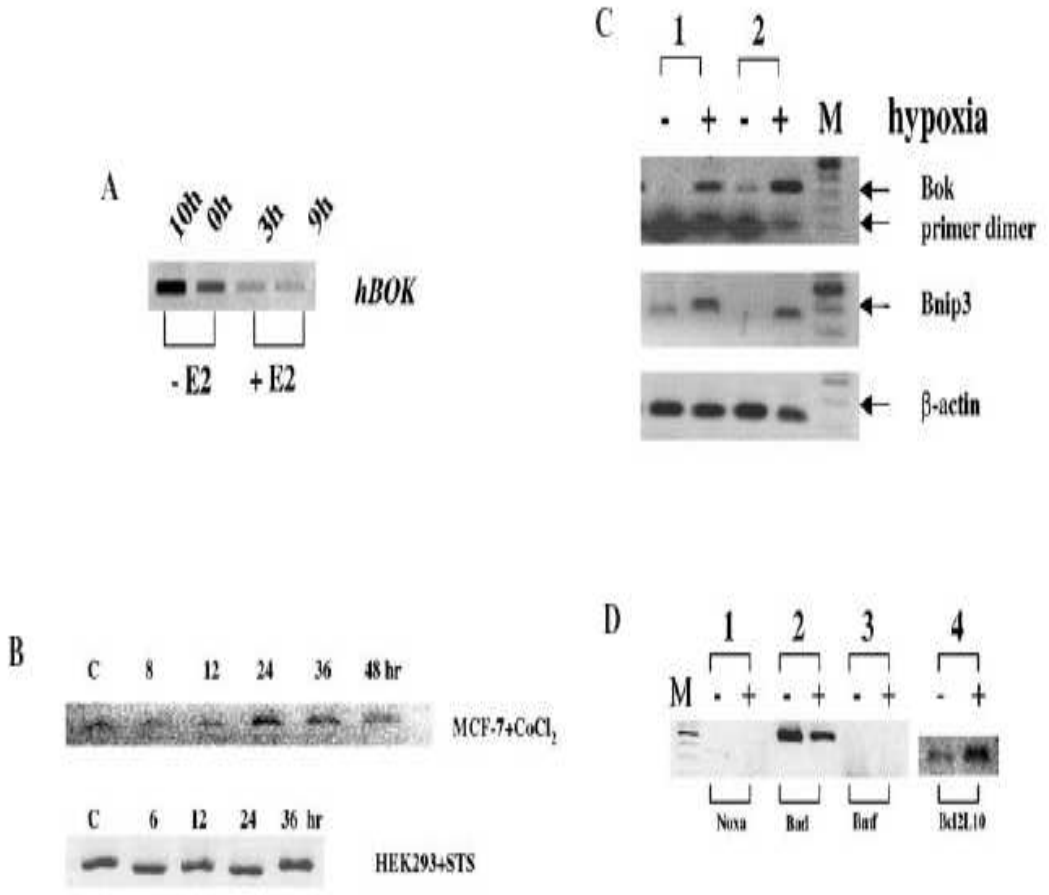


Figure 9: Regulation of hBok mRNA expression in various conditions studied by semi-Q-RT-PCR. (A) MCF-7 cells were cultured in an estrogen (E2)-free, serum-free medium for 48 h. 10 h indicates further culture for 10 h in the estrogen-free medium (-E2) or addition of 50 nM E2 for 3 or 9 h. (B) Expression of hBok mRNA was examined in 2X105 MCF7 cells treated with 500 mM CoCl2 for the indicated times. Expression of hBok mRNA was also examined in HEK293 cells after treatment with 100 nM staurosporine (STS). (C) Examination of Bok expression in dissected mouse hearts from two animals. Each dissected mouse heart was divided into two parts and incubated in either 21% O2 condition (–, normaxia) or 1% O2 (+, hypoxia). Total RNA was normalized by determination of concentration at 260 nm. One microgram of RNA was used for reverse transcription. As controls, the expression of Bnip3 and the housekeeping gene b-actin were examined in parallel. (D) Expression of four members of the Bcl2 family, Bad, Noxa, Bmf and Bcl2L10 were examined in the same condition as in C.

4.1.4. Death signalling modify the localization of hBok

PSORT II predicted that the possibility of localization of hBok were 39.1 % cytoplasmic and 34.8 % mitochondrial. To assess subcellular localization of hBok experimentally, we

examined hBok in MCF-7 cells by confocal laser scanning microscopy using two different anti-Bok antibodies. As shown in Fig. 10A, the expression of endogenous hBok was rather diffuse thoughout the cell.

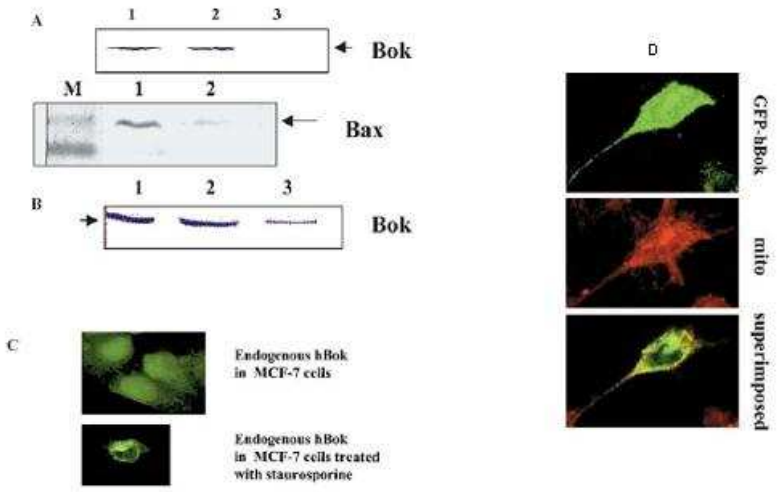


Figure 10: Intracellular localization of hBok. (A&B) Subcellular localization of hBok and Bax by cell fractionation. $5x10^6$ MCF-7 cells were collected, lysed and fractionated for high-speed cytosolic and membrane fractions as described in Materials and methods. The protein samples were separated by 15% SDS-PAGE and analyzed by Western blot with a polyclonal antibody against human Bok (Santa Cruz) or Bax (BD/Pharmingen). The proteins were fractionated into cytosolic (1) and membrane (2) fractions. 3 indicate alkaline-treated membrane fractions. (B) MCF-7 cells were treated with 100 nM staurosporine for 12 h. Cell lysates were prepared and analysed as in A. (C) Immunolocalization of endogenous hBok in MCF-7 cells treated with or without 100 nM staurosporine. Micrograph showing a single, confocal image plane of MCF-7 cells labelled with a rabbit anti-Bok antibody (Sc-11424) followed by an Alexa-Fluor-488-conjugated secondary antibody (green). (D) Subcellular localization of GFP-Bok by confocal microscopy with a magnification of X63. Hela cells were transiently transfected at GFP-Bok as described in Materials and methods. MitoTracker was used to label mitochondria (Mito).

The examination of hBok-GFP fusion proteins by transient transfection in HeLa cells revealed that it is associated with mitochondrial membrane (Figure 10B). We also assayed its localization by subcellular fractionation studies. Cells were lysed and fractionated into a cytosolic and a membrane fraction after removal of the low-speed nuclear pellet. The endogenous protein of hBok can be detected in both cytosolic and membrane fraction on subcellular fractionation of healthy MCF-7, whereas Bax is detected mainly with the cytosotic fraction (Figure10B and Hsu et al., 1997; Zhang et al., 1999). Similar results were obtained in HEK293 or Hela cells lysed in a buffer with or without detergent (data not shown). Furthermore, detergent or an alkali treatment is effective at extracting hBok from the membrane indicating that in healthy cells, Bok appears to be bound only weakly to the membranes (Figure 10B). However, after treatment of the cells with apoptotic stimuli, the membrane-bound hBok could not be released by alkali treatment (Figure 10C). Our results suggest that in healthy cells hBok is associated loosely with membrane and the insertion and accumulation of hBok on membranes is enhanced by death signals and the membrane-bound hBok protein became integrated into the membrane.

4.1.5. Identification of characterization of two hBok deletion variants: hBok-404 and hBok-424.

In addition to the Bcl-2 homology (BH) domains 1 and 2 and the transmembrane sequence, Bok also possesses a BH3 domain which is believed to be important for dimerization with selective anti-apoptotic Bcl-2 proteins and cell death (Huang et al., 2000), because

conserved BH3 domains are known to be important for apoptosis induction in a variety of proapoptotic Bcl-2 proteins. Mutations in the BH3 domain of several proapoptotic proteins abolished their heterodimerization with antiapoptotic partners and dampened their cell-killing activity (Reed et al., 1997). We constructed two other deletion mutants: Bok-404 carries a C-terminus transmembrane domain-deletion and BH3 only domain, whereas Bok-424 carries an N-terminus domain-deletion lacking a BH3 domain (Figure11a) in order to study the functional roles of the BH3 domain.

Recent studies using crystallography, computer modelling, and membrane potential recording have established that the amphipathic BH3 domain in some proapoptotic Bcl-2 proteins might regulate apoptosis by binding to a hydrophobic cleft formed by the conserved BH1, 2, and 3 domains in the antiapoptotic Bcl-2 proteins, represented by Bcl-2 and Bcl-xL (Muchmore et al., 2000; Aritomi et al., 1997). Furthermore, the region spanning BH1 and BH2 domains of Bcl-2 proteins is important for pore formation in the artificial membrane and could function as ion channels in the mitochondria and other subcellular membrane organelles (Reed et al., 1997; Aritomi et al., 1997; Schlesinger et al., 1997) As shown in Fig. 11b, Bok-404 was detected in the nucleus predominantly, but only partially also in the cytosolic fraction, but not in the membrane fraction of cellular lysates. Cells with transient or stable expression of GFP-Bok-424 showed a diffuse pattern (Figure11D). In contrast, Bok-404 was detected mostly in the nuclear fraction (Figure11C, 11E, 11F). Thereupon, we performed a triple organelle stainings of the mitochondria (Mito), the endoplasmic reticulum (ER) and the Golgi complex (Golgi) in order to visualize the shift of location of these two variants throughout the transfected cells. The results, together with the observation depicted in Figure 11E, suggest that the N-terminus of hBok-424 inhibits the insertion of hBok into

the membrane. The BH3-only variant, hBok-404, dysplayed a strong signalling in the nucleus of the HEK 293 transfected cells (Figure11F). Because the hBok-404 BH3-only subgroup members lack the region spanning BH1 and BH2 domains important for pore formation and mainly reside in the cytoplasm, they are believed to serve as ligands or facilitators of the pore forming Bcl-2 proteins. The BH3 sequence 71LLRLGDELEQIR82 has also a short leucine-rich stretch of amino acids corresponding to a putative NES (Nuclear Export Sequence). The most prevalent NESs including a similar stretch, in which the leucine residues are critical for function (Gorlich et al., 1999). A similar leucine-rich sequence is not present in the BH3 domains of any other member of the Bcl-2 family (Adachi et al., 2002; Nakano et al., 2001; Han et al., 2001).

The loose association of hBok to the mitochondrial membranes of healthy cells prompted further investigations into the localization of hBok during apoptosis. The amount of hBok-424 and hBok-404 in the cytosolic fraction decreased and translocates into nucleus and activation of apoptosis after addition of 100mM staurosporine for 12 hours (Figure11G, F). This indicates that hBok was relocalized from the cytosol to nucleus during apoptosis, similar to Bax in thymocytes, HL-60 leukemia cells, splenocytes (Hsu et al 1997; Zhang et al, 1999). This analogy suggested that the change in the subcellular distribution of hBok is a common step for all cells undergoing apoptosis in response to apoptotic stimuli. The results obtained after treatment of the cells with apoptotic stimuli STS suggested that the insertion and accumulation of hBok on membranes at this stage is enhanced by death signals and the membrane-bound hBok protein became integrated into the membrane. Bok also has a BH3 domain believed to be important for dimerization with selective antiapoptotic Bcl-2 proteins and cell killing.

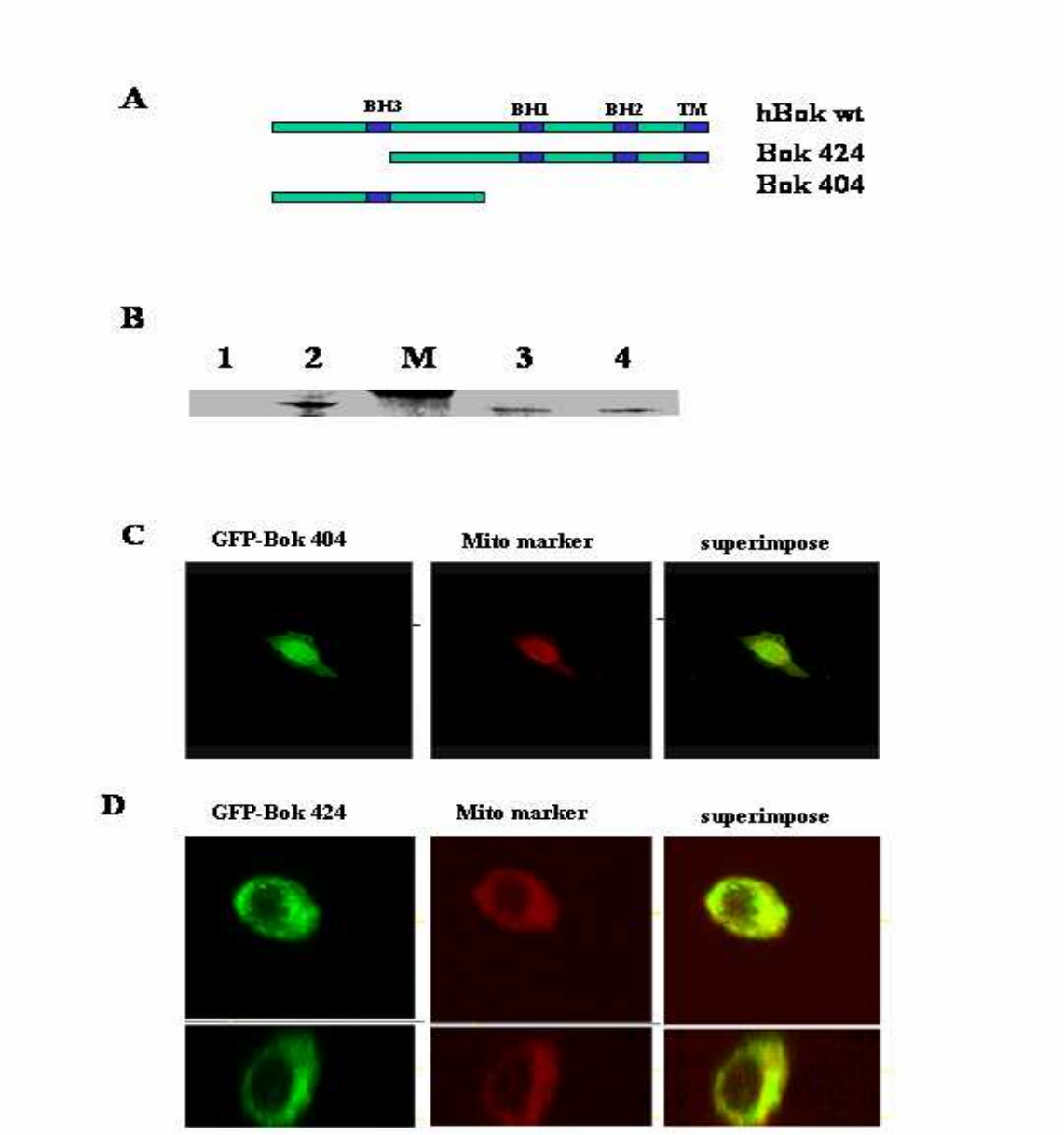


Figure 11: Inhibition of hBok to mitochondrial membrane by N-terminus sequence

A: hBok-424 carries a C-terminus transmembrane domain-deletion, hBok-404 carries an N-terminus domain-deletion, **B:** Subcellular localization of Bok constructs by cell fractionation: The protein samples 1 and 2 were obtained from cells expressing Bok-404 whereas 3 and 4 were from Bok-424. 1 and 3 were protein samples fractionated in the cytosolic fractions but 2 and 4 were from the membrane fractions. Western blots were performed with a monoclonal antibody against GFP. **C&D**: Subcellular localization of Bok constructs by confocal microscopy with an original magnification of ×63 (Hela cells).

