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Regulation of Cell death in Human Fetal and Adult Ovaries - Role of Bok and Bcl-X_L

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Abstract

Of eight million oocytes formed in fetal ovaries, only 400 are ovulated and the rest are degraded via apoptosis. Studies in rodents suggest an important role for Bok and Bcl-X_L in ovarian apoptosis, but their expression patterns and roles in human ovaries are not known. Protein expression of Bok and Bcl-X_L as well as the death pathway effectors TNF and caspase-3 were determined in an important collection of samples consisting of human fetal and adult ovaries. A penetrant expression of Bok, Bcl-X_L, TNF and full length and cleaved caspase-3 were characterized in fetal ovaries, with specific patterns in oocytes and pre-granulosa/granulosa cells. Bok and Bcl-X_L were detected also in adult ovaries. Lentiviral shRNA delivery demonstrated that loss of Bok markedly reduces vulnerability to apoptosis and, conversely, loss of Bcl-X_L increases apoptosis in human granulosa tumour cell line. The results suggest important roles for Bok and Bcl-X_L in human ovarian development, follicle maturation and apoptosis.

Introduction

Apoptosis is a central mechanism regulating human ovarian development, follicle maturation and function from fetal to adult life. By mid-pregnancy nearly eight million oocytes are formed, but only around 400 of these will survive until ovulation during fertile life and most oocytes undergo atresia through apoptotic cell death (Baker 1963, Vaskivuo et al. 2001). This process has been proposed to serve in the selection of oocytes with best promises of producing offspring (Krakauer and Mira 1999).

Ovarian apoptosis takes place mainly among either the oocytes or follicular granulosa cells and oocyte demise culminates in the destruction of the follicle, leading to follicular atresia. The destiny of an ovarian follicle might not depend on a single granulosa cell but rather on a threshold level of granulosa cells required for the wellbeing and survival of the follicle as whole. It is likely that oocyte cell death is the principal form of apoptosis during ovarian development and in resting follicles, while granulosa cell apoptosis dominates during fertile life and in growing follicles. However, both mechanisms are possible at any point of the ovarian life-span (Vaskivuo et al. 2001, Hsueh et al. 1994, Rolaki et al. 2005).

Apoptotic signals can be processed through two main pathways: the cell extrinsic, and the cell intrinsic or mitochondria-associated pathway, with significant crosstalk and feedback occurring between them (Khosravi-Far and Esposti 2004). The most extensively characterized mediators of the cell extrinsic pathway are the soluble or membrane-bound death ligands: FAS ligand, tumour necrosis factor-α (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL). The apoptotic process is initialized by these ligands binding to the TNF superfamily of death receptors on a cellular membrane (Schultz and Harrington 2003) and apoptotic signals are transmitted to the cell by amino acid tails of the receptors called "death domains" (DDs) (Zimmermann et al. 2001, Zimmermann and Green 2001). The cell intrinsic apoptosis pathway, on the other hand, is operated by the Bcl-2 family of cytoplasmic and mitochondrial proteins (Adams and Cory 1998). The Bcl-2 family consists of both anti- (e.g. Bcl-X_L, Bcl-2, Mcl-1) and proapoptotic (e.g. Bid, Bax, Bok, Bad) factors, and their apoptosis-regulating effects are dependent on the balance of these competing family members (Adams and Cory 1998, Gross et al. 1999). Apoptosis is induced by proapoptotic members of the Bcl-2 family forming pores in the mitochondrial layer and changing the mitochondrial membrane potential, which allows apoptosis-mediating proteins, such as cytochrome-c, Smac (mitochondria-derived activator of caspases) and ICE (Interleukin-1beta-Converting Enzyme) to be released into the cytoplasm. Both apoptosis pathways ultimately end with activation of executioner caspase-3, followed by DNA degradation and apoptotic elimination of the cell.

Bok (Bcl-2-related ovarian killer) belongs to the group of channel-forming proapoptotic members of the Bcl-2 family (Adams and Cory 1998) containing Bcl-2 homology (BH) domains 1, 2 and 3 but lacking the BH4 domain, which is typically present in antiapoptotic Bcl-2 family members (Hsu et al. 1997). In rodents Bok is expressed mainly in hormonally regulated reproductive tissues, particularly the ovary, uterus and testis. In adult rat ovary Bok mRNA has been detected in primary to antral follicles, where it is mainly localized to granulosa cells (Hsu et al. 1997). Bok has also been demonstrated to be expressed in adult human ovaries (Gao et al. 2005). However, the exact expression pattern and role of Bok in human ovaries from fetal development to fertile life are not known.

The antiapoptotic Bcl-2 family member Bcl-X_L contains four BH domains including BH4, vital for its apoptosis-preventing effect (Chao and Korsmeyer 1998). Bcl-X_L mRNA is expressed in rodent primordial germ cells and follicular granulosa cells (Watanabe et al. 1997, Tilly et al. 1995) and it has an important role in preventing ovarian cells from undergoing apoptosis. Bcl-X_L hypomorphic mice show massive apoptotic loss of oocytes during fetal development and they have markedly decreased numbers of primordial and primary follicles and severely impaired fertility in adult life when compared with wild-type mice (Rucker et al. 2000). In humans, Bcl-X_L mRNA is present in adult ovarian tissue and granulosa-lutein cells obtained from women undergoing fertility treatment (Kugu et al. 1998). However, the cellular localization and function of Bcl-X_L in human fetal and adult ovaries have not yet been characterized.

Both the cell extrinsic and mitochondria-associated pathways are active in regulating ovarian apoptosis. However, in human ovaries, their roles and mechanisms are not well characterized. In the present study the expression patterns of two key apoptosis regulators, TNF and caspase-3, were evaluated in human ovaries during fetal development. In addition, protein expression of antiapoptotic Bcl-X_L and proapoptotic Bok was studied in human fetal and adult ovarian tissues. For these studies we utilized biopsy and obduction material from fetuses and newborns collected systematically over several years at Oulu University Hospital. The possible regulatory roles of Bok and Bcl-X_L in granulosa cell apoptosis were further evaluated by silencing their protein expression in a human tumour-derived granulosa cell line (KGN).

Materials and methods

Tissue samples

Fetal and adult human ovarian tissues were collected from autopsy and biopsy samples at the Department of Pathology, Oulu University Hospital. Ovaries from 13 fetuses after spontaneous or therapeutic abortion and from 15 fetuses after intrauterine death followed by spontaneous or induced delivery or Caesarean section were collected. In addition, ovaries from 10 neonates who died as a result of perinatal asphyxia or infection within 15 min–7 days after birth were studied. Adult ovarian tissues were obtained from 11 pre-menopausal women aged 29–49 years undergoing ovariectomy because of endometriosis. The fetuses and neonates were stored until autopsy at + 4°C. The autopsy was performed as soon as possible during the next 24 hours after abortion or delivery. The removed ovaries of fetuses and neonates as well as the surgically removed adult ovarian tissue specimens were put immediately into 10% phosphate-buffered neutral formalin. The formalin fixation time for ovarian tissue of fetuses less than 22 weeks of gestational age and for the ovarian tissue samples of adults was 24 hours. The fixation time was 7 days for ovarian tissue removed from neonates and from fetuses older than 22 gestational weeks. After fixation the samples were routinely processed (tissue processor Leica ASP 300) and embedded in paraffin. Sections, 4 micrometers in thickness, were stained with hematoxylin and eosin. All fetuses and neonates with chromosomal abnormalities and samples with detectable autolysis were excluded from the study.
The study was approved by the Ethics Committees of Oulu University Hospital and the University of Oulu. A permit to study human autopsy tissues and resection material was obtained from the Finnish National Authority for Medicolegal Affairs.

Immunohistochemistry

Immunohistochemical staining was performed following standard protocols. Briefly, tissue sections were deparaffinised in xylene and rehydrated through graded alcohols. The tissues were pre-treated in 10 mM sodium citrate (for TNF, Bcl-X_L and caspase-3 immunohistochemistry) or in Tris-HCl (for Bok immunohistochemistry) in a microwave oven (800 W for 2 min and 300 W for 15 min). Endogenous peroxidase activity was blocked in 3% H_2O_2. Primary antibodies against human TNF (cat. no. sc-8301), Bok (cat. no. sc-11424), Bcl-X_L (cat. no. sc-7122) or caspase-3 recognising both full length and cleaved caspase-3 (cat. no. sc-1226)(Santa Cruz Biotechnology, Santa Cruz, CA, USA) at concentrations of 1:100, 1:50, 1:100 and 1:100, respectively, were used. In addition, primary antibody against cleaved caspase-3 (cat.no. #9661, Cell Signaling Technology, Beverly, MA, USA) in a concentration of 1:100 was used. To visualize the bound antibodies, Vectastain elite ABC kits (Vector Laboratories, Burlingame, CA, USA) were used for TNF, Bcl-X_L, caspase-3 and cleaved caspase-3 immunostainings. Bok was visualized by using an Envision plus Rabbit HRP system (Dako, Glostrup, Denmark). Colour reactions were brought about by using commercial diaminobenzidine tetrahydrochloride (DAB, DakoCytomation Ltd., Ely, UK) at 15 µL/mL and the samples were counterstained with haematoxylin. Peptide blocked antibody was used as negative control for Bcl-X_L and Caspase-3 stainings following instructions of the manufacturer. Shortly, primary antibodies were combined to five- fold excess of specific blocking peptide (Cat nos. sc-7122P Bcl-X_L blocking peptide and sc-1226P Caspase-3 blocking peptide) and incubated for 2 h in room temperature. The peptide blocked antibody was then used similarly to primary antibody in negative control samples. For Bok, TNF and cleaved caspase-3 antibody diluent (PBS for Bok and TNF and TBST/5% normal goat serum for cleaved caspase-3) was applied instead of primary antibody in negative control samples

Preparation of shBok- and shBcl-X_L-silenced cell lines

The KGN cells were infected with lentiviral shRNA produced by Biomedicum Genomics (http://www.helsinki.fi/biomedicumgenomics/) and after 72 hours the cells were selected in hygromycin (100 ug/mL). Gateway pDSL_hpUGIH (AFCS) lentiviral vector was used for lentiviral production and expression of shRNA targeting the sequences against Bok at 5´-GACTGCAGGGAGATGTGCag-3´ and Bcl-x_L at 5´-TCCATCTCCTTGTTGACACtt-3.