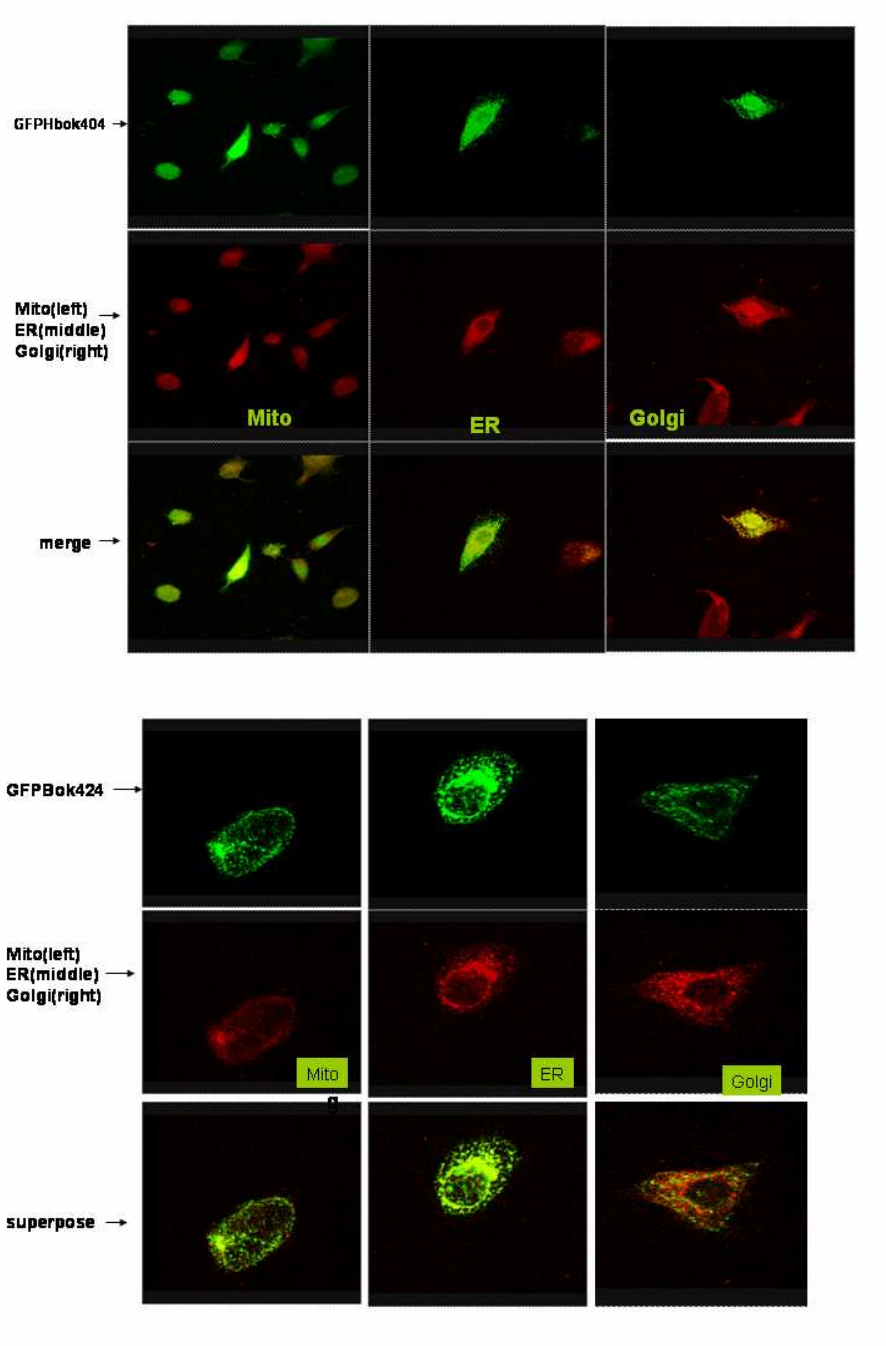


Figure 11E: CLSM visualization o f bok-424, bok-404 Mitochondria, Endoplasmic reticulum (ER), Golgi complex staining images 63 x.

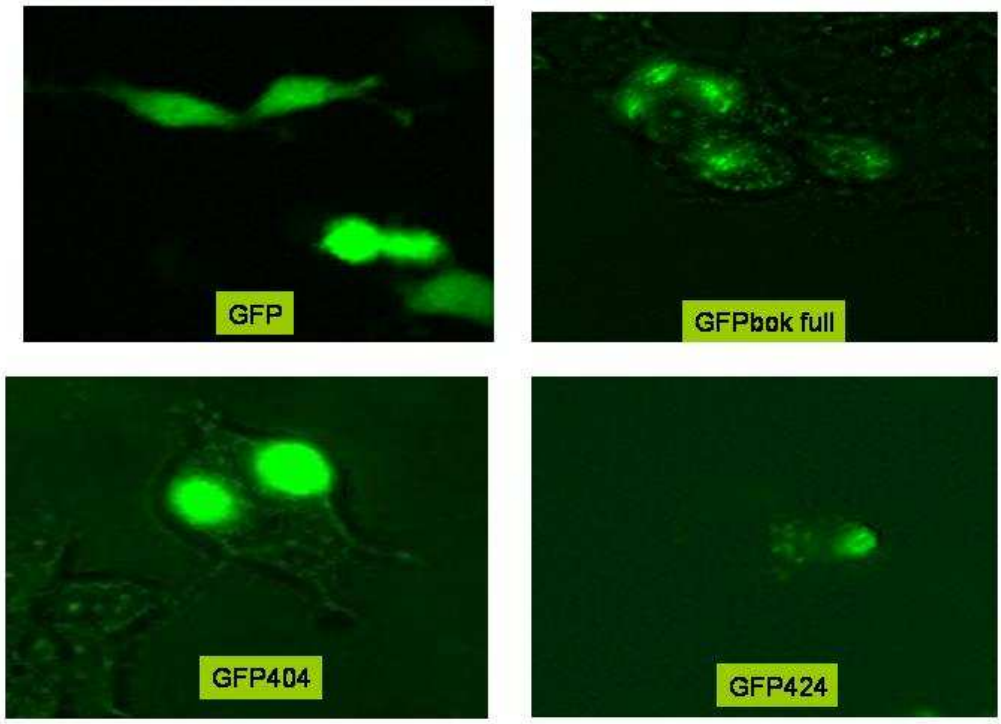
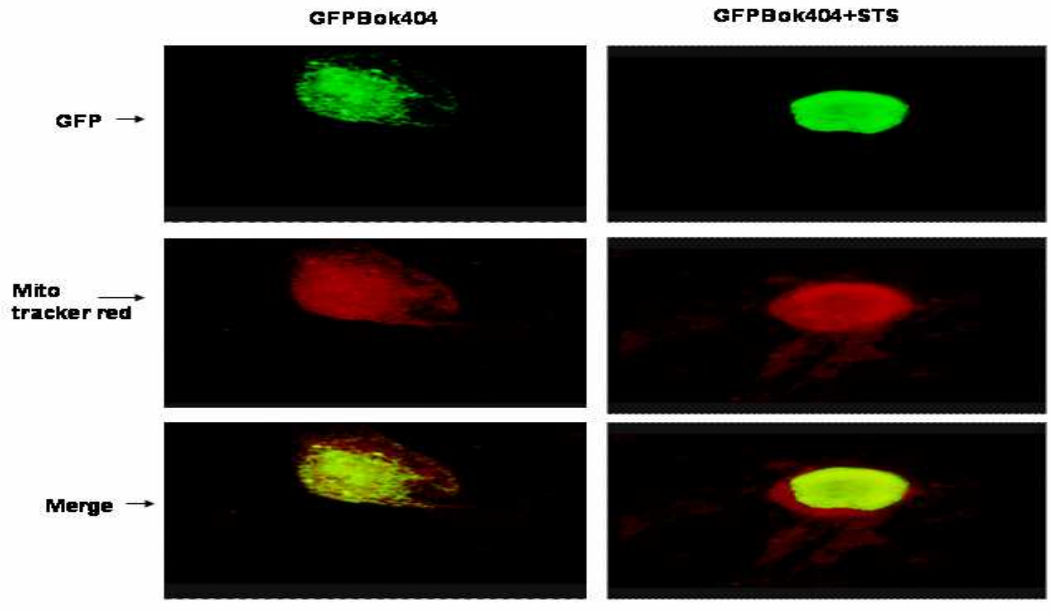


Figure 11F: GFP, GPF Bok full and GFP Bok424, GFP Bok404 transfected to HEK 293 cells 24 hour later visualized in immunofluoresence microscope (Leica), the original is 40 x magnified.



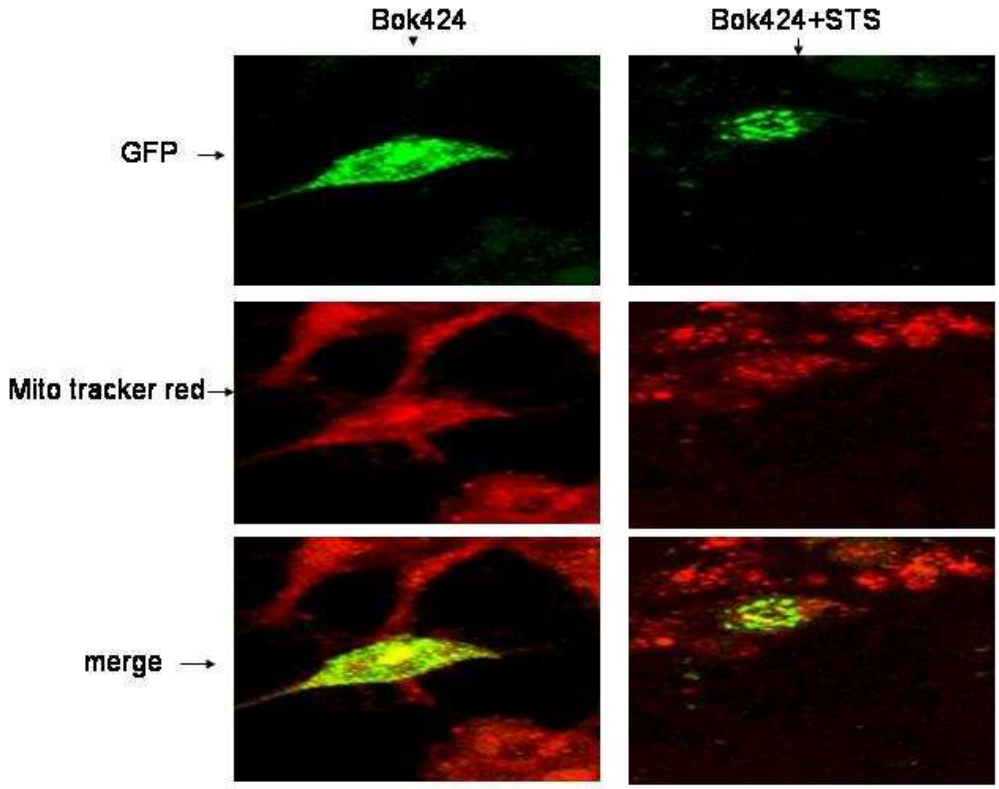


Figure 11G: STS induced Bok 404 and Bok424 apototic CLSM images. plasmids encoding GFP-Bok404bp or GFP Bok 424bp were transfected into HEK 293 cells. At 12 h, 100 nM STS treated cells for 12 h and 24 h after transfection the cells were incubated with MitotrackerRed, then fixed and imaged.

4.1.6. Oligomerization of hBok is promoted by bnip3 and p53.

Since the expression of bnip3 and p53 were induced together with hBok during hypoxia (Figure 12) (Bruick et al., 2000) and because bnip3, p53 and hBok share several properties, such as their common cytoplasmic and mitochondrial localization and their pro-apoptotic function, we explored the possibility of a functional cooperation of these proteins during

apoptosis. Apoptotic stimuli and BH3-like proteins enhanced the pro-apoptotic activity of hBok. HEK293 cells were either treated with 100 nM staurosporine or transient transfected with GFP-hBok with or without constructs indicated for 12 hours. Viability was determined at various time points by trypan blue exclusion, counting at least 100 cells from each individual culture. The percentage of cell survival was calculated as the number of surviving cells per total cell count. The results represent a least two separated experiments. The membrane fraction of cell lysates was incubated with 10 mM bismaleim-idohexane (**BMH)** cross-linker for 30 min at the room temperature. Mitochondrial proteins were extracted with a Chaps buffer and subjected to SDS-PAGE under a non-denatured condition. MCF-7 cells were transfected with Bnip3, p53 or tBid.

As shown in Figure12a, expression of p53 and Bnip3 increased the sensitivity of cells to apoptotic stimuli, which could be encountered by expression of Bfl-1 and partially by Bcl-2. Furthermore, oligomerization of hBok could be detected in all transfectants with p53 and bnip3, indicating that p53 and bnip3 were able to induce translocation and oligomerization of hBok (18 hr). Again oligomerization of Bok was prevented by the transfection of bfl-1. (Figure12a). Next, we explored the mode of action of p53 and Bnip3 by examining their interaction with Bok. However, hBok could be detected using the His pull-down assay with His-Bnip3 (Figure12b). These results indicate that hBok interacts with Bnip3, but not with p53, suggesting that regulation of hBok activation is mediated, at least in part, by two distinct mechanisms. Oligomerization of hBok can be directly triggered by Bnip3 but only be formed by p53 via a specific unknown element.

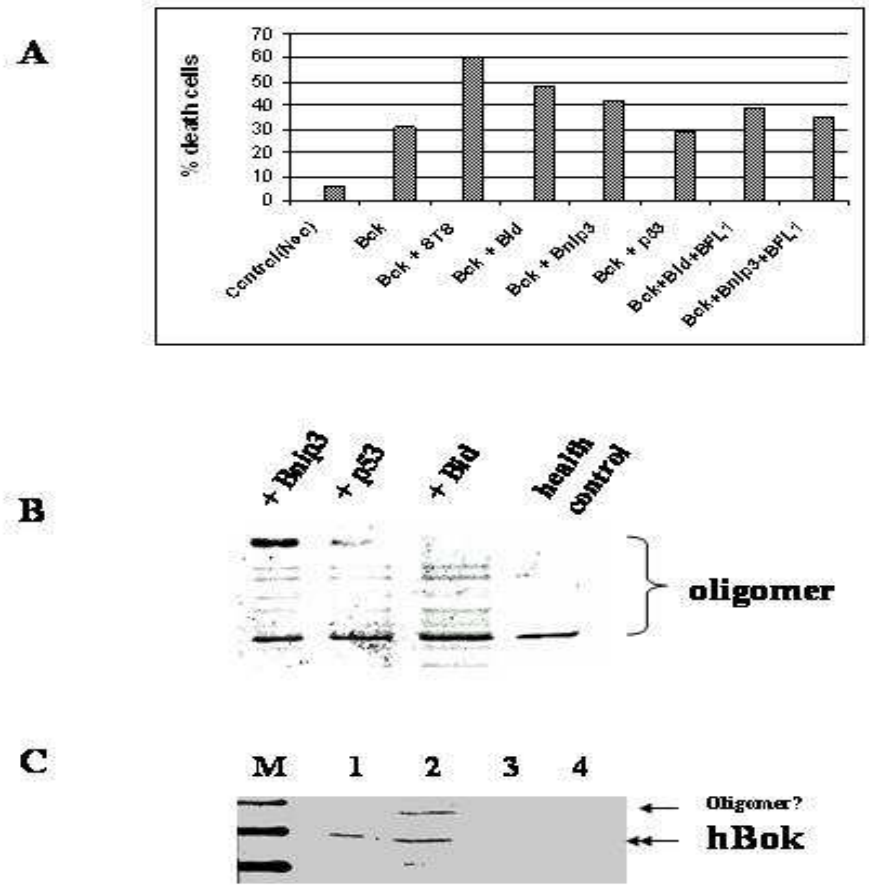


Figure 12: Oligomerization of hBok by BH3-like proteins and interaction of hBok with Bnip3. (A**)** Cell viability assay of hbok and with STS, Bid Bnip3 P53,bfl-1 (B) Oligomerization of hBok. The membrane fraction of cell lysates was incubated with 10 mM BMH cross-linker for 30 min at room temperature. Mitochondrial proteins were extracted with a Chaps buffer and subjected to SDS-PAGE under non-denaturing conditions. Cells were transfected without (health control) or with the genes indicated or treated with 100 nM staurosporine (STS). Western blot was performed with a rabbit anti-Bok antibody (Sc-11424, Santa Cruz). (c) HEK293 cells were transfected with Bnip3, p53 or tBid with non-transfected cells as a control. Western blot was performed with a polyclonal antibody against human Bok (Sc-11424, Santa Cruz).With non-transfected cells as a control. Western blot was performed with a polyclonal antibody against human Bok (Santa Cruz).

Table 1: **PCR primers used to generate fragments for hBok constructs and mouse**

		PCR product size (bp)
	a. 5'-Forward primer-3' b. 5'-Reverse primer-3'	
GFP-hBok	a. 5'-TCTCGAGCAAGCTTGAATTCTATGGAGGTGCTGC GGCGCTCCTCGGT-3' b. 5'-CGGGATCCTCATCTCTCTGGCAGCAGCACGAAG-3'	636
hBok	a. 5'-CCTCGCGGGTCTGAATGGAAGG-3' c. 5'-CGAGCGGTCAAAGGCGTCCAT-3'	164
mBok	a. 5'-CTCCCTCACTCAAATTGGGA-3' b. 5'-ACTGAGGCCAGCTCATGTCT-3'	267
mNip3	a. 5'-GGGTTTTCCCCAAAGGAATA-3' b. 5'-GACCACCCAAGGTAATGGTG-3'	179
mBad	a. 5'-TTCCAGATCCCAGAGTTTG-3' b. 5'-GGAGATCACTGGGAGGGGGTGG-3'	488
mNoxa	a. 5'-CGTCGGAACGCGCCAGTGAACCC-3' b. 5'-TCCTTCCTGGGAGGTCCCTTCTTGC-3'	335
mBmf	a. 5'-CCCTTGGGGAGCAGCCCCCTG-3' b. 5'-CAAGACAGTATCTGTCCTCCCAGAC-3'	218
mBcl2L10	a. 5'-TGAGGAAGTCCTCCAGCCTA-3' b. 5'-GCAGCTCAAGAACCCAGAAC-3'	240

4.2. Gene cloning, regulation and the functional studies of Bcl2-L12

4.2.1. The intracellular nuclear localization of Bcl2-L12

Using the TBLASTN program by searching the GenBank and EST database, following by PCR we have cloned and identified another novel human Bcl-2-related gene, Bcl2-L12. Full length Bcl2-L12 contains 828 bp. At present, there is no any report or reference about Bcl2-L12‘s cellular localization. A 828 bp fragment of Bcl2-L12 was connected to a pLEGFP vector (GFP828) and transfected (see Material and Method section). Fluorescent cells transfection followed by microscopy clearly demonstrated that GFP-Bcl2-L12 is predominantly located in the nucleus (Figure 13), whereas in some cells a week cytosolic signaling was also found (Figure 13, middle). To further confirm the localization of Bcl2-L12, mitochondrial, endoplasmic reticulum, and Golgi immunostaining and DAPI staining were used separately. CLSM images also showed the nuclear signalling of GFP-Bcl2-L12 (Figure14A). The DAPI staining of GFP-Bcl2-L12 demonstrated a clearcut nuclear signalling (Figure14B). The nuclear localization of Bcl2-L12 was also detected in other transfected cells such as HEK293T (Figure 29), 293, NIH/3T and MCF7 cells (data not shown). This conclusion was further confirmed by Western blot analysis of cytoplasmic and nuclear fractions of GFP-tagged Bcl2-L12 in transfected HEK 293 cells. HeLa cells mainly showed the protein in the nuclear fraction (Figure 15).