Western Blot analyses

Primary antibodies against human Bok (Cell Signaling Technology, Danvers, MA, USA, cat. no. 4521) and Bcl-X_L (BD, Franklin Lakes, NJ, USA, cat. no. 610211) at a concentration of 1:1000 were used for detecting downregulation in KGN cells as described previously (Nieminen et al. 2007). Ovarian tissue samples were lysed in Precellys tube with ceramic beads in lysis buffer (10 mM Tris.HCl pH 7.4, 150 nM NaCl, 1 % Triton X-100, 1 mM EDTA, 1 mM EGTA, 0,5 % NP-40) with protease inhibitor coctail (Roche). Subsequently the samples were

homogenized in Precellys homogenizer (Bertin technologies) for 6500 rpm twice for 45 s and kept on ice for 30 min. Unbroken cells were removed by centrifugation at 13 000 rpm for 30 min at +4°C. Ovarian samples were analyzed in SDS-PAGE with primary antibodies against Bok (cat. no. sc-11424) and Bcl-X_L (cat. no. sc-7122). Primary antibodies against B-tubulin (Abcam, Cambridge, MA, USA, cat. no. ab6046) and actin (cat. A4700, Sigma, St. Louis, MO, USA) were used as loading control.

The intensity of Bok and Bcl-X_L blots in Western analyses were measured using Quantity One program. The results were adjusted to β-tubulin and compared to corresponding KGN control blots.

Cell culture

Human tumour-derived granulosa cells (KGN cells) (Nishi et al. 2001) and shBok- or shBcl-X_L silenced KGN cells were cultured in DMEM/F-12 supplemented (10%) with fetal bovine serum (FBS). The cells were distributed in 96-well plates at approximately 20 000 cells/well and allowed to adhere overnight at 37 °C in an incubator. The cells were then treated with rTRAIL (Chemicon International Inc., Billerica, MA, USA) at 10, 25, or 50 ng/mL (Jaaskelainen et al. 2009), TNF (R&D systems, Minneapolis, MN, USA, cat. no. 210-TA-010) at 10 or 100 ng/mL or 10 or 100 nM staurosporine (Sigma, St. Louis, MO, USA, cat. no. S5921) to induce apoptosis. Caspase activity was measured at 6 and 24 hours after treatment. All experiments were performed in triplicate and repeated at least three times.

Caspase Glo 3/7 activation assay

Caspase assays were performed by using Caspase Glo 3/7 activation assay kits (Promega Corporation, Madison, WI, USA) following the instructions of the manufacturer. Room temperature Caspase-Glo 3/7 substrate and buffer were mixed and fresh reagent was applied to the cells. Luminescence was analysed by means of a Labsystems Luminoskan RT reader (Labsystems, Helsinki, Finland) after 1 hour of incubation.

Apoptosis in situ 3´-end labelling

For TUNEL apoptosis detection assay the cells were distributed at approximately 50 000 cells/chamber on 8-chamber slides. The cells were allowed to adhere overnight and were then treated with rTRAIL at 25 ng/mL, TNF at 100 ng/mL or 100 nM staurosporine. Apoptosis *in situ* 3´-end labelling was performed after 24 hours of incubation using an Apoptag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA, cat. no. S7100) following the instructions of the manufacturer. Briefly, the cells were washed with PBS and fixed with 1% paraformaldehyde in PBS for 10 minutes at room temperature and post-fixed and permeabilized in pre-cooled ethanol/acetic acid (2:1) at -20 °C for 5 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS. DNA 3´-end-binding TdT enzyme was pipetted onto the samples and they were incubated under plastic cover slips at 37 °C for one hour. A colour reaction was achieved by using a Sigma Fast™ DAB peroxidase substrate tablet set (Sigma, St. Louis, MO, USA) and the samples were counterstained by brief incubation in haematoxylin.

Microscopy analysis and statistics

Immunohistochemical stainings were evaluated by two independent observers (TNF, Bok and Bcl-X_L by M.J. and T.V.; caspase-3 and cleaved caspase-3 by M.J. and J.T.).
For statistical analysis of apoptosis 3'-end labelling, positively labelled cells in shBok and shBcl-X_L silenced cell lines were counted in ten visual fields and compared to KGN control cells.
Apoptosis 3'-end labelling and caspase activation assay data were analysed by using SPSS 15.0 software (SPSS, Chicago, IL, USA). Student's *t* test (normally distributed groups) or the Mann–Whitney test (groups that were not normally distributed) were used to compare different treatment groups. A value of $p < 0.05$ was considered significant. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Results

TNF, Bok, Bcl-X_L and caspase-3 are expressed in human fetal ovaries

Tumour necrosis factor-α, Bok, Bcl- X_L and caspase-3 were studied in human ovarian samples collected from fetuses/newborns from the 16th week onwards. These proteins were expressed in fetal ovaries at all gestational ages studied (Table 1, Fig. 1). TNF was expressed in pre-granulosa cells and oogonia of young ovaries (gestational age under 20 weeks) (Table 1, Fig. 1 a). In addition, at this age diffuse staining was detected on the cellular membranes and in the intercellular spaces. Later during development (gestational age 20 weeks onwards) oocyte cytoplasm was moderately stained, while granulosa cells of the developing follicles showed weak or moderate TNF expression (Table 1, Fig. 1 b–d).

The pro-apoptotic Bcl-2 family member Bok was expressed mainly in oocytes, but weak staining was also detected in granulosa cells of the developing follicles (Table 1, Fig. 1 e–h). Bok staining was observed in both cell cytoplasm and the nucleus (Table 1, Fig. 1 e–h).

Bcl-X_L was expressed constantly in all fetal ovaries studied (Fig. 1 i–l). Before follicular formation, diffuse staining was observed in ovarian stroma and pre-granulosa cells (Table 1, Fig. 1 i). Later during fetal development Bcl-X_L expression was mainly localized to the cell cytoplasm of oocytes and granulosa cells, oocyte expression being dominant (Table 1, Fig. 1 j–l).

Weak/moderate expression of caspase-3 protein was detected in human fetal ovaries at all gestational ages (Fig. 1 m–p). The staining was most profound in the ovarian cortex, where follicular structures were not yet formed. Weak/moderate expression could also be detected in oocytes and granulosa cells of small developing follicles (Table 1, Fig. 1 m–p). Caspase-3 was not expressed in secondary or small antral follicles in fetal ovaries. Cleaved caspase-3 was strongly and specifically expressed in fetal ovarian cells. In young ovaries (16-17 weeks) individual oogonias and pregranulosa cells outside the follicle structures were stained (Table 1, Fig. 1 q). After midpregnancy cleaved caspase-3 could be detected also in individual oocytes and granulosa cells of primordial follicles (Table 1, Fig 1 r-t).

Expression of Bok and Bcl-X_L in adult human ovaries

In adult human ovaries Bok was strongly expressed in oocyte cytoplasm and nuclei of primordial, primary and secondary follicles (Table 2, Fig. 2 a–c). Granulosa cells expressed Bok at all follicular stages, but in primordial/primary follicles granulosa cell expression was weak (Fig. 2 a and b) and staining intensity was more profound in secondary and antral follicles (Fig. 2 c and d). Western analysis revealed a specific expression of 23kDa Bok in adult human ovary (Fig 3).

The anti-apoptotic protein Bcl-X_L was detected in oocytes and granulosa cells of adult ovaries (Table 2, Fig. 2 g–j). Oocyte expression was strong in primordial to secondary follicles (Fig. 2 g–i), and granulosa cells of primordial, primary, secondary and antral follicles expressed Bcl-X_L moderately (Fig. 2 g–j). Western analysis revealed a specific expression of 26kDa Bcl-X_L in adult human ovary (Fig. 3).

Bok operates as a proapoptotic factor in human tumour-derived granulosa cells

To study the possible effects of (proapoptotic) Bok in human granulosa cell apoptosis, shRNA-expressing constructs targeting Bok were lentivirally transduced into KGN cells. Transduction led to silencing effiency of >60% in Bok protein expression (Fig. 4 A). Bok-deficient KGN cells were subsequently treated with various apoptosis-inducing factors (TRAIL, TNF and staurosporine) and caspase- 3/7- activation was measured at 6 and 24 hours of treatment. The results in Figure 4 B and C show that silencing Bok inhibits caspase- 3/7 activation in different experimental conditions. No marked difference was observed in caspase activation of shBok-silenced and control KGN cells when incubated only in media for 6 or 24 hours (Fig. 4 B and C). Treatments with rTRAIL, TNF and staurosporine led to marked induction of caspase- 3/7 activation and this induction was significantly lower in the shBok-silenced cells than in control KGN cells (Fig. 4 B and C).
To confirm the results obtained from caspase activation assay, Bok-deficient cells were treated with media only, rTRAIL, TNF or staurosporine and apoptotic cells were recognized by TUNEL assay after 24 hours of treatment. At this time point considerably lower numbers of Bok-deficient cells were undergoing apoptosis in wells treated with TNF or staurosporine when compared with control KGN cells (Fig. 5 A a–d and e–h, respectively, Fig. 5

B). A similar trend was also detected in shBok cells treated with media only or rTRAIL (Fig. 5 A a–d and e–h, respectively, Fig. 5 B).