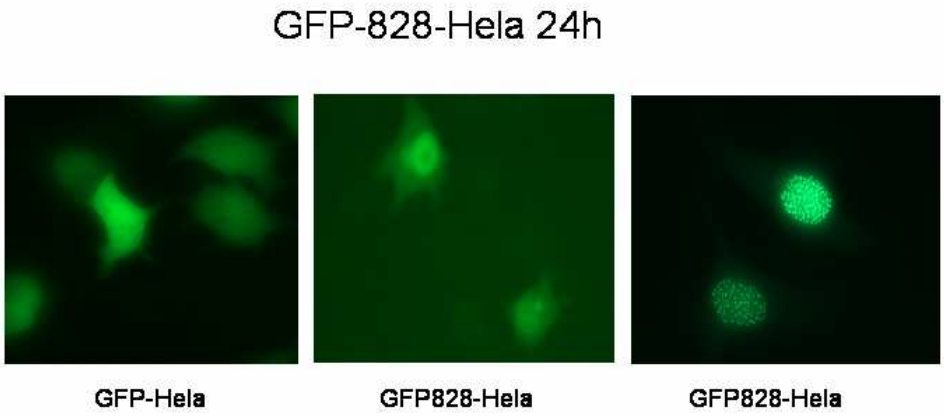


Figure 13: Intracellular nuclear localization of Bcl2-L12. Hela cells were transiently transfected with GFP or GFP-Bcl2-L12 (828) (0.3 µg), images were obtained 24 h after transfection by Fluorescence Microscope, 400x Magnification.

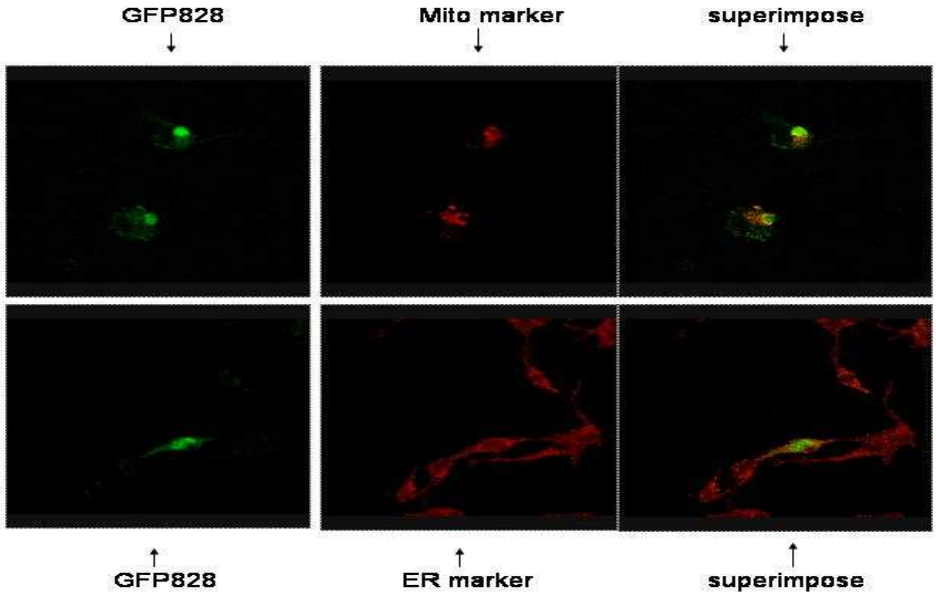


Figure 14A: Mito tracker red, ER staining showing nuclear localization of Bcl2-L12. Hela cells was transiently transfected with GFP-Bcl2-L12(828), images were obtained 24 hours after transfection,Mito tracker red and ER staining were used and visualized by CLSM, 63x.

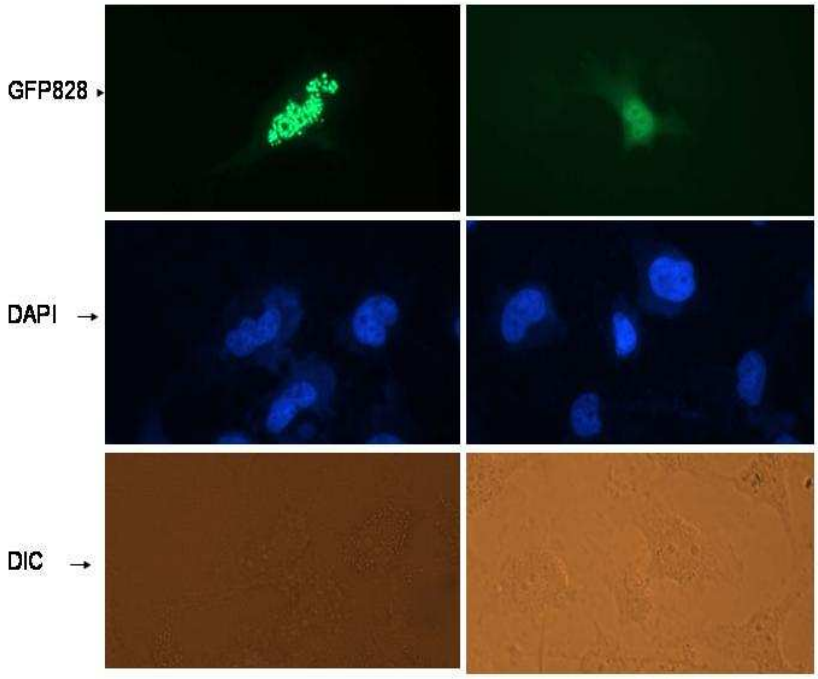
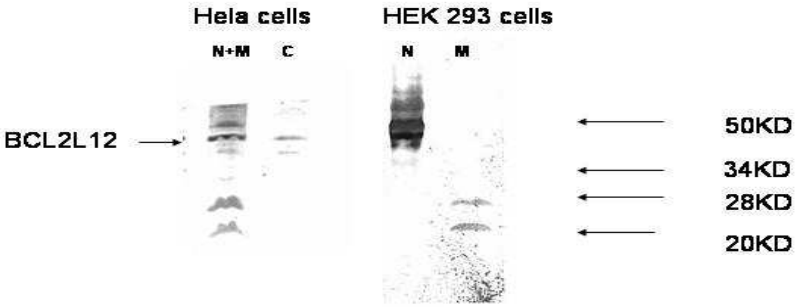


Figure 14B: DAPI immunofluorescence staining clearly demonstrates the nuclear localization of BCL2L12. GFP- Bcl2-L12(828) was transiently transfected to Hela cells and the results were visualized by Fluorescence Microscope at 400x magnification



western blotting of Bcl2l12 with fractionated cell lysates.
N+M: Nuclear and membrane fractions.
C: cytoplasmic fraction
N: Nuclear fraction
M: membrane

.

Figure 15: **Bcl2-L12 Weston bloting results.** Hela and 293 cells were transfected with GFP- Bcl2-L12, after 2 weeks G418 selection cells were collection and protein were purified by unclear extraction kit.

4.2.2. Bcl2-L12 can enhance the S phase of the cell cycle.

Individual Bcl2 family members may combine regulation of apoptosis and control of the cell cycle in unique ways, but nothing is known about the involvement of Bcl2-L12 both in apoptosis and cell cycle control. FACS analysis of HEK 293 cells transfected with GFP-Bcl2-L12 24 hours and 36 hours after PI staining revealed that GFP- Bcl2-L12 increases G1/S phase. As compared with HEK293 transfected with the empty vector (pLEGFP) as a control, GFP-Bcl2-L12 cell fraction of S phase proceeded about 8 % at 24 h and 5 % at 36 h (Fig.16& Fig.17), whereas G1 decreased at 24 h from 52.7 % to 40.7 %, from 51.4 % to 51.9 % at 36 h, but G2 from 15.6 % growth to 20.4 % at 24 h and decreased at 36 h from 13.6 to 11.6 (Figure 16& Figure 17) respectively. The S phase represents the phase with DNA synthesis. Therefore, increased S phase content means that the replication of DNA is promoted. Taking the localization of Bcl2-L12 in the cell´s nucleus, we may thus speculate that Bcl2-L12 could be involved in the regulation of cell cycle control and DNA replication.

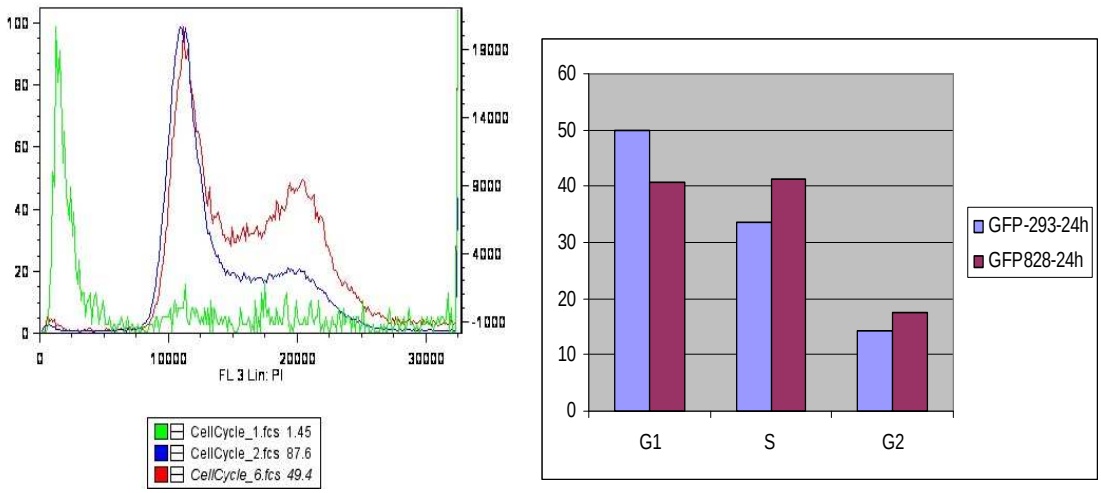


Figure 16: Bcl2-L12-GFP positive fraction 24h transfection FACS analysis date. Left: The red line represents GFP-Bcl2-L12. The blue line represents is the 293-GFP-positive control, the green line is 293 negative control. Right; Blue column is GFP-293 control; Red column is GFP-Bcl2-L12.

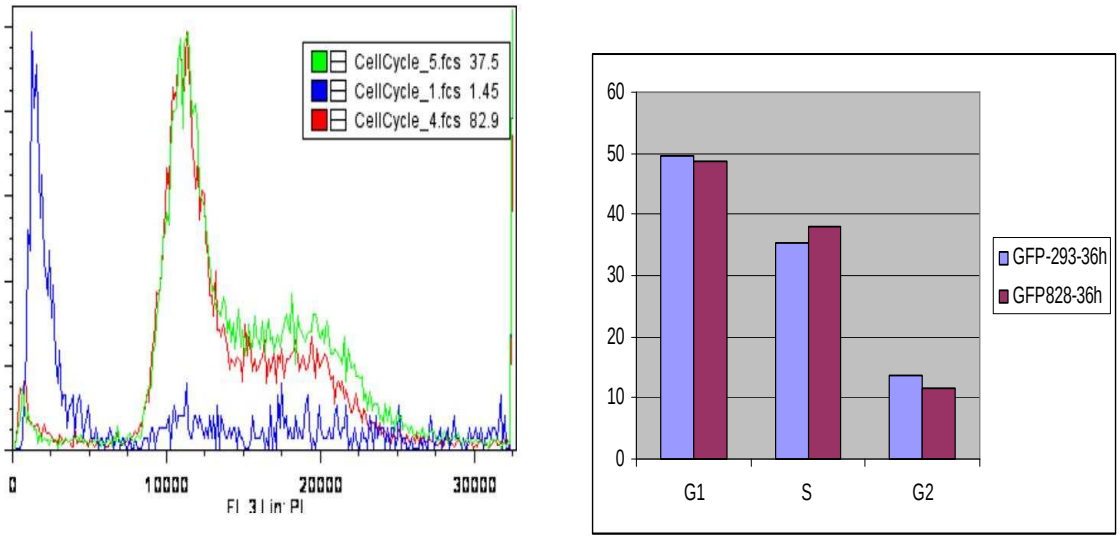


Figure 17: FACS analysis of GFP-Bcl2-L12 (828) transiently transfected to HEK 293 cells and gated in GFP-positive fraction. Blue: GFP control; Red: GFP-828.

4.2.3. Bcl2-L12 has an anti-apoptotic effect in MG 132-induced HEK 293 cell apoptosis.

In order to determine Bcl2-L12's function in apoptosis, we induced apoptosis using staurosporine (STS) or MG 132 (carbobenzoxy-L-isoleucyl-gamma-t-butyl-L-glutamyl-L-alanyl-L-leucinal to treatment HEK 293 cells. First, we tried with various concentrations of STS (1 μM, 500 nm, 200 nM, 100 nM) and MG 132 (500 μM, 250 μM, 100 μM). 12 hours after transfection with GFP-Bcl2-L12, we started to treat the transfected cells with STS or MG 132 for an additional 16 hours. Then, these cells were collected by trypsinization, stained with PI and analyzed with FACS. The FACS results demonstrated that treatment with 100 μM MG132 for 12 h, the sub G1 population decreased through apoptosis from 53.2 % to 44.9 % after transfection with Bcl2-L12. (Figure 18) Therefore, the results achieved

with FACS suggest that Bcl2-L12 inhibits the proteasome-induced apoptosis (MG 132) and thus protects the cell against apoptosis. Furthermore, we were able to demonstrate the effect of Bcl2-L12 on the cell cycle after treatment of transfected HEK293 cells with MG 132. As Table2 and Fig. 19 both demonstrate, the transfection of HEK293 cells with Bcl2-L12 provokes a shift in the cell cycle from the S phase to the G2 phase, when treatment with MG 132. These datas (Figure 18 & Figure 19) conclusively indicate that Bcl2-L12 possesses an inherent anti-apoptotic activity. This pathway seems to be ubiquitin-mediated as demonstrated by the involvement of a proteasome-proteolytic system.

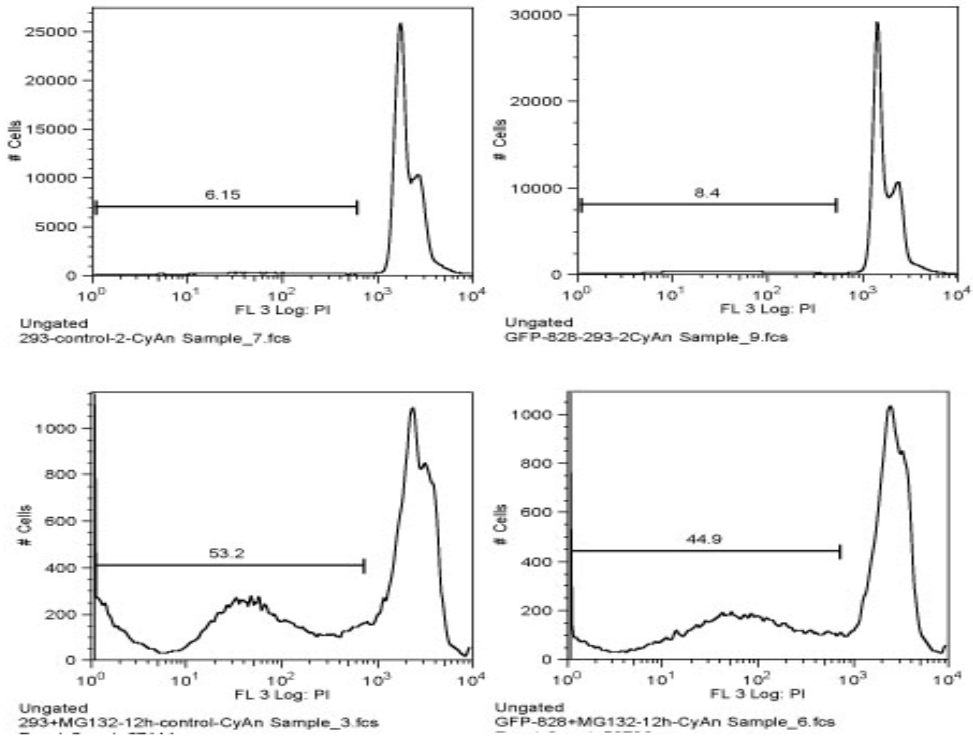


Figure 18: Bcl2-L12 has an anti-apoptotic effect in HEK 293 cells treated with 100 µM MG 132 to induce apoptosis. HEK 293 cells were transfected with GFP-Bcl2-L12 and treated with 100 µM MG 132 for 12 h. FACS analysis was performed and the size of the sub G1 population was analyzed in 4 different treatment groups.

Measurement of the cell cycle with FACS after treatment with STS of empty vector-transfected HEK 293 cells demonstrated that the subG1 population approximated 38.4 % after 24 hours. After transfection of the HEK 293 cells with GFP-828 (Bcl2-L12) the subG1 population approximated 40.8 %, indicating that Bcl2-L12 did not exert any anti-apoptotic effect (Figure 20). Whereas Bcl2-L12 exerted an anti-apoptotic effect in MG 132 induced apoptosis, no such an effect in STS-induced apoptosis was observed.