Bcl-X_L protects human tumour-derived granulosa cells from apoptosis

The shRNA construct targeting human Bcl-X_L was lentivirally transduced into KGN cells to create a Bcl-X_L-deficient granulosa cell line. Immunoblotting showed >60% decrease in Bcl-X_L protein expression in shBcl-X_L-expressing cells (Fig. 4 A). Bcl-X_L –deficient cells were significantly more susceptible to apoptosis than control KGN cells (Fig. 4 B and C, Fig. 5). At 6 hours of incubation a marked increase in caspase activation was detected in Bcl-X_L-deficient cells in wells treated with media only, TRAIL and TNF when compared with control KGN cells. At this time point Bcl-X_L-deficient cells also tended to be more vulnerable to staurosporine-mediated caspase activation, but this tendency did not reach statistical significance (Fig. 4 B; 8549 cpm *vs.* 6905 cpm, $p = 0.257$ with 10 nM staurosporine and 18079 cpm *vs.* 12903 cpm, $p = 0.15$ with 100 nM staurosporine). At 24 hours, however, staurosporine treatment also resulted in a significantly increased activation of caspases 3/7 in Bcl-X_L-deficient cells (Fig. 4 C).
Apoptosis *in situ* 3´-end labelling was applied to rTRAIL-, TNF- and staurosporine-treated shBcl-X_L and control KGN cells to confirm the caspase assay results further. Apoptosis was measured after 24 hours of treatment and a markedly higher number of Bcl-X_L-deficient cells undergoing apoptosis were observed when compared with similarly treated control KGN cells (Fig. 5 A i–l and e–h, respectively, Fig. 5 B).

Discussion

Oocyte and granulosa cell apoptosis are essential mechanisms for the regulation of ovarian development and follicle maturation. The exact roles of the apoptosis-regulating factors in human ovarian cell death are, however, still largely unknown. In the present study we elucidate the cellular expression patterns of TNF, Bok, Bcl-X_L and caspase-3 in an important material consisting of human fetal ovaries at 16–40 weeks of gestation. In addition, novel data concerning the expression of Bok and Bcl-X_L in adult human ovaries and their participation in granulosa cell apoptosis is presented.

We found that TNF was widely expressed in the developing ovary, with most of the follicles showing positive staining. Although TNF has an apoptosis-regulating role in developing mammalian ovaries (Marcinkiewicz et al. 2002), it also has many non-apoptotic functions (Terranova 1997) that may explain its wide expression pattern in healthy follicles. In rat ovaries, expression of TNF has been detected only after the formation of primordial follicles (Marcinkiewicz et al. 1994), while in humans it was observed not only in the intercellular space and cellular membranes, but also in immature oocytes and pre-granulosa cells before follicular development. These findings may reflect species-specific developmental differences between humans and rats. In a previous study on human ovarian tissue, TNF was found to be expressed in oocytes of all follicular stages, but only maturing antral follicles showed expression in granulosa cells (Kondo et al. 1995). The present results extend these findings by demonstrating weak/moderate TNF staining in follicular granulosa cells and moderate or strong expression in oocytes of small developing follicles.

In the present study we found that caspase-3 is expressed in ovarian tissue throughout gestation indicating the potency of fetal ovarian cells to undergo caspase-3 mediated apoptosis. In addition, primary antibody against cleaved caspase-3 was used to detect the cells undergoing apoptosis. Cleaved caspase-3 was strongly present in fetal ovarian pregranulosa cells and oogonias outside the follicle structures but in older fetuses (after midgestation) also in individual oocytes and granulosa cells of primordial/primary follicles. Many of the positively labelled cells had features of the apoptotic morphology: shrunken cytoplasm, chromatin condensation and apoptotic body formation. These results are in agreement with an earlier study on human fetal ovaries before the 20th week of gestation (Fulton et al. 2005). Furthermore, a high number of cleaved caspase-3 labeled cells were detected at midgestation which coincides with the high rate of oocyte apoptosis observed in earlier studies (Vaskivuo et al 2001, Baker 1963).

In addition to participating in apoptotic processes, active caspase-3 may have non-apoptotic functions in the developing ovary. It has previously been shown to have a role in controlling cell cycle, cell differentiation and migration for instance in T-lymphocytes, platelets, nervous tissue and ovarian cancer cells (Rosado et al. 2006, Gulyaeva 2003, Zhao et al. 2006). Furthermore, studies in caspase-3 knockout mice have demonstrated that these animals are smaller and born at lower frequency which may be a result of disturbed development or reduced cell number caused by non-apoptotic or apoptotic mechanisms (Matikainen et al. 2001, Los et al. 2001). The exact role of caspase-3 in ovarian apoptosis is, however, somewhat unclear, since caspase-3 deficiency has not been shown to affect oocyte death or the size of the follicle pool in prenatal or postnatal mouse ovaries (Matikainen et al. 2001, Los et al. 2001).