Table2: Cell cycle analysis of MG132 induced apoptosis in HEK 293 and transfected with GFP-Bcl2L12

Group	Cell cycle		
	G1 phase	S phase	G2 phase
293 control	52.4%	32.2%	13.4%
293+MG 12h	40.1%	38.5%	20.8%
GFPBcl2L12-293	50.2%	38.6%	10.6%
GFPBcl2l12+MG 12h	30.5%	45.9%	23.4%

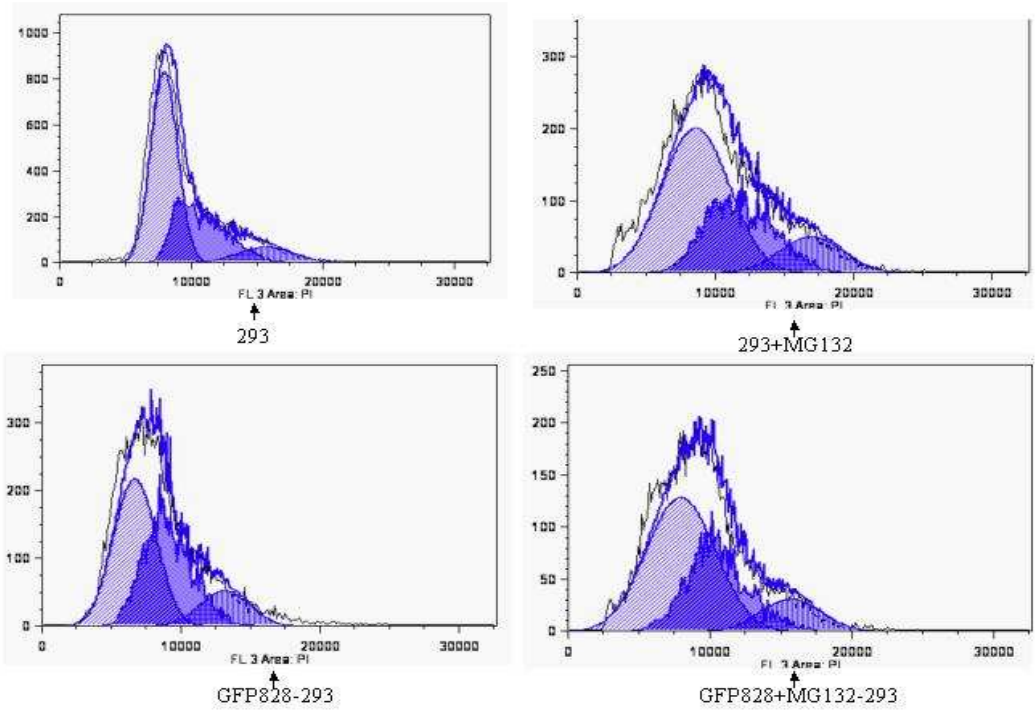


Figure 19: Cell cycle comparison of MG 132 treatment in HEK 293 cells. FACS data were analyzed with the FlowJo Version 8.1 software and Watson Pragmatic model special for cell cycle was used. G1, S, G2 phase have been auto-calculated in different blue shadow as shown in Table 2.

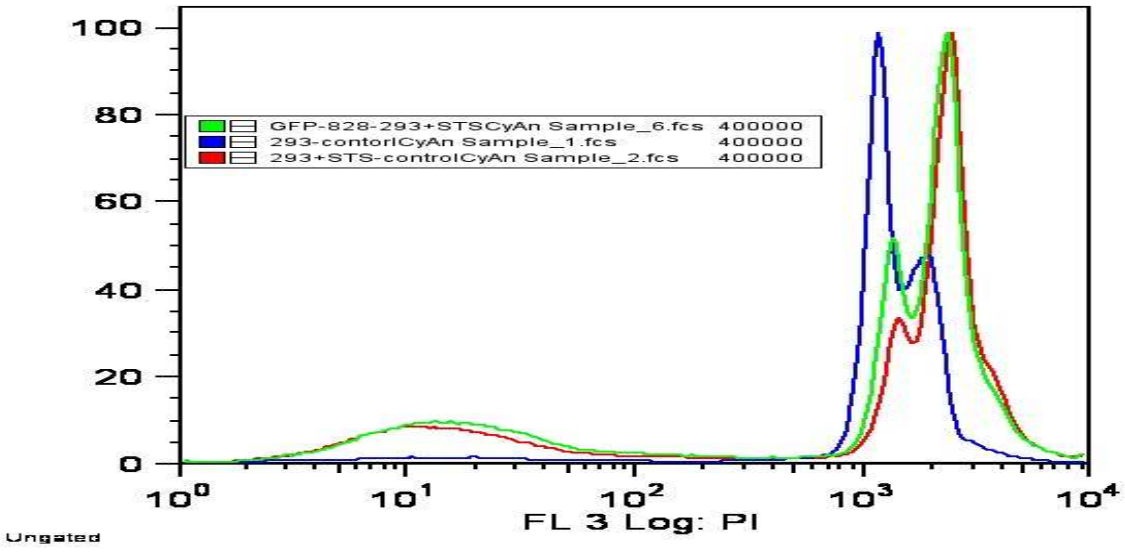


Figure 20: FACS assayed for sub-G1 DNA content of STS treated 293 cells.

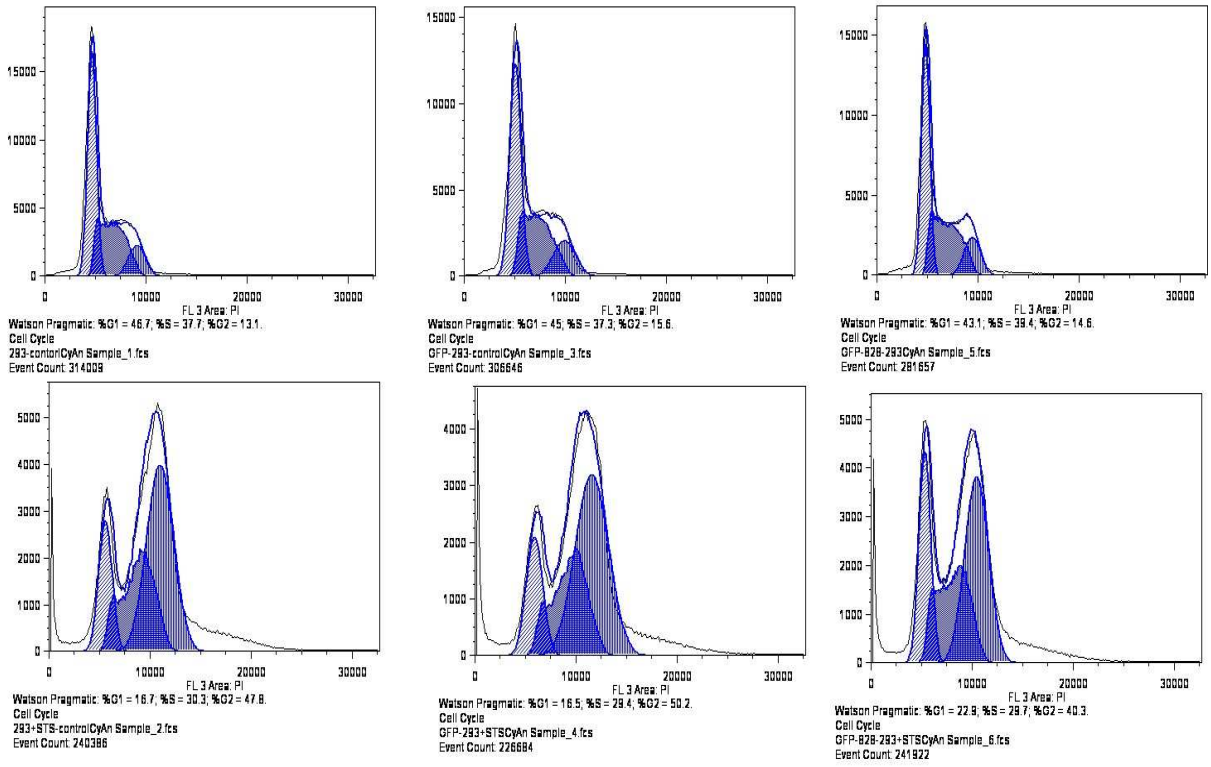


Figure 21: Cell cycle analysis of the STS-treated pEGFP-Bcl2-L12 transfected HEK 293.

4.2.4. Regulation of Bcl2-L12 by other members of the Bcl-2 family

The study of the interaction of Bcl2-L12 with the other members of the Bcl2 family could provide a better understanding of its role in regulating apoptosis. Therefore, we co-transfected GFP-Bcl2-L12 with other anti-apoptotic proteins, such as Bcl2, Bcl2-xL, with pro-apoptotic proteins, such as Bax, Bid, tbid, hBok. The effects of co-expression of these genes of the Bcl2 family with that of Bcl2-L12 was demonstrated with immunofluorescence staining.The results are depicted in Figure 22. Except for bid, co-transfection of Bcl2-L12 with Bcl-xL, tbid or Hbok induced the export of Bcl2-L12 from the nucleus to the

cytoplasm and into the cytosolic organelles. In order to further identify into which organelle the signal was exported, special stainings of the ER, the mitochondria and the Golgi apparatus were made (Fig.23). When we co-transfected GFP-Bcl2-L12 with Bcl2-xL and performed staining of the mitochondria, ER and the Golgi apparatus, the CLSM images results clearly showed the original nuclear signalling has moved to outside the nucleus. However, when we used STS to induce apoptosis in cells co-transfected with both GFP-Bcl2L-12 and Bcl-xL, the cytosolic GFP-Bcl2-L12 signalling was depleted and the GFP-Bcl2-L12 was translocated into nucleus again (Fig. 24). Co-transfection of Bcl2-L12 with other Bcl2-family members, such as Bcl2 and Bax, lead to a redistribution of Bcl2-L12 from the nucleus into the cytoplasm (Fig. 23). All these findings clearly demonstrate that Bcl2-L12 can interact with both anti- and pro-apoptotic members of the Bcl2 family. The same experimental setting was also used to examine whether Bcl2-L12 is able to interact with other important genes of the apoptotic pathway, such as p53, E2F and Rad9. However, we did not find any evidence for an interaction of Bcl2-L12 with these proteins (data not shown).

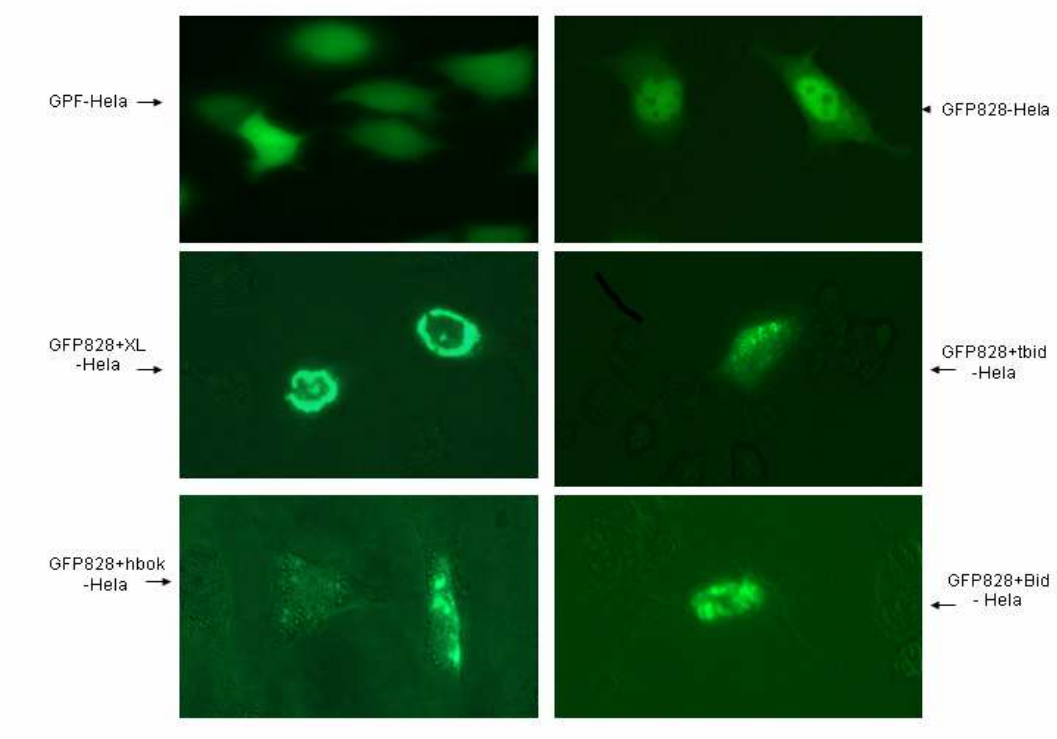


Figure 22: GFP828 co-transfection with $Bcl2_{XL}$, Bid, tbid, Hbok for 24 h. Subcellular localization was observed in Hela cells. The original magnification is 40x.

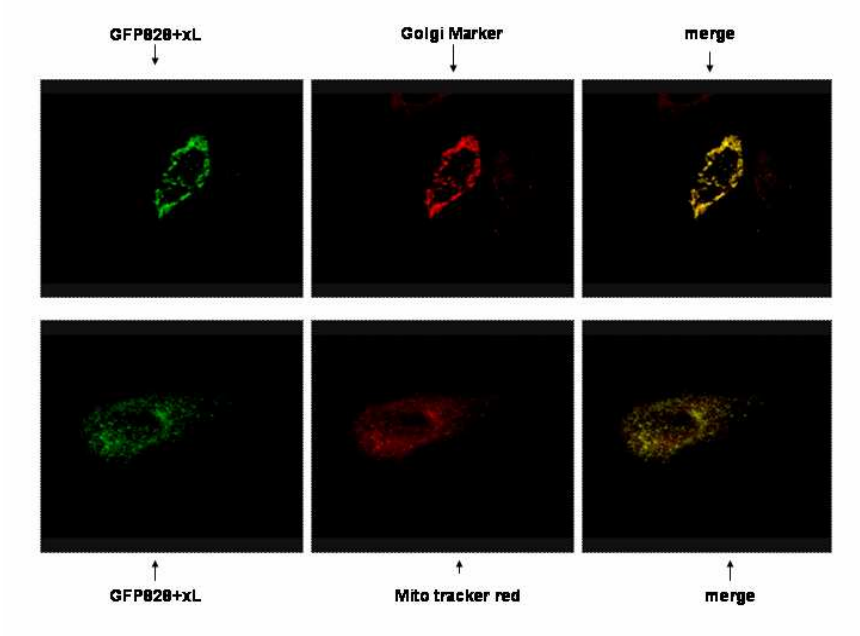


Figure 23: GFP828 co-expression $Bcl2_{XL}$. Mitochondria (Mito tracker red), endoplasmic reticulum (ER) and Golgi staining. HEK 293 cells were transfected with GFP828 and $Bcl2_{XL}$ for 24 h. Different stanings were performed and images were taken using a CSLM. Original magnification were 400x and 63x.

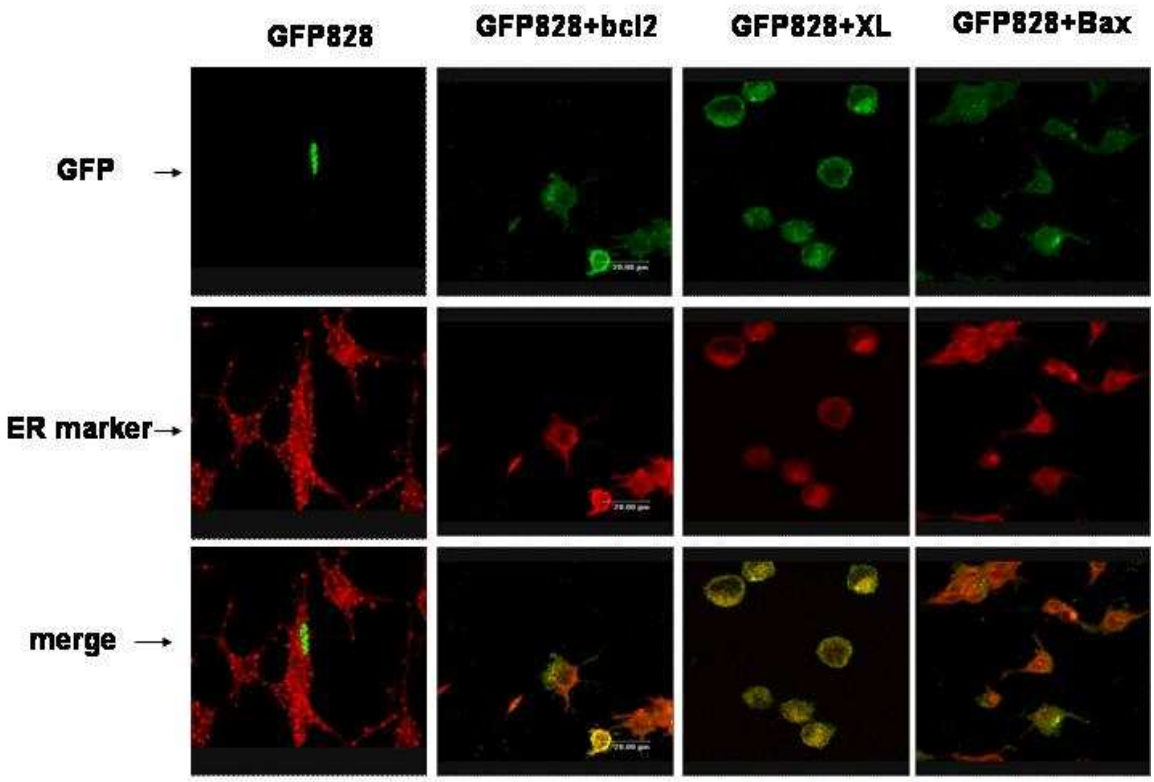


Figure 24: Bcl2-L12 co-expression with Bcl_{XL}, Bax and Bcl2: CLSM images of ER (63x)

To evaluate the anti-apoptotic function of Bcl2-L12, we transfected pro-apoptotic genes such as Bax, Bid, tbid, hBok together with GFP-Bcl2-L12 and we then counted the number of apoptotic cells under the microscope both after 24 h and after 48 h. The percentage of viable cells was determined by trypan blue exclusion, thereby counting at least 100 cells from each individual culture. The percentage of cell survival was calculated as the number of surviving cells per total cell count in three separate fields. The percentage of cell death was quantitatively analyzed by comparing the number of viable cells in the co-transfected cells to the number of viable cells transfected with Bcl2-L12 only. The results are depicted

in Figure 25. We observed that Bcl2-L12 indeed possesses an inherent anti-apoptotic effect, especially 48 hours after co-transfection with bid. Similar results were achieved with tbid and hBok (data not shown).