In concordance with earlier observations in rat (Hsu et al. 1997) and human (Gao et al. 2005) the expression of Bok was detected in granulosa cells and oocytes of human fetal and adultovaries and it was localized to both cell cytoplasm and nucleus. Nuclear localization of Bok has previously been identified in cells derived from human breast, ovarian and pancreatic cancer tissues (Bartholomeusz et al. 2006). In contrast to other Bcl-2 family members, Bok has a unique nuclear export signal enabling it to transfer into the nucleus, which has been suggested to be required for the apoptosis-inducing effect of Bok (Bartholomeusz et al. 2006). The localization pattern of Bok in the present study and the results of previous studies (Hsu et al. 1997, Bartholomeusz et al. 2006) suggest that follicular cells with distinct nuclear staining may have a greater susceptibility to apoptosis than cells expressing Bok only in the cytoplasm.

The protein expression of Bok and Bcl-X_L was silenced in KGN cells by means of a lentivirus-based system that has been successfully used in studying apoptosis-regulating genes in cells of an origin other than the ovary (Nieminen et al. 2007). The present results demonstrated that down-regulation of Bok had a marked protective effect on granulosa cells in experimental conditions that differentially activated the cell extrinsic and intrinsic pathways. These important *in vitro* observations in human ovarian cells are in line with previous studies in rodents showing the proapoptotic role of Bok in female reproductive tissues (Hsu et al. 1997, Hsu and Hsueh 1998).

In accordance with the results of previous studies on mammalian and avian ovaries (Watanabe et al. 1997, Tilly et al. 1995, Johnson et al. 1996), Bcl-X_L was widely expressed in human oocytes and granulosa cells. Silencing of Bcl-X_L had a significant apoptosis-enhancing effect in response to various apoptotic stimuli in KGN cells, indicating that human granulosa cell sensitivity to apoptotic signals coming from outside the cell are dependent on the expression level of Bcl-X_L. The wide expression pattern of Bcl-X_L in human ovaries during fetal and adult life, and a marked contribution of its expression level to human granulosa tumour cell survival, suggests that Bcl-X_L has an active role in protecting human ovarian cells from apoptosis, as earlier demonstrated in rodents (Rucker et al. 2000).

The origin and nature of KGN cells have to be taken into consideration when interpreting results obtained from *in vitro* experiments, since the expression patterns of Bcl-2 family members and the susceptibility to apoptosis of ovarian cancer cells may differ from those in normal ovary (Marone et al. 1998). However, KGN cells have many functions of normal cells such as hormone responsiveness and a similar pattern of Fas-mediated apoptosis as demonstrated in normal human granulosa cells (Nishi et al. 2001). Thus, we believe that the KGN cell line is a good experimental model for studying the regulation of apoptosis in human granulosa cells.

The highest rate of oocyte apoptosis in humans is detected at midgestation (Vaskivuo et al 2001, Baker 1963) when the expression of apoptosis regulating proteins TNF, Bok, Bcl-X_L, caspase-3 and cleaved caspase-3 was significant. This suggests that these factors may be involved in the regulation of fetal ovarian development and apoptosis. Furthermore, the expression of Bok and Bcl-X_L in follicular cells of adult human ovaries and the markedly changed apoptosis rate in Bok- and Bcl-X_L –deficient KGN cells suggests that these two factors play a role in the survival and death of ovarian follicles.

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Table 1. Summary of TNF, Bok, Bcl-X_L and caspase-3 protein expression in human fetal ovaries.

Protein	Stroma	Oocytes	Granulosa cells	Cytoplasm	Nucleus
TNF	Diffuse staining in stroma and intercellular space before follicle formation	Moderate to strong expression in extra-follicular cells of young ovaries. Moderate staining in oocytes	Strong or moderate staining in extra-follicular cells of young ovaries. Weak to moderate expression in granulosa cells	Strong staining	Negative
Bok	Negative	Moderate or strong expression	Weak or negligible expression in follicular granulosa cells	Strong staining	Strong staining
Bcl-X_L	Diffuse staining before follicle formation	Moderate or strong expression	Weak to moderate expression	Strong staining	Negative
Caspase-3	Weak staining	Expressed mainly in extra-follicular cells on ovarian cortex. Weak/moderate staining detected also in primordial/primary follicles	Expressed mainly in extra-follicular cells located on ovarian cortex. Weak/moderate staining in follicular granulosa cells.	Strong staining	Staining detected in some cells
Cleaved caspase-3	Negative	Expressed in oogonias and individual oocytes inside the primordial follicle structures.	Expressed in pregranulosa cells and individual follicular granulosa cells.	Strong staining	Strong staining

Table 2. Summary of Bok and Bcl-X_L expression in human adult ovaries.

Protein	Stroma	Oocytes	Granulosa cells	Cytoplasm	Nucleus
Bok	Weak staining	Strongly expressed in oocytes in primordial to antral follicles	Weak expression in primordial and primary follicles. Staining increased to moderate or strong in secondary and antral follicles	Strong staining	Strong staining
Bcl-X_L	Weak staining	Strong expression at all follicular stages	Moderate expression at all follicular stages	Strong staining	Negative

Figure legends

Figure 1. Protein expression of TNF, Bok, Bcl-X_L and caspase-3 in human fetal ovaries.

In the young ovaries (16–17 weeks) the most intensive TNF staining was detected in small non-follicular cells. More diffuse staining was also observed in the intercellular space (a). Later in fetal development, TNF localizes preferentially to oocyte cytoplasm, but weak/moderate staining was also detected in granulosa cells (b-d).
Bok was strongly expressed in oocytes in all fetal ovaries studied. Staining was localized to the cell cytoplasm and nucleus (e–h). Staining intensity in different follicles varied from negative to strong. The expression of Bok in fetal granulosa cells was weak or negligible (e–h).
Before follicular formation Bcl-X_L was detected in stroma and pre-follicular cells of the developing ovary (i). Later, strong or moderate Bcl-X_L protein expression was detected in follicular cells. Expression was strong in follicle-encapsulated oocytes and weak/moderate in granulosa cells (j–l).
Caspase-3 staining was limited mainly to extra-follicular cells located in the cortex area of the ovary (m–p). Weak/moderate staining could also be detected in follicular oocytes and granulosa cells (n, o).
Cleaved caspase-3 was strongly and specifically detected in individual oogonias and pregranulosa cells before primordial follicle assembly (q). After midgestation cleaved caspase-3 was also present in some follicular oocytes and granulosa cells (r-t).
Controls: negative controls using peptide blocked antibody (Bcl-X_L and Caspase-3) or antibody diluent instead of primary antibody (TNF, Bok and cleaved caspase-3).
* = Oocyte, arrows indicate granulosa cells. Scale bar 100 μm.

Figure 2. Protein expression of Bok and Bcl-X_L in adult human ovaries.

Bok was strongly present in the oocytes of primordial/primary (a and b) and secondary (c) follicles. Bok staining in the granulosa cells of small primary follicles was weak (a and b), but in secondary follicles (c) it was moderate and in antral follicles (d) strong granulosa cell staining was observed. Bok expression was localized to cell cytoplasm and the nucleus (a–c).
Bcl-X_L was strongly expressed in oocytes at all follicular stages (g–i). Granulosa cells were also moderately stained (g–j). Bcl-X_L staining was mainly localized to the cell cytoplasm.
Controls: negative controls using peptide blocked antibody (Bcl-X_L) or PBS instead of primary antibody (Bok) – primordial/primary follicles (e and k) and antral follicles (k and l).
* = Oocyte, arrows indicate granulosa cells. Scale bar 100 μm.

Figure 3. Western analyses in adult human ovary.

Western analyses revealed a specific expression of 26 kDa Bcl-X_L and 23kDa Bok in adult human ovary.

Figure 4. Protein expression and caspase activation assays in shBok- and shBcl-X_L-deficient and control KGN cells.