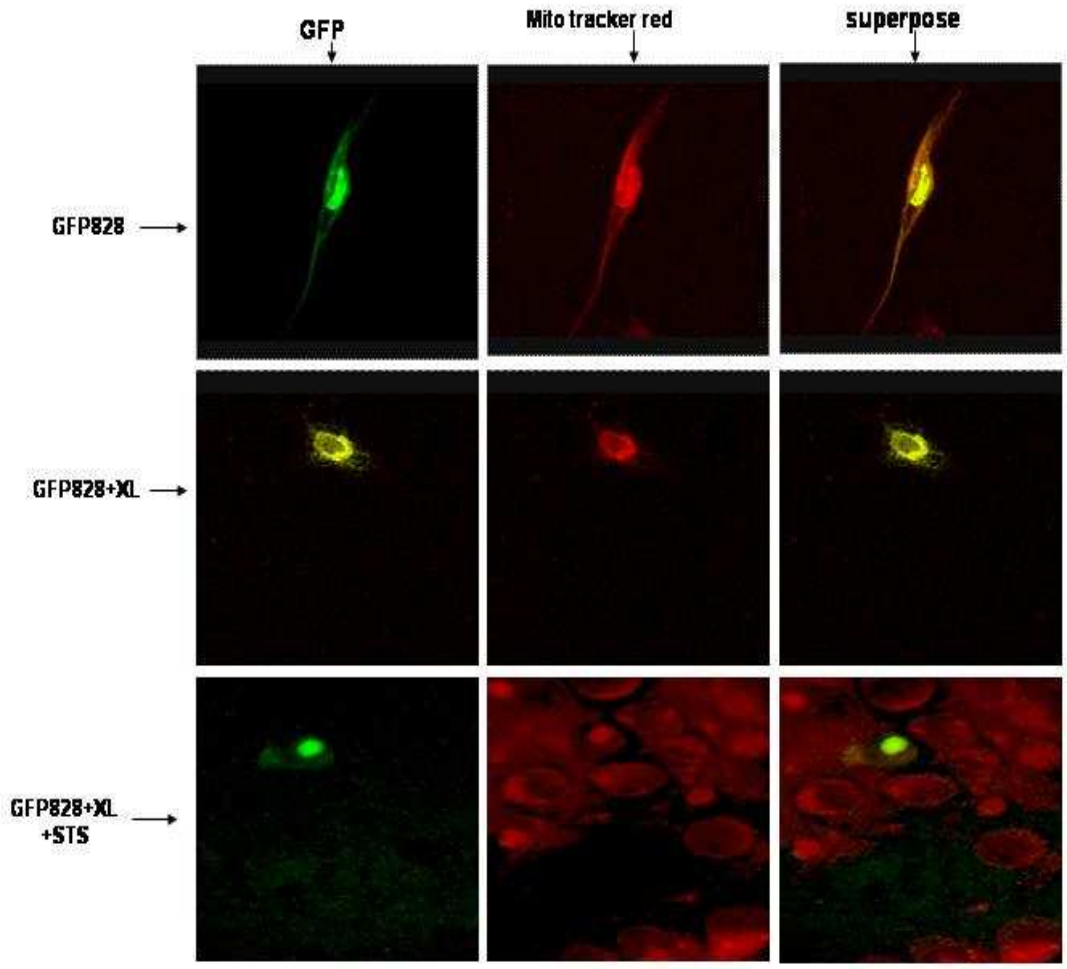


Figure 25: GFP828 co-transfection with $Bcl2_{XL}$. MitoTracker Red CLSM. Magnification 63x

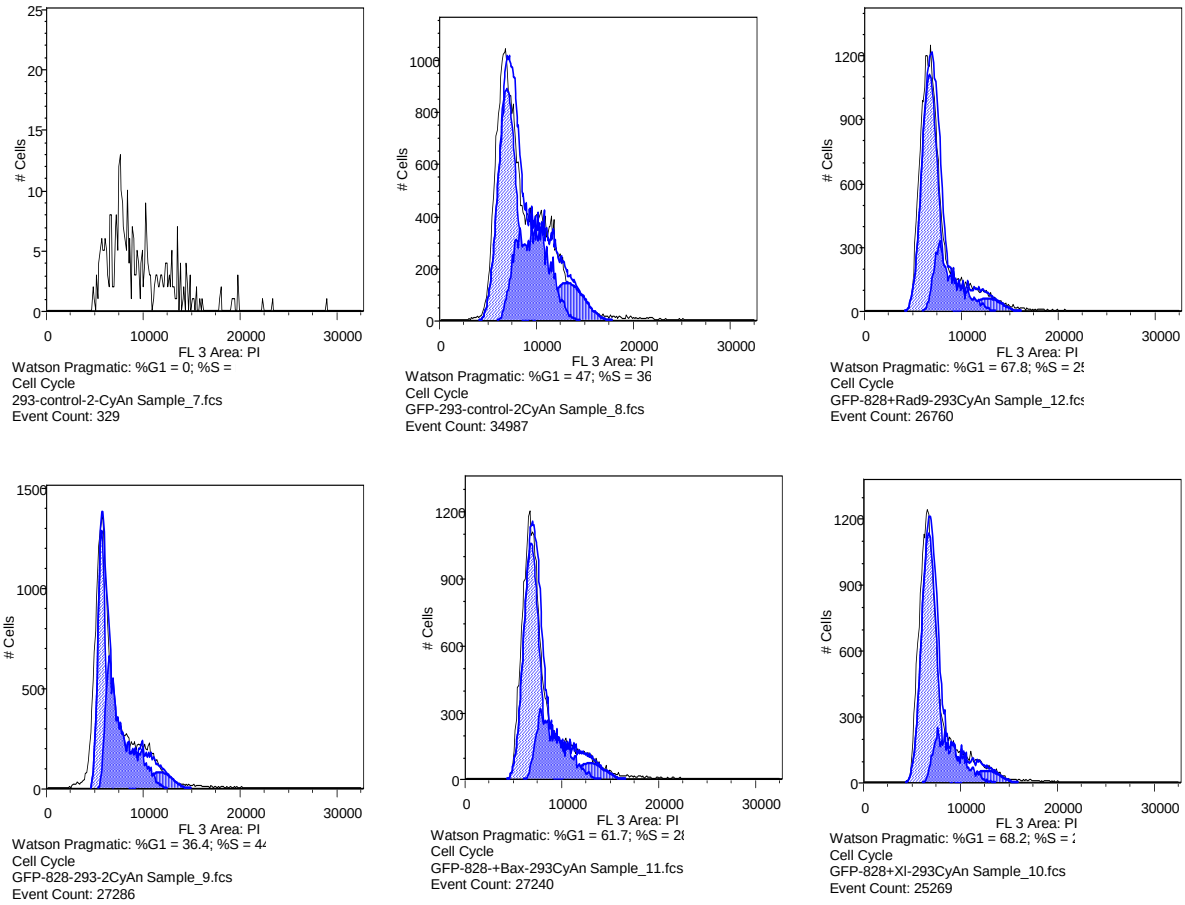


Figure 26: FACS analysis with FlowJo Version 8.1 software and Watson Pragmatic for cell cycle modell was used. Bcl2-L12 co-transfection with $Bcl2_{XL}$, Bax, Rad9 to HEK293 cells, 24 h later $5x10^5$ cells were PI-stained and gated GFP-positive fraction for cell cycle analysis.

By using FACS analysis we examined whether Bcl2-L12 co-transfection with other Bcl2 family members would also affect the cell cycle. The FACS results of GFP-tagged Bcl2-L12 co-transfection together with $Bcl2_{XL}$, Bax or Rad9 in HEK293 cells indicated that a subfraction of the transfected cells underwent a shift of the S phase to G1 (Figure 26). Consequently, the results provided by the co-transfection of Bcl2-L12 together with $Bcl2_{XL}$ and others clearly demonstrated that the nuclear signalling of Bcl2-L12 can be inhibited by

other members of the Bcl2 family. Bcl2-L12 is transported to various cytosolic organelles such as the mitochondria and others. Consequently, Bcl2-L12 can interact with most of the Bcl2 family members implicating that Bcl2-L12 possesses a functional domain with docking site for the transmission of nuclear signals. But what is this critical functional interaction domain is needed further investigation. In addition, in order to determine Bcl2-L12's anti-apoptotic function, we co-transfected Bcl2-L12 with proapoptotic protein Bid and afer 48h transfection, cell viability was performed (Figure 27) the result showing that Bcl2L-12 have the anti-apoptotic function when using apoptotic reagents.

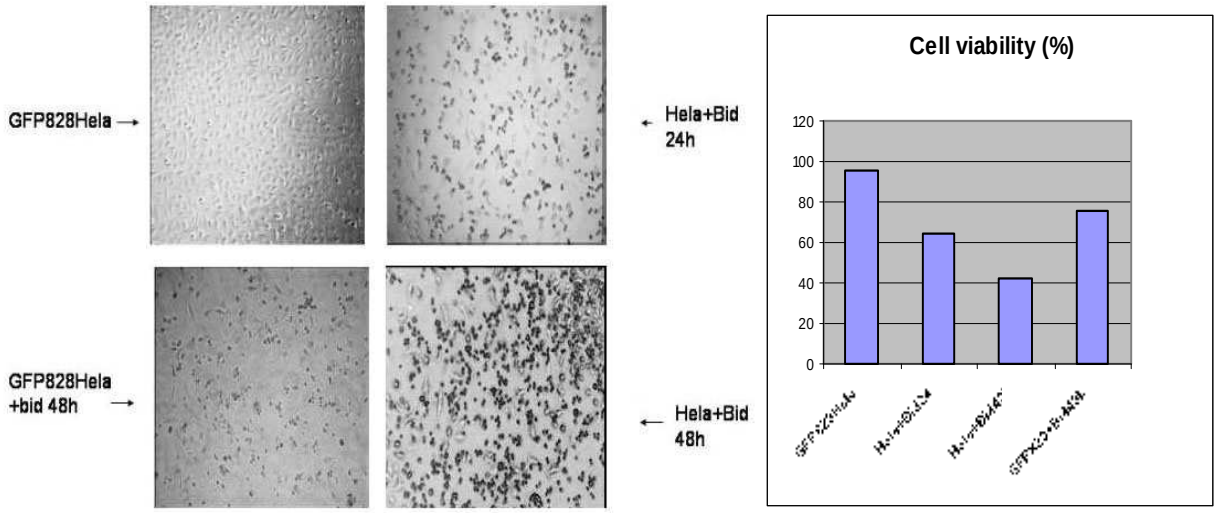


Figure 27: DIC images of GFP- Bcl2-L12(828) co-transfection with Bid 24 h, 48 h (left) apoptotic cells is in darker colour and percentages of survival cells were determined by trypan blue exclusion. Viability was determined at various time points by trypan blue exclusion, counting at least 100 cells from each individual culture (right).

4.2.5. The Bcl2-L12 deletion mutants and their functions

The Bcl2-L12 protein was found to have proline-rich sites. One PPPP site as well as five PP amino acid sites was found to be present in this protein. Eight putative PXXP motifs were also identified (Figuer 28). In order to further elucidate each domain‘s function, we cloned five deletion mutants of Bcl2-L12 (Figure 28). Full length Bcl2-L12 contains 828 bp. In the so-called “deletN mutant” is the N terminal domain was deleted and contains only the BH2 domain and one PPPP domain (Bcl2-L12-deletN). In the socalled “Delet 12cc” mutant we deleted 202 to 230 amino acids leading to a coiled-coli structure-like domain (Bcl2-L12 delet 12cc). The deletBH2 is truncated in the C-terminal, the N-terminal, and BH2-domain, contains only PGPPPPSP and IRF-FID (Bcl2-L12-delet BH2). The PxxP mutant was constructed using mutagenesis PCR and the PGPPPPSP sequence was mutated to PGPXXPSP(Bcl2L12mutePxxP)(Figure28).

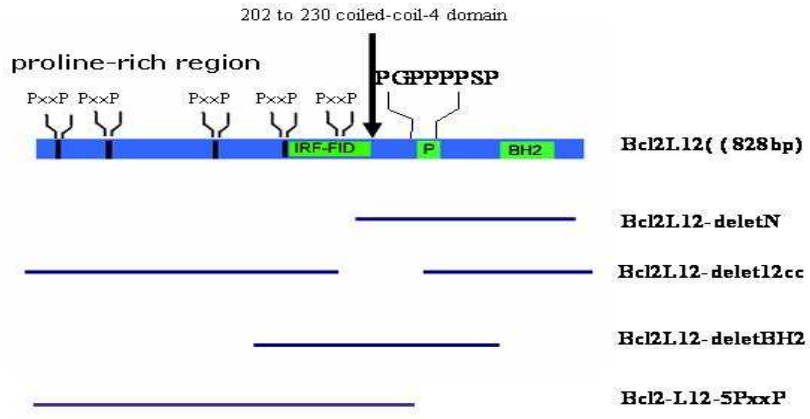


Figure 28: Representation of Bcl2-L12 and the 4 deletion mutant constructs

In order to determine the relationship between the structure of Bcl2-L12 and its localization within the cell, we transfected four deletion mutants into HEK 293T cells. The results are presented in Figure 29. Except for Bcl2-L12, which is expressed strongly only in the nucleus, the Bcl2-L12-deleted N-terminal resides also weakly both in the nucleus and the cytosol (Fig. 29) and both of these have a commonBH2 domain. The BH2delet showed pointed signalling in the cell center region and 12cc mutated variants are found both only in the cytosolic compartments of the transfected cells. The 5 PXXP without BH2domain is localized specifically within the nucleus region or close to the nuclear membrane (Figuer 29). The result from 12cc (coiled-coil domain) deletion mutants showed that the target suppressed nuclear signalling, therfore the domain we deleted is a key domain structure for the NES.

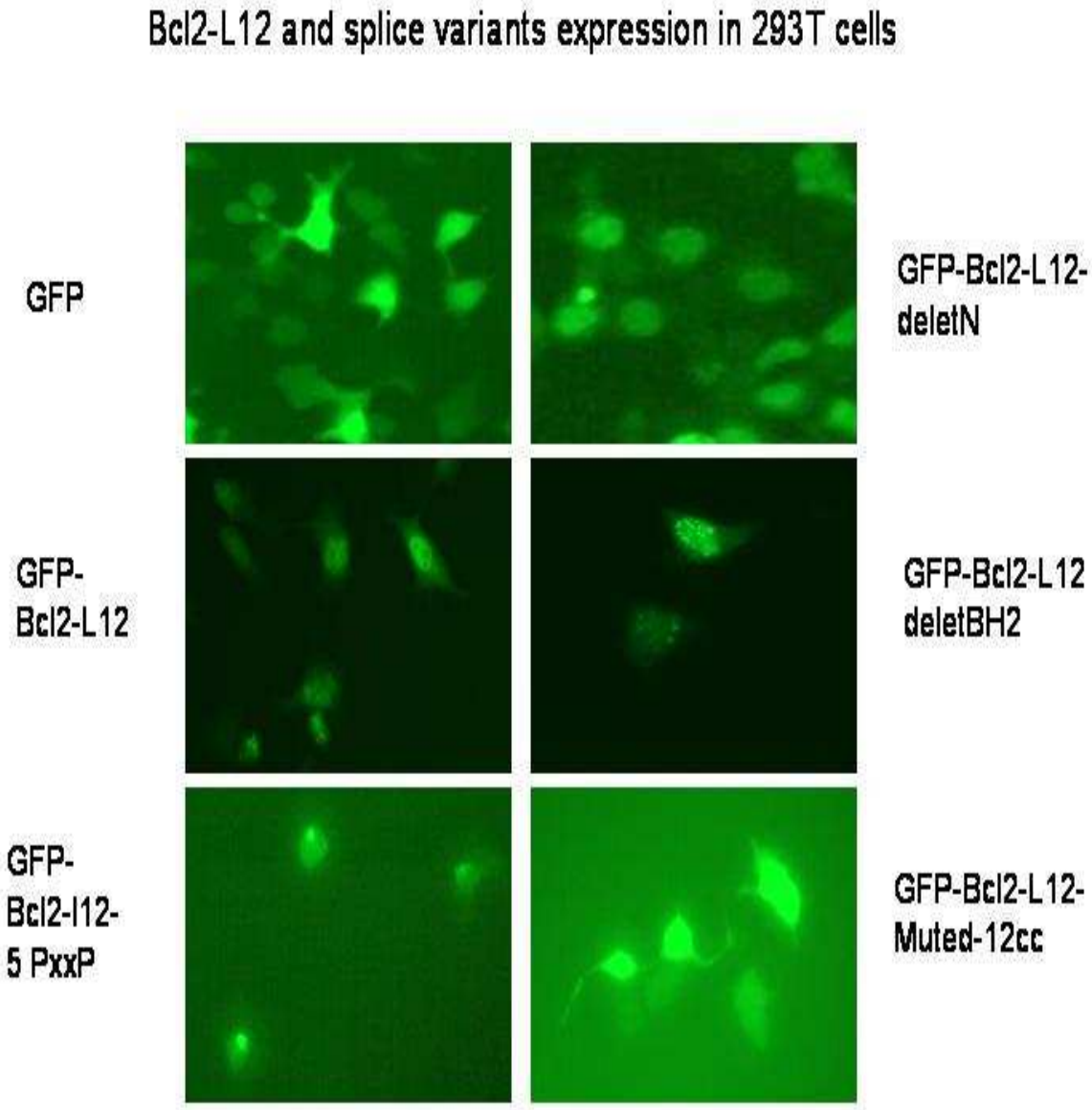


Figure 29: Subcellular localization of Bcl2-L12 their splice variants. HEK 293T cells were transiently transfected with GFP or GFP- Bcl2-L12 and 4 variants, fusion protein was viewed 24 h later by fluorescence microscopy.

Table 3: Bcl2-L12 and its splice variants transfected to HEK 293T and GFP-positive fraction cell cycle G1, S, G2 phase distribution (from Fig. 30)

Bcl2L12 deletion mutants	G1	S	G2
GFP 828 (Bcl2-L12)	29.8	59.2	11.0
GFP 828 deleted N	22.9	62.7	14.4
GFP 828 Muted 12cc	10.0	74.1	15.9
GFP 828 5 PXXP	27.6	64.6	8.9
GFP 828 deleted BH2	13.9	71.4	14.7
GFP-293T control	35.5	53.9	10.6

Further, we analyzed the effect of these Bcl2-L12 deletion mutants on the cell cycle using FACS. The results demonstrated that the splice variants induced a shift in the cycle cycle to the S phase. This experiment suggests that the splice variants of Bcl2-L12 induce more DNA synthesis.

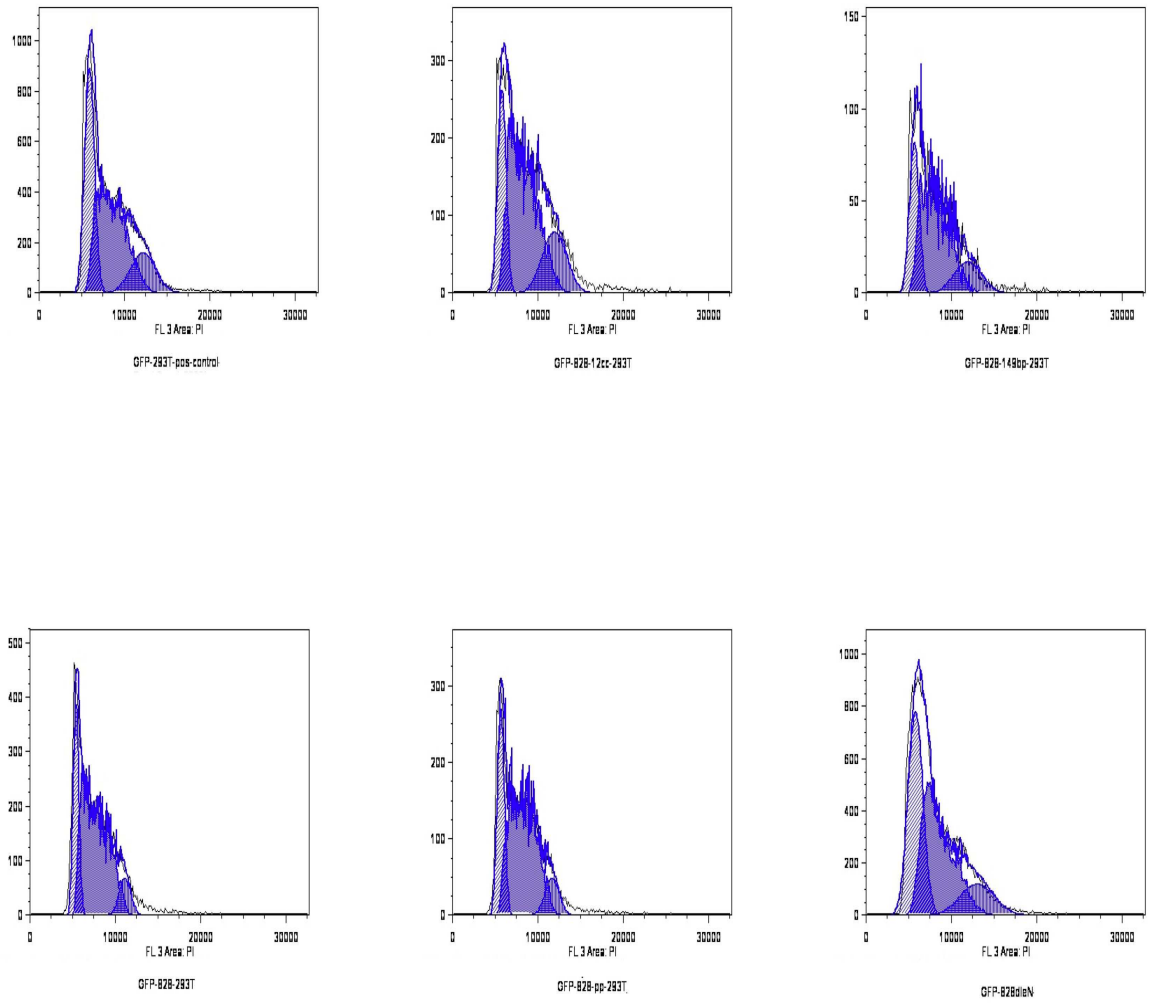


Figure 30: Bcl2-L12 and its splice variants transfected to HEK 293 and GFP-positive fraction cell cycle analysis data (similar as Figure 5.4)

4.3. Cloning, expression analysis, and structural characterization of a novel human ADAMTSs, Adamts-16 , a family of metalloproteinases with disintegrin and thrombospondin-1 domains and induction of its expression with FSH

4.3.1 Molecular Cloning of Human ADAMTS-16 and its variant ADAMTS-16s

During the search of our ovarian-rich gene expression database, OR0021 was identified based on its high expression levels in the ovary (see below). This gene has been designated as ADAMTS-16, a member of the family of proteases, jointly denominated ADAMTS. By screening of the EST database followed by PCR, we identified the full length cDNA in which 3' sequence was missed previously (Cal et al., 2002). In addition to that, we identified a novel splicing variant (ABB70405) and termed it ADAMTS-16s for the short splicing form of the ADAMTS-16 transcript. Sequence analysis revealed that the full length ADAMTS-16 exibihts the typical ADAMTS modular structure (containing signal sequence, propeptide, metalloproteinase domain, disintegrin-like domain, central TS1 motif, cysteine-rich region and a C-terminal module with several TS1 submotifs) and that ADAMTS-16s encoded an open reading frame of 570 amino acids, containing only the peptidase domain (Figure31). Sequencing of AK031314 from NCBI provided an ORF encoding the full length mouse ADAMTS-16. The human and mouse nucleotide and predicted amino acid sequences have an overall identity of 86 and 91 % respectively (Figure 31).

The full-length cDNA sequence of human ADAMTS-16 was mapped by BLAST comparison to BAC clones CTD-2297D10 (GenBank acc. no. AC022424) and CTC-485I21 (AC010269) on human chromosome 5p15. ADAMTS-16 appears to consist of 23 exons spanning 180 kb of genomic DNA (Table 4). Thus, ADAMTS-16 s is an alternative splicing form, that arises from the read-through of the exon 11 splice donor to a site within the ensuing intron.

Table 4: Human ADAMTS-16 gene structure

Exon No.	Exon size (bp)	Position in cDNA	Intron size (bp)	Splice acceptor	Splice donor
1	72	1-72	124	5' UTR	AGCAG**gt**gag
2	103	73-175	5363	cgc**ag**GCACC	GGGCG**gt**aag
3	326	176-501	35588	ttc**ag**AATAT	GCTTG**gt**gag
4	262	502-763	3746	tcc**ag**TCAGG	GAAAT**gt**atg
5	200	764-963	1473	cat**ag**ACATG	ACATG**gt**agg
6	84	964-1047	2162	ttc**ag** GTATC	AACAG**gt**agt
7	160	1048-1207	1554	cac**ag**CCAGG	TTTGG**gt**gag
8	106	1208-1313	8341	cac**ag**GATTT	CACAA**gt**aag
9	138	1314-1451	8823	cat**ag**CTTTG	CTAAG**gt**agg
10	154	1452-1605	13542	ttc**ag**CACCG	AAAAG**gc**aag
11	96	1606-1701	9483	ttt**ag**GACAT	ACATG**gt**aag
12	149	1702-1850	2497	ttt**ag**TGGTG	CCCAA**gt**aag
13	173	1851-2023	1782	tcc**ag**GCCAT	AGAAG**gt**aaa
14	131	2024-2154	2.051	tta**ag**ATCAG	GTGAG**gt**aat
15	124	2155-2278	406	tcc**ag**AGAGT	CAACC**gt**gag
16	245	2279-2523	2127	tct**ag**AGTAT	TGGAG**gt**aaa
17	139	2524-2662	20465	tgt**ag**CTGCT	AGGGG**gt**agg
18	127	2663-2789	40484	tgc**ag**GACAG	CCCAG**gt**aag
19	202	2790-2991	102	ccc**ag**CTGGT	CAGAG**gt**aac
20	195	2992-3186	2737	tgc**ag**TGCTC	CCCAG**gt**agg
21	225	3187-3411	11405	ttt**ag**TGCTC	CTCAG**gt**agg
22	148	3412-3559	741	cac**ag**TGCAC	GAAAG**gt**gag
23	116	3560-3676	-	ttc**ag**ATGCC	3' UTR

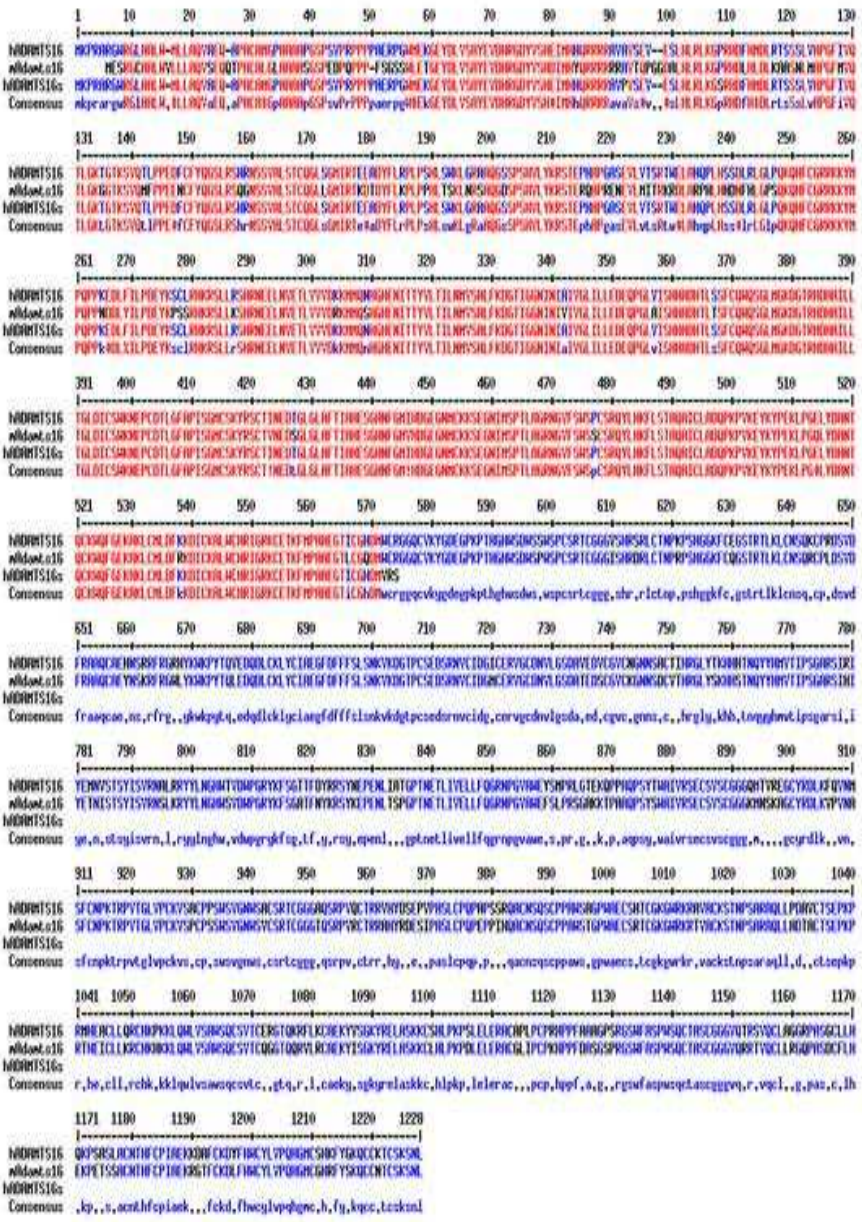


Figure 31*:* The human and mouse nucleotide and predicted amino acid sequences alignment

4.3.2. ADAMTS-16 mRNA is highly expressed in the ovary

To determine the tissue specific expression of ADAMTS-16 in the human, semiquantitative RT-PCR analysis (semi-Q-RT-PCR) was performed by using a pair of primers corresponding to sequences flanking to the first intron. As shown in Figure 32A, ADAMTS-16 was expressed mainly in adult kidney, pancreas and ovary. We subsequently analyzed expression of the ADAMTS-16 transcript in the UniGene (Build #184), expression of ADAMTS-16 was also present in ESTs from brain medulloblastoma, brain amygdala, Lung Focal Fibrosis, lung large cell carcinoma, endometrial adenocarcinoma, retinoblastoma (Wheeler et al., 2003).

These results obtained from RT-PCR analyses were also supported by microarray expression analysis collected from GNF (Genomics Institute of the Novartis Research Foundation) Expression Atlas Chips U133A, GNF1H and Affy U95, confirming that ADAMTS-16 is highly expressed in the ovary, but that it is also expressed to some extend in other tissues (Figure 32B, http://symatlas.gnf.org/SymAtlas).

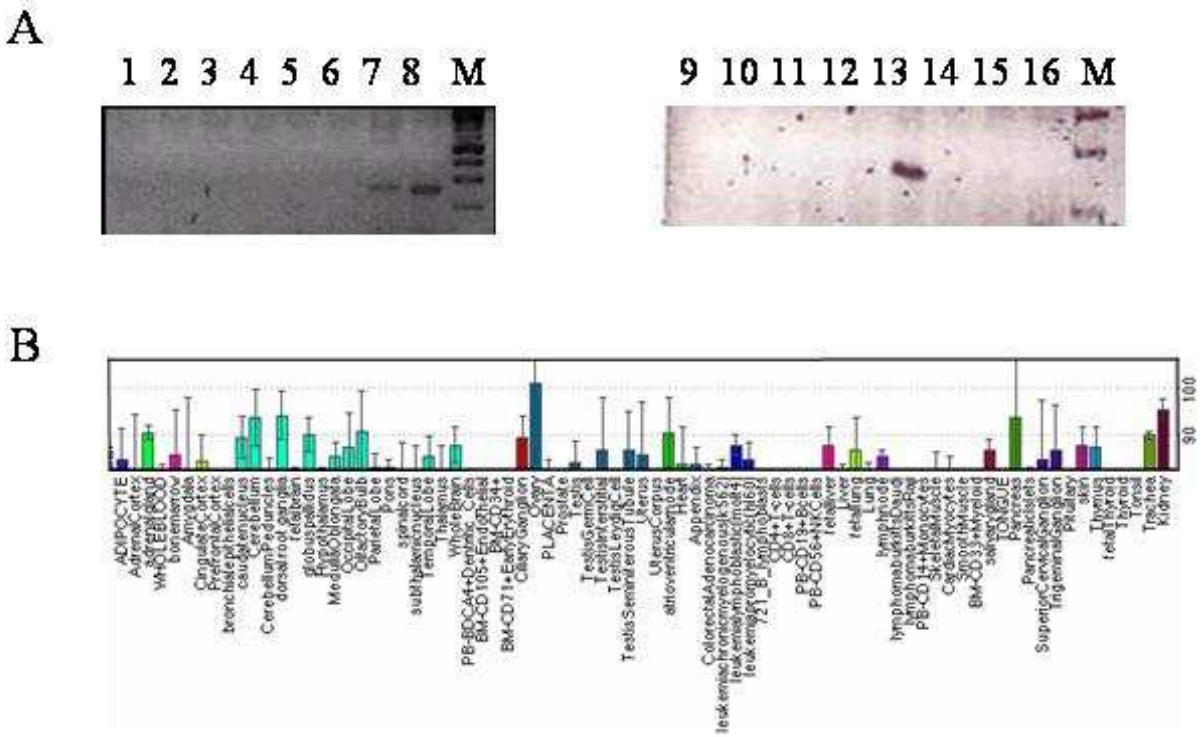


Figure32. The expression level and distribution of *ADAMTS16* mRNA species vary in different tissues. (A) PCR was performed on a panel of first strand cDNAs prepared from different human tissues (Origene). The PCR products obtained from each reaction following 30 cycles were separated on a 1.2 % agarose gel and visualized by serber green I. As a control of the cDNA from each tissue, PCRs (30 cycles) of the housekeeping gene β-actin were performed in parallel. The amounts of the β-actin PCR products are nearly equivalent in the different tissues (not shown). (1) brain, (2) heart, (3) placenta, (4) lung, (5) liver, (6) muscle, (7) kidney, (8) pancreas, (9) spleen, (10) thymus, (11) prostate, (12) testis, (13) ovary, (14) small intestine, (15) colon, (16) PBL. **(B)**. microarray expression analysis collected from GNF (Genomics Institute of the Novartis Research Foundation) Expression Atlas Chips U133A, GNF1H and Affy U95 (http://symatlas.gnf.org/SymAtlas).

4.3.3. Identification of the promotor region of ADAMTS-16

Analysis of the genomic region at the 5′ end of ADAMTS-16 indicates that it has a CpG island (AC022424, nt 28,391– 27,251; %GC = 66.6; O/E = 0.848; no. of CpGs = 112), suggesting that it is likely to be a tissue-specific gene (Figure 33A). The programme PromoterInspector was used to analyze the genomic sequences at the 5′ end of the ADAMTS-16 genes for putative promoter regions. The presence of one strong promoter region of ADAMTS-16 was predicted at nt 27,728–27,478 of AC0022424. Two sp1 sites and one egr-1 site are present in the promoter (Figuer 33B) suggesting that they may mediate the expression during development.

4.3.4. Hormonal regulation of ADAMTS-16 expression

We subsequently examined the effects of both the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) on the induction of ADAMTS-16 mRNA expression in both luteinizing granulosa cells and cumulus cells *in vitro*, both collected from patients treated with exogenous gonadotropins for in-vitro fertilization or intracytoplasmic sperm injection. As shown in Figure33C, ADAMTS-16 mRNA was predominantly detected in luteinizing granulosa cells but a little in cumulus cells. A weak but distinct expression pattern was also found in cells of an immortalized granulosa cell line developed from a granulosa cell tumor (KGN, Nishi et al., 2001). The increase in ADAMTS-16 expression was only detected in the presence of FSH and forskolin (Figure 33D, lane 3-5), whereas LH failed to induce any change in the expression levels of ADAMTS-16 (Figuer 33D, lane 1).

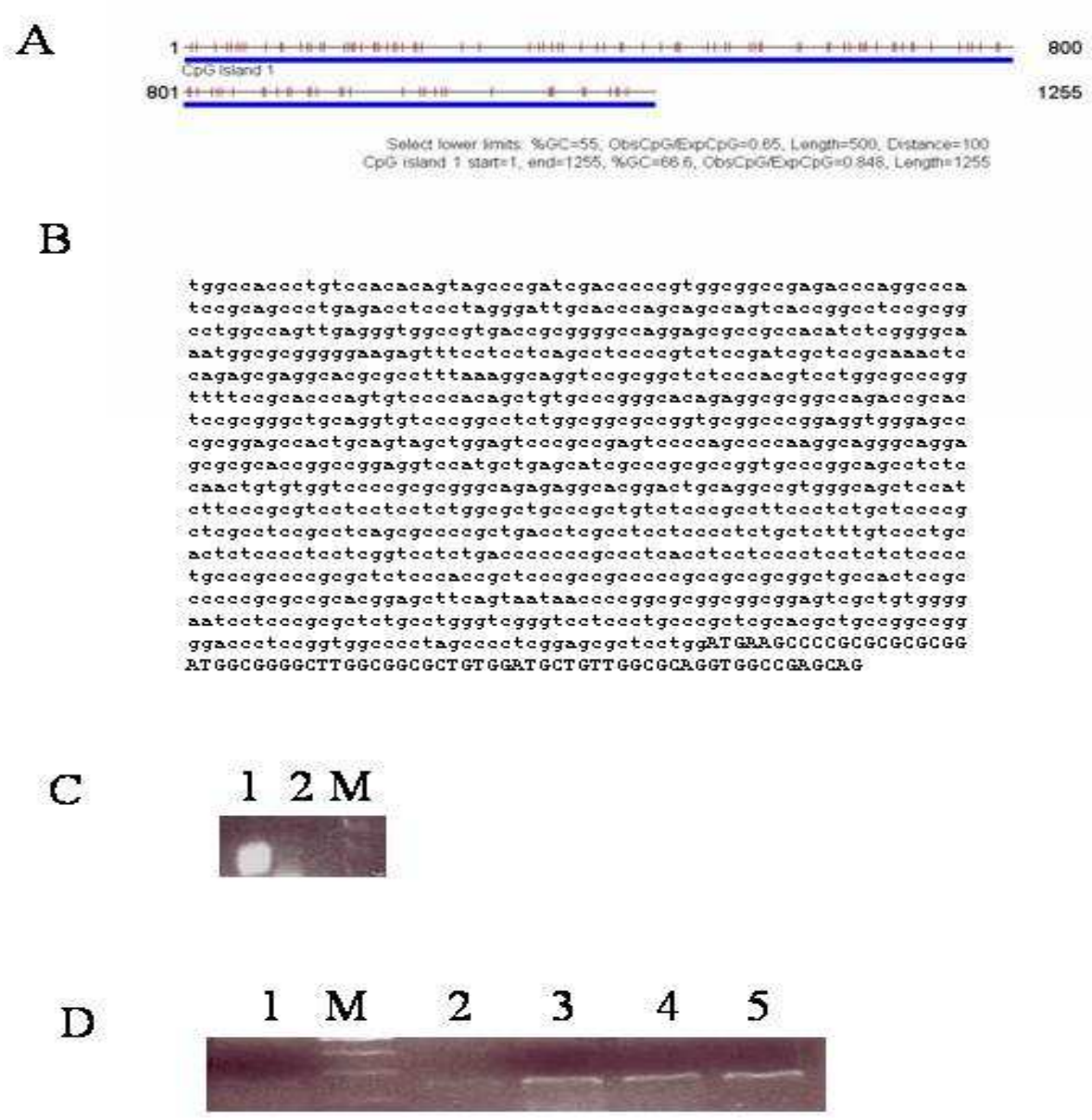


Figure 33: Induction of ADAMTS-16 expression. (A). Analysis of the genomic region at the 5′ end of *ADAMTS-16* using CpGp lot . **(B).** The program PromoterInspector was used to analyze the genomic sequences at the 5′ end of the ADAMTS-16 genes for putative promoter regions. **(C).** PCR was performed on first strand cDNAs prepared from cells isolated from IVF (1) and is not in cumulus. Semiquantitive-based PCRs were performed.

The PCR products obtained from each reaction following 30 cycles were separated on a 1.2 % agarose gel and visualized by serber green I **(D)**. human granulosa cells were collected from infertile women treated with IVF. After 7days in DMEM culture, treatment was carried out. PCR was performed on first strand cDNAs prepared from the cells treated with LH (1), forskolin (3), FSH (4), and FSH+Forskolin (5), (2) served as a control. PCRs (30 cycles) of the housekeeping gene β-actin were performed in parallel. The amounts of the β-actin PCR products are nearly equivalent in the different tissues (not shown).

4.3.5. α_2-macroglobulin is a substrate of ADAMTS-16

Several sequence features of the human ADAMTS-16 are very similar to those of other of the ADAMTS´s family of proteases (Figure 34) including the number of cysteine residues (Porter et al., 2005). Thus, this "trapping" mechanism induces a delayed migration of a_2-macroglobulin in SDS-PAGE upon incubation with a protease and is considered an experimental evidence of proteolytic activity, which is different from the cleavage seen by typsin attack (Kuno et al, 1999). As shown in Figure 34A & B, in the presence of ADAMTS-16 produced either by E. coli (Figure 34A, lane 1 & 2) or by HEK293 (Figure 34B), migration of α_2-macroglobulin on SDS-polyacrylamide gel was altered and conversion of intact α_2-macroglobulin to the 90-kDa species was also observed, indicating the formation of α_2-macroglobulin-ADAMTS-16 complexes and proteolysis of α_2-macroglobulin by ADAMTS-16, although α_2-macroglobulin-ADAMTS-16 complexes were mainly detected in HEK293 (Figure 34B, lane 1). In contrast, high levels of α_2-macroglobulin could not form high order complexes in the absence of ADAMTS-16 (Figure 34B, lane 2). In the control experiments, trypsin attacks sites different to ADAMTS-16 and does not form complexes with ADAMTS-16 (Figure 34C). These studies demonstrate that ADAMTS-16 was able to cleave α_2-macroglobulin.

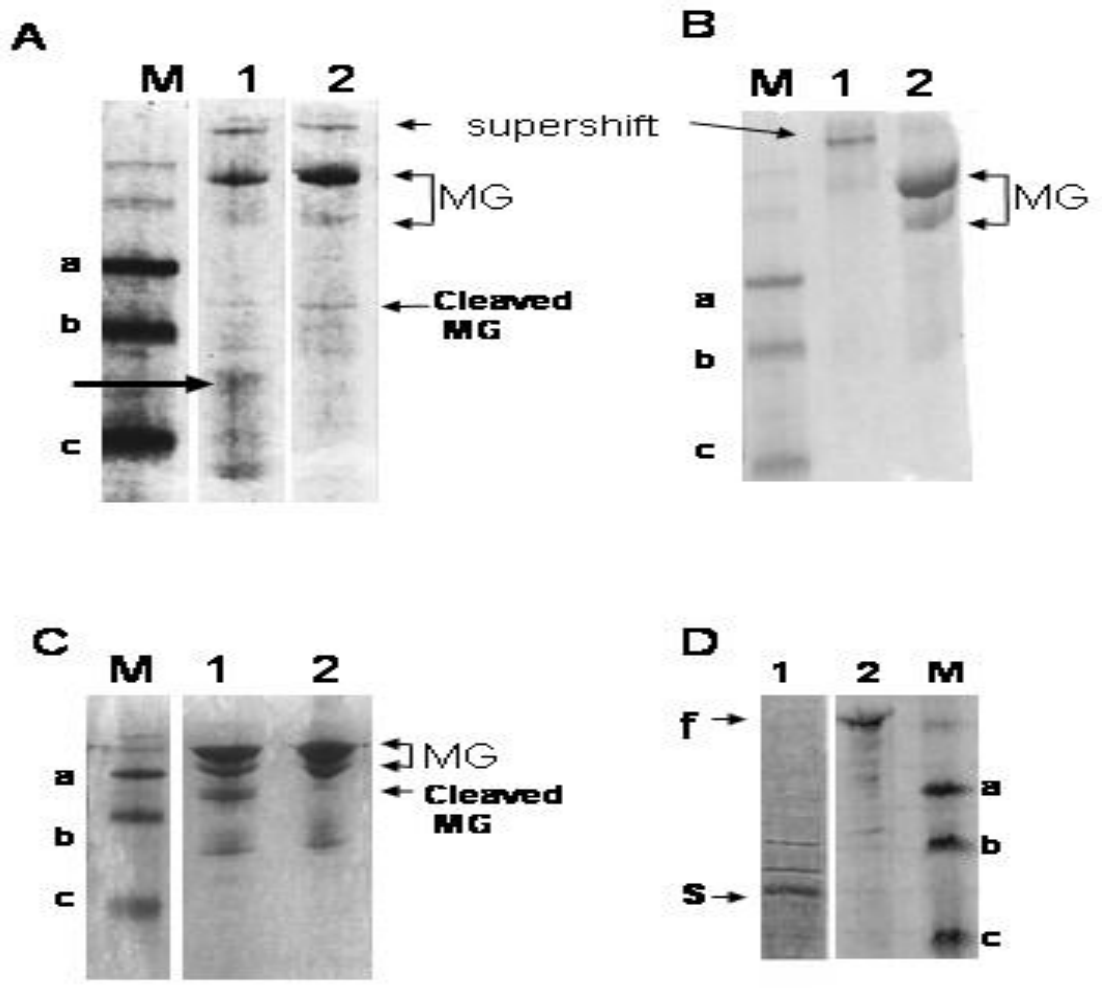


Figure34: Cleavage of macroglobulin by ADAMTS16. (A) Trapping and cleavage of α2-macroglobulin (MG) by incubating MG with ADAMTS-16s (1) or ADAMTD-16 (2). In all experiments, equivalent amounts of GST-ADAMTS-16 coupled to GSH beads were used. M indicates a protein marker with a (103 KD), b (77 kD) and c (50 kD). **(B).** trapping of MG by incubating MG with His-ADAMTS-16 (1). **(C).** Cleavage of α2-macroglobulin (MG) by trypsin (0.5 μg). **(D)** GST-ADAMTS-16s (1) and GST-ADAMTS-16 (2) produced in E. coli. f indicates full length GST-ADAMTS-16 and s indicates GST-ADAMTS-16s.

5. Conclusions and discussions

5.1. hBok (Bcl2-L9)

We initially embarked on studying the role of human Bok, because both in the rat and in the mouse the expression of Bok is highly restricted to reproductive tissues (Hsu et al., 1997). The human homologue of Bok was first identified in our research laboratory (Zhang et al., 2000). Indeed, human Bok (hBok) is expressed in human ovarian granulosa cells. In addition to that, it has been reported that human Bok is expressed in pronucleate oocytes, in 2 cell and 8 cell stage embryos and that its expression is downregulated during the blastocyst stage, suggesting a role of bok in the preimplantation development of embryos (Metcalfe et al., 2004). However, the analysis of gene expression of hBok mRNA in various human tissues has clearly demonstrated that hBok is characterized by expression in many more tissues than its mouse or rat homologues (Hsu et al.,1997), suggesting that human Bok may play a more general role in the homeostasis of many tissues. We were able to show that the expression of hBok is regulated by estrogen withdrawal and hypoxia in various tissues, such as the breast and myocardial cells. In consistance with our observations, a recent study has also shown that DNA damage induced the expression of Bok in neuroblastoma cells (Yakovlev et al., 2004).

The restricted expression of Bok in the gonads and uterus suggests its potential role in the regulation of apoptosis in these tissues. It is likely that selective pairs of Bcl-2 agonists or antagonists may play tissue-specific roles in the regulation of apoptosis. Similar to many of

the other Bcl-2 family proteins, hBok possesses a hydrophobic COOH-terminal domain. HBok can be readily isolated from membrane fractions and the cytosolic fraction in healthy cells, indicating that in the absence of apoptosis hBok is not an integral membrane protein. Alkali treatment revealed that hBok is associated loosely with the mitochondrial membrane, which distinguishes it from Bcl-x_L and Bak, the latter being integral membrane proteins of the mitochondria (Kaufmann et al., 2003) or Bax, which resides prodominantly in the cytoplasm of healthy cells (Oda et al., 2000; Putcha et al., 1999). The distribution of the various members of the Bcl-2 family in cells appear to be notably distinctive from that of hBok.

The weak association of hBok with the mitochondrial membranes of healthy cells is dramatically altered in dying cells, in which hBok becomes firmly integrated into the membrane. Therefore, similar to Bax and Bak (Suzuki et al., 2000), hBok is an essential signal during many forms of apoptosis (Lindsten et al., 2000). The conformational alteration of hBok represents a critical step during apoptosis. Structural studies revealed that the hydrophobic COOH-terminal residues of Bax and Bcl-w form a helix that tuckes into and occupies a hydrophobic groove, into which a BH3 domain may bind. Damage signals induce the expression of a BH3 domain-only protein, which then replaces the hydrophobic COOH-terminal helix and so Bax translocates from the cytosol to mitochondrial membranes, where it forms oligomeres (Antonsson et al., 2001; Lindsten et al., 2000; Nechushtan et al., 1999; Nechushtan et al., 2001). However, unlike Bax which is a cytosolic protein, hBok is associated only weakly with the membranes. Our studies on the deletion mutants revealed that N-terminus inhibits the insertion of the hydrophobic C-terminus into the membrane.

Results of similar experiments with other deletion mutants also demonstrated the role of the N-terminal of some other proapoptotic members of the Bcl-2 family, such as Bax (Gao et al., 2000; Hsu et al., 1998), Bad (Condorelli et al., 2000) and Bid (Li et al., 1998), suggesting that the removal of inhibition by the N-terminal amino acids from Bok may expose the hydrophobic BH3 domain and induce a conformational change. We consider that this conformational change facilitates the insertion of Bok into the mitochondrial membrane leading to its oligomerization into the outer mitochondrial membranes resulting in the leakage of cytochrome c into the cytosol thereby initiating the activation of the caspases. Consequently, enzymatic removal of the N-terminus may represent a common activation/amplification signal for the regulation of apoptosis. It has been proposed that the acidic phospholipid cardiolipin is the receptor for Bid, one of BH3-only Bcl-2 family members, which is a cytosolic protein that lacks the hydrophobic C-terminal domain (Lutter et al., 2000). Homology modeling of the Bok sequence strongly suggests that it belongs to the pore-forming group of pro-apoptotic Bcl-2 family proteins, along with Bax, Bak, Bok/Mtd and Bid. The ability of the Bok-BH3 mutant to induce apoptosis further supports this hypothesis.

Furthermore, other studies have demonstrated that a domain that encompasses the hydrophobic helices H6 and H7 represents the minimal region necessary for strong mitochondrial binding activity (Desagher et al., 1999; Hu et al., 2003). All these observations have stimulated us to speculate that there is an additional domain in hBok, which would be responsible for membrane association. However, additional structural studies will be needed to verify this hypothesis. Our present data suggest that the weak

binding to the mitochondrial membrane represents a primary contact in preparation for activation of apoptosis. Interaction of other BH3-domain proteins with Bok induce a secondary membrane insertion step resulting in firm integration of hBok into the membrane.

Considering the regulation of hBok expression, we were able to demonstrate that hypoxia–induced activation of hBok, bnip3 and p53 and their similar oligomerization effect suggest that these factors work cooperatively. Bnip3 is a BH3-only protein. Bnip3 regulates activation-induced cell death of effecter cytotoxic T lymphocytes (Wan et al., 2003). It has been demonstrated previously to interact with Bcl-2-xL (Ray et al., 2000) and CD47 (Lamy et al., 2003). We now found that hBok is another protein interacting with Bnip3 and that this binding with Bnip3 activates hBok by triggering its oligomerization thereby initiating apoptosis. It has been suggested that BH3-domain proteins are able to replace the hydrophobic groove which tuck the COOH-terminal hydrophobic residues (Sattler et al., 1997; Petros et al., 2004).

p53 is a key tumour suppressor, which induces cell cycle arrest, DNA repair and apoptosis. p53 is normally ascribed to reside localized within the nucleus and to regulate numerous p53-responsive genes (Liang et al., 2001; Fei et al., 2002). However, recent studies have also revealed that p53 has an additional function in the cytoplasm in order to induce apoptosis (Leu et al., 2004; Chipuk et al., 2004). Our studies showed that although p53 and R273H mutant were unable to interact directly with hBok, it was able to activate hBok by triggering oligomerization. Many pro-apoptotic Bcl-2-related genes, such as Bax, Bad, Bid, Puma and Noxa, are p53-dependent target genes (Miyashita et al., 1995; Sax et al.,2002;

Villunger et al.,2003; Sheikh et al.,1998). Activation of hBok is not a direct target of p53, since DNA BD mutated is in r273h mutant (Sauter et al., 1995). Therefore, it is most likely that p53 functions to activate hBok in the cytoplasm, although we could not exclude some additional function of nuclear p53. Unlike the direct p53-bak interaction (Leu et al., 2004), p53 could not interact with hBok directly. p53 may sequester Bcl-2/Bcl-xL to release pro-apoptotic Bcl-2 proteins or BH3-only proteins, such as Bid or Bim as suggested with Bax (Chipuk et al., 2004).

The precise mechanism by which the Bcl-2–related proteins are targeted to these membranes remains unknown. Based on the data presented here, together with the observation mentioned above, we propose that structurally Bcl-2–like proteins have three categories:

1 Proteins with exposed C-terminus, localized constitutively in the membrane, such as Bak.

2 Proteins with the C-terminus hindered by their N-terminus but with certain amino acid residues for loose membrane binding. These proteins will have a loose association to the membrane, such as Bok.

3 Proteins with the C-terminus hindered by their N-terminus and also without certain association to the membrane, such as Bax. These proteins are cytosolic.

Activation of later two categories is triggered by interaction of BH3-like proteins, such as bid, Bnip3 or p53, presumably by thwarting physiologically important death signals delivered through upregulation (Figure 35).

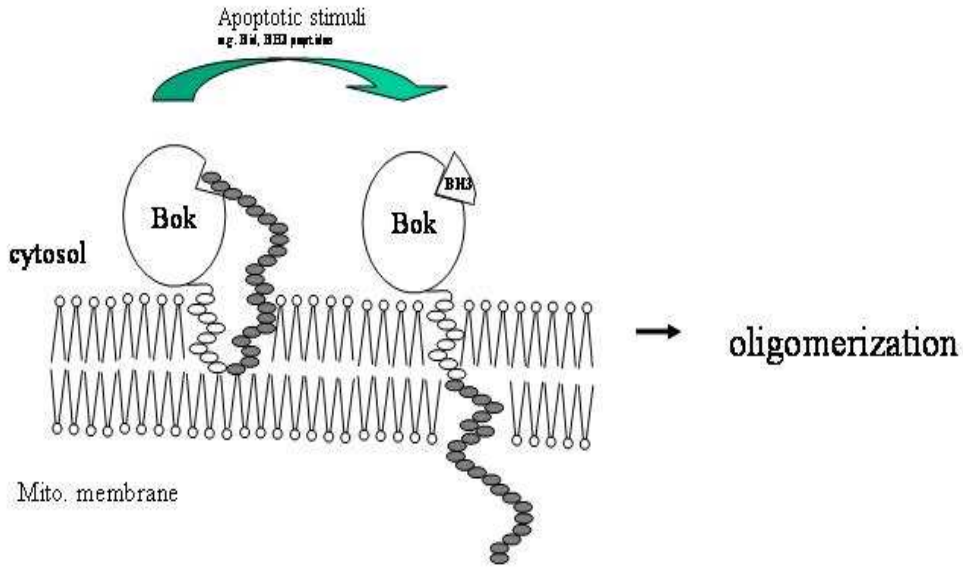


Figure 35: Suggested scheme of BH3-like proteins binding to Bok thereby inducing oligomerization.

Upon the identification of four novel hBok isoforms, we have analyzed their distribution in the various tissue compartments in order to identify their intrinsic roles in tissue homeostasis. The effects of the various Bok isoforms upon cellular apoptosis were distinct from each other. The Bok BH4 & BH3 domains can induce apoptosis as transfected into otherwise Bok-free cells, whereas the variants containing the BH1-BH2 and TM domains showed no apoptotic effects upon transfection. None of the Bok isoforms were able to interact functionally with other Bcl-2 family members, indicating the particular role of hBok in

apoptosis. Cells expressing a GFP-hBok variant (BH1-2-h6-9) showed co-localization with fluorescent mitochondrial staining. Surprisingly, when the cells were transfected with constructs for GFP-hBok (H1-H3) and GFP-hBok (H6-H9), the fluorescence was similarly distributed throughout the cell, suggesting that the BH3 domain was not necessary for mitochondrial targeting. The additional sequence in H1-5 seems to encode an additional functional targeting region as a mitochondrial-targeting motif. Furthermore, we found that TM is necessary but not sufficient for mitochondrial localization of hBok indicating that hBok may exhibit distinct features for mitochondrial localization of hBok and that H5 and H6 are required for the mitochondrial localization of hBok.

5.2. Bcl2-L12

For the first time we were able to demonstrate that Bcl2-L12 an anti-apoptotic member of the Bcl-2 family resides predominantly in the nucleus of cells and that it is of functional relevance there. Although some other members of the Bcl-2 family, such as Bax, may transiently reside in the nucleus, but this not been associated with any particular function (Mandal et al., 1998). It has also been reported that Bcl-2 and Bcl-xL are present in the membranes of the endoplasmic reticulum, mitochondria, and nucleus (Borner et al., 1994; Nguyen et al., 1994; Kaufmann et al., 2003), but their major function is at the mitochondrial membrane, where they oppose the mitochondrial changes initiated by Bax or Bak.

Our findings suggest that Bcl2-L12 plays a critical role in regulating the cell cycle, because Bcl2-L12 provokes a shift in the cell cycle from the G1 to the S phase. Other members of the Bcl2 family of proteins, such as Bcl2, Bcl2-$_{XL}$, are able to inhibit cell proliferation. However, Bcl2-L12 enhances the proliferation of cells, so Bcl2-L12 possesses the ability to modulate both mitosis and apoptosis. Anti-apoptotic Bcl2 and BCL-$_{XL}$ are antiproliferative by facilitating G0. BAX is pro-apoptotic and accelerates the progression of the S-phase (Anuja et al., 2001). G1 to S transition is related to Cdk2 activity. An initial, transient response leads to rapid inhibition of Cdk2 activity due to degradation of Cdc25A (Mailand et al., 2000). A second, sustained response is carried out by the p53-dependent production of p21, which binds to and blocks activity of Cdk2/cyclin E complexes (Stewart et al., 2001). If this Cdk2/cyclin E complexes or other factors are indeed involved in this cell cycle transition we decided to provide the evidence needed for the interaction of Bcl2-L12 in cell cycle control.

It is reported that cells exposed to the proteasome inhibitor (MG 132) become arrested at the G2/M phase followed by internucleosomal DNA cleavage, chromatin condensation, and formation of apoptotic bodies. STS-induces apoptosis mainly through the release of cytochrome *c* from the mitochondria, thereby causing a loss of mitochondrial membrane potential (Tanimoto et al., 1997). Using sequence analysis tools various putative posttranslational modification sites were found in Bcl2-L12. There are numerous potential sites for O-glycosylation. Furthermore, several possible sites of phosphorylation have been identified for cAMP-dependent protein kinase, protein kinase C, and casein kinase 2. In addition, several N-myristoylation sites have been predicted (Andreas et al., 2000).

The ratio of anti- and pro-apoptotic Bcl-2 members and their hetero- and homodimerization are believed to determine whether a cell will respond to an apoptotic signal (Yin et al., 1994; Chittenden et al., 1995; White et al., 1996) (Figure 36). Through co-transfection of Bcl2-L12 with other members of the Bcl2 family the involvement of this protein in the apoptotic pathway was investigated.

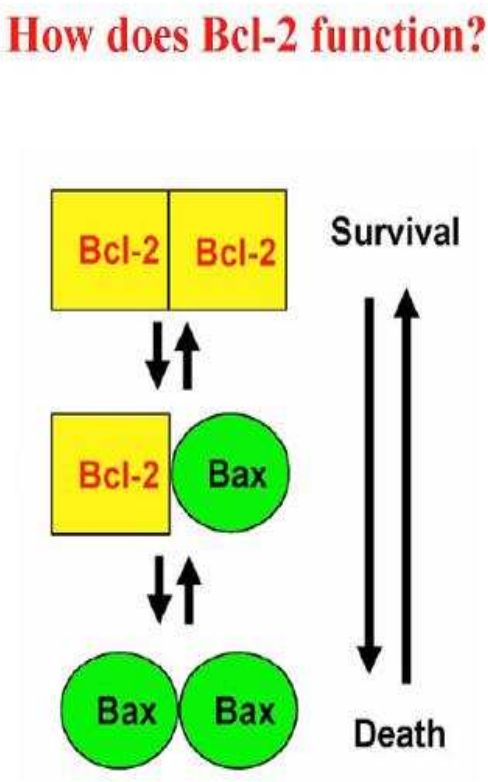


Figure 36: The Kundson cell fate model

Recent studies suggested that the region spanning BH1 and BH2 domains of Bcl-2 proteins are important for pore formation in an artificial membrane and could function as ion channels in the mitochondria as well as in other subcellular membrane organelles. By

deleting the BH2 domain of Bcl2-L12 and by creating the BH2 domain-only fragment (deleted N) variant subcellular location both have outside nuclear signalling showing that BH2 is necessary for NES (Nuclear Export Seqence), and also have function in subcellular membrane , because all Bcl2 apoptotic-related members have similar structure (Figure 37) (Reed et al., 1997).

Figure 37: Alignment of the BH2 deduced amino acid sequence of BCL2L12 with members of the Bcl-2 multagene family. Identical amino acids are highlighted in black and similar residues in grey.

In particular, the PXXP motif has been described as a docking site for interaction with SH3 domains (Lee et al., 1996). A variety of intracellular signaling pathways are linked to cell surface receptor signaling through their recruitment by Src homology 2 (SH2)/SH3-containing adapter molecules. Bcl2-L12 have 5 proline-rich SH3 binding site and one special PGPPPPSP domain. After we mutated this PPPP to PXXP domain, the subcellular location of the protein changed together with its effect on cell cycle control, which demonstrated that it is a functional domain. This PXXP site is the p21-activated kinases (PAKs) adapter. The PAKs are important effector proteins of the small GTPases, Cdc42 and Rac, and they control cytoskeletal rearrangements and cell proliferation. The coiled-coil

structure at the C terminus of Bcl2-L12 deletion also clearly demonstrated that this domain is a crucial domain for NES. However, to entirely ascertain the structure-related function of the various domains of Bcl2-L12, the crystal structure of this protein is needed.

Independent of its anti-apoptotic function, Bcl2-L12 can, through a yet undetermined mechanism, modify and provoke a shift in the cell cycle. Cell cycle progression requires the phosphorylation by cyclin-dependent kinases (Cdks) of the retinoblastoma protein (pRB) family members to free E2F transcription factors. Among the genes induced by p53 the most important candidate to mediate cell cycle block is p21/waf1 (Harper et al., 1993; Xiong et al., 1993). Increased levels of p21/waf1 have been shown to interfere with the cell cycle machinery through the combined inhibition of the cyclin/cdk kinases and the replicative functions of the DNA polymerase-associated an anti-proliferative transcription factor which enhances the rate of transcription of several genes. (Levine et al., 1997; Tilly KI et al., 1995).

The role of BH3-only molecules in the cell cycle control is more variable. BAD antagonizes both the cell cycle and anti-apoptotic functions of Bcl2 and BCL-$_{xL}$ through BH3-binding. BID has biochemically separable functions in apoptosis and S-phase checkpoint, determined by post-translational modification. Individual Bcl2 family members couple apoptosis regulation and cell cycle control in unique ways. The dual functions of Bcl2-L12 in both apoptosis and cell cycle are co-ordinately regulated by the multiple Bcl2 family members and suggest that survival is maintained at the expense of proliferation. (Zinkel et al., 2006)

The Bcl2-L12 gene is constitutively expressed in many tissues, mainly in breast, thymus, prostate, fetal liver, colon, placenta, pancreas, small intestine, spinal cord, kidney, and bone

marrow and to a lesser extent in many other tissues (Andreas et al., 2000), suggesting that the encoded protein serves an important function in different cell types. Recent reports showed that the expression levels of Bcl2-L12 may be regarded as a new independent favourable prognostic marker for breast cancer (Thomadaki et al., 2006). Pathological elevations in expression of Bcl-2 or Bcl-$_{XL}$ are commonly observed in many leukaemias, lymphomas and solid tumours, including prostate, breast, lung and colorectal cancers. mRNA expression analysis of Bcl2-L12 and other members of the Bcl2 gene family may serve as useful molecular markers predicting chemotherapy response in breast cancer. Cory and co-workers proposed that the anti-apoptotic members could function as oncogenes and the pro-apoptotic members as tumor suppressors (Cory et al., 2003).

5.3. ADAMTS-16

Various members of the ADAMTS protease family have been implicated in the process of ovulation. The liberation of an oocyte from its mature follicle during ovulation is the climax of complex remodelling processes occurring both in the follicular wall and in the cumulus-oocyte complex. This involves remodelling of various components of the extracellular matrix (ECM) through the action of a multitude of proteases. The efficacy of each of these processes results from the equilibrium between the specific components of the ECM and each of the proteases. Both the amount of various components of the ECM and the expression of specific proteases seem to be hormonally regulated during the entire process of follicular development and ovulation. Due to the dependency of the expression and secretion of each component on a particular endocrine signal, which may be conveyed to the tissue both locally and during particular time intervals, the whole process becomes tightly

regulated. The stringent control on of these processes and the specificity of the various substances involved carries the risk of making ovulation – and with this also the success of reproduction - crucially dependent on the exact functioning of each individual process. Therefore, the involvement of a large number of substrates and proteases adds an aspect of redundancy to this system (Richards et al., 2005), permitting the ongoing of successful ovulations – altghough quantitatively reduced - even in the absence of one of the players (for example ADAMTS-1, Shindo et al., 2000; Shozu et al., 2005).

One of the components of the ECM of the cumulus oophorus, versican, is induced in the granulosa under the influence of LH and is a substrate of various members of the ADAMTS protease family, mainly ADAMTS-1 (Russell et al., 2003). In the progesterone receptor knockout mouse, which fails to ovulate in response to LH, the expression of ADAMTS-1 is significantly reduced (Robker et al., 2000), suggesting an important role of the ADAMTS protease family in ovulation.

ADAMTS-16 is another member of this family and has not yet been studied in detail. Phylogenetically it is most related to ADAMTS-18 (Cal et al., 2002) and has been implicated in the pathogenesis of osteoarthritis (Kevorkian et al., 2004). We identified high expression levels ADAMTS-16 in the ovary during searching an ovary-specific gene expression database. ADAMTS-16 was now shown to consist of 23 exons spanning 180 kb of genomic DNA and is located on the human chromosome 5p15. ADAMTS-16 was expressed only in luteinizing granulosa cells, not in the cumulus oophorus, both previously collected from patients treated with exogenous gonadotrophins for assisted reproduction.

The increase in ADAMTS-16 expression in luteinizing granulosa cells was only detected in the presence of FSH and forskolin. LH failed to provoke a change in the expression levels of ADAMTS-16, suggesting a distinct role of ADAMTS-16 in ovarian function as compared with that of other members of the ADAMTS protease family, such as ADAMTS-1, ADAMTS-4 and ADAMTS-5.

α_2-macroglobulin is a highly conserved proteinase inhibitor present both in human serum and in ovarian follicular fluid at high concentration. Our results have for the first time demonstrated that ADAMTS-16 and its variant were able to cleave α_2-macroglobulin, indicating that ADAMTS-16 is an active protease. Although the entire spectrum of its cognate substrates in vivo is still unknown, the interaction between ADAMTS-16 and α_2-macroglobulin may well be a physiological, because both are present in high concentrations in the follicular fluid of mature ovarian follicles. Alpha 2-macroglobulin has been demonstrated to be involved in the regulation of oestradiol production by the granulosa cells (Gaddy-Kurten *et al.*, 1989; Ireland et al., 2004). Alpha 2-macroglobulin interacts with the vascular endothelial growth factor (VEGF) leading to the inactivation of the latter (Bhattarjargee et al., 2000) and is thought to restrict the development of small capillary blood vessels to the thecal layer until final follicular maturation (Gruemmer et al., 2005). ADAMTS-16, which is expressed in the granulosa cell layer of the follicular wall under the influence of FSH, may participate in regulating the local concentration of α2-macroglobulin.

Recently, it has been reported that ADAMTS-16 among many genes was aberrantly regulated in the absence of the nuclear co-repressor RIP140 (Tullet et al., 2005). In the

ovary RIP140 is essential for ovulation, but is not required for follicle growth and luteinization. By comparing ovarian gene expression profiles in untreated immature wild-type and RIP140-null mice and after treatment with pregnant mare serum gonadotropin and human chorionic gonadotropin, ADAMTS-16 among many genes was aberrantly regulated in the absence of RIP140.

The interaction of ADAMTS-16 with α2-macroglobulin may be of interest to reproductive medicine, as it suggests that ADAMTS-16 is involved in the pathogenesis of the ovarian hyperstimulation syndrome. The inadvertent administration of excessive amounts of FSH may lead to an overproduction of ADAMTS-16 by the granulosa, thereby prematurely inactivating the locally stored alpha 2-macroglobulin, which in turn would induce an excessive production of VEGF followed by the stimulation of endothelial proliferation, particularly in the presence of human chorionic gonadotropin after ovulation induction (Neulen et al., 1998). However, further experimental and clinical evidence is necessary to substantiate this hypothesis.

Further prospects

For the further investigation into the function of hBok, we have constructed many deletion variants and mutation mutants. The function of these deletion mutants and their potential role in processes such as to cell cycle and apoptosis pathway need to be further investigated into. In addition, we needed to produce a hBok knockout mouse model and confirm its functional role in the ovary, but also in other organs.

We are considering the potential role of Bcl2-L12 in both the diagnostics and treatment of cancer. We have to examine the expression of Bcl2-L12 in different cancer cell lines and determine its functional role in the progression of the disease.

In addition to ADAMTS-16 and ADAMTS-16s, the HUGE database of the Kazusa DNA Research Institute and PubMed provided additional data of transcripts of genes of both known and unknown function that were ovary-specific or ovary-enriched. Further characterization of these genes has already lead to the identification of other novel genes, which were found to be associated with the putative inhibin B-receptor (Kiaa 0364), inhibin-binding protein (INhBP) (kiaa 1464) and E3 ligase (Kiaa 1331). We have cloned these 3 genes and also made some deletion and mutation constructs and some expression experiments have been done.

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List of abbreviations

ADAM	**A disintegrin and metalloprotease;**
ADAMTS	**ADAM with thrombospondin type I modules;**
AIF	**Apoptosis-inducing factor**
AP-1	**Activating protein-1**
Apaf-1	**Apoptotic proteaseactivating factor-1**
AR	**Androgen receptor**
ATP	**Adenosine triphosphate**
BAC	**Bacterial artificial chromosome;**
BH	**Bcl-2 homology domains**
BLAST	**Basic local alignment search tool**
BSA	**Bovine serum albumin**
cAMP	**Cyclic adenosine monophosphate**
cDNA	**DNA complementary to RNA**
CTL	**Cytotoxic T lymphocytes**
Cyt c	**Cytochrome c**
DD Death domain	**DED Death effector domain**
DR	**Death receptor**
DTT	**Dithiothreitol**
ECM	**Extra celullar Matrix**
EDTA	**Ethylenediamine tetra-acetic acid**
EGF	**Epithelial growth factor**
ER	**Estrogen receptor**

EST	**Expressed sequence tag;**
FADD	**Fas-associated death domain protein**
FasL	**Fas ligand**
FLIP	**FLICE inhibitory protein**
FSH	**Follicle stimulating hormone**
GnRH	**Gonadotrophin-releasing hormone**
HCG	**Human chorionic gonadotropin**
HSP	**Heat shock protein**
IAP	**Inhibitor of apoptosis protein**
IGFBP	**Insulin-like growth factor binding protein**
IRF3	**Interferon regulatory factor 3;**
JNK	**C-Jun N-terminal kinase**
LH	**Luteinizing hormone**
MAPK	**Mitogen-activated protein kinase**
MG132	**Carbobenzoxy-l-leucyl-l-leucyl-l-leucinal**
NES	**Nuclear export seqence**
PBS	**Phosphate buffered saline**
RT–PCR	**Rreverse transcriptase–polymerase chain reaction;**
STS	**Staurosporine**
TRADD	**TNFR1-associated death domain protein**
TRAF	**TNF-receptor-associated factor**
TRAIL	**TNF-related apoptosis-inducing ligand**
TRAIL-R	**TNF-related apoptosis-inducing ligand receptor**

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