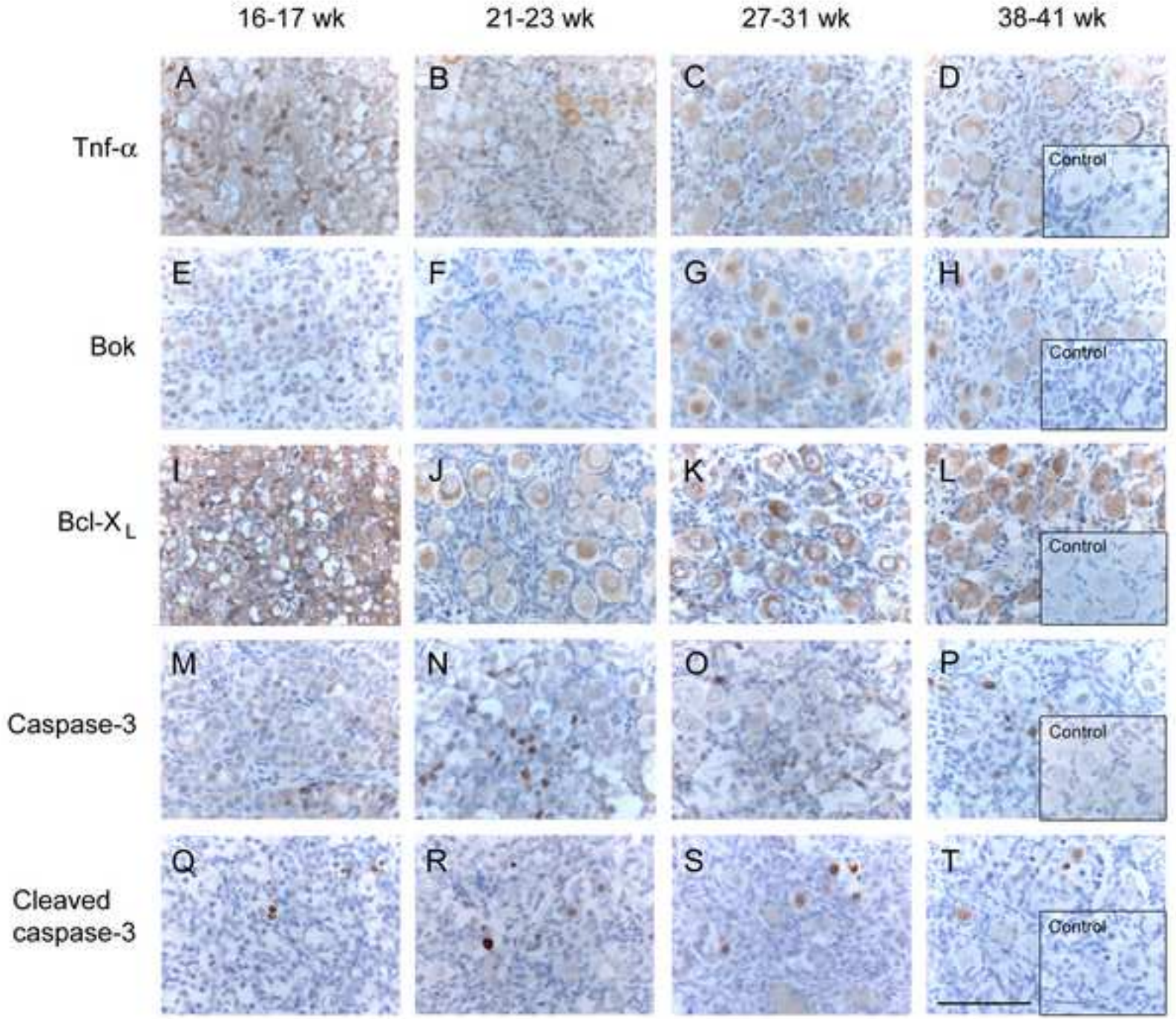
Protein expression of Bok and Bcl-X_L was detected by immunoblotting. Both proteins were present in control KGN cells and lentiviral shBok- and shBcl-X_L delivery resulted in >60% silencing efficiency in Bok and Bcl-X_L protein expressions (A).
The cells were treated with rTRAIL at 10, 25 or 50 ng/mL, TNF at 10 or 100 ng/mL and 10 or 100 nM staurosporine (STS). Apoptosis induction was measured by caspase- 3/7 activation assay after 6 (B) and 24 (C) hours of treatment and the results compared with that in similarly treated control KGN cells. Significantly decreased caspase- 3/7 activation was detected in Bok-deficient KGN cells response to treatment with rTRAIL, TNF and STS when compared with control KGN cells. This difference was detected at both time points (B and C). Increased caspase- 3/7 activation was observed in shBcl-X_L-expressing cells in all treatment groups, even in wells treated with media only. This elevated caspase activation was observed at 6 hours (B) and it remained so at 24 hours (C) of treatment. The results are shown as arithmetic means + SD. All experiments were performed in triplicate, and repeated at least three times (n = 3–5). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

Figure 5. Apoptosis *in situ* 3´-end labelling in Bok- and Bcl-X_L-deficient and control KGN cells.

The cells were cultured in media only (a, e and i), rTRAIL at 25 ng/mL (b, f and j), TNF at 100 ng/mL (c, g and k) or 100 nM staurosporine (STS) (d, h and l) and apoptosis *in situ* 3´-end labelling was performed after 24 hours of treatment. In all treatment groups fewer apoptotic granulosa tumor cells were detected in Bok-deficient cultures (A: a-d, B) although the difference was not statistically significant in media only or TRAIL treated cells.

The number of apoptotic cells was increased in shBcl-X_L-expressing cell cultures treated with media only, TNF or STS (A: i, k-l, B) when compared with control KGN cells (A: e-h, B). The change was not significant in TRAIL treated shBcl-X_L- deficient cells (A: j, B). Apoptotic cells are highlighted with circles. Scale bar 100 μm (A).
The number of apoptotic cells in each treatment groups are shown in relative to KGN control cells (100%, corresponding following absolute values: 10,9; 13,3; 7,1 and 16,6 respectively). Error bars represent SDs. The statistical analysis is performed using arithmetic means * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ (B).

Figure 1


16-17 wk
21-23 wk
27-31 wk
38-41 wk
Tnf-α
Bok
Bcl-X$_L$
Caspase-3
Cleaved caspase-3
A
B
C
D
E
F
G
H
I
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K
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Control
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Figure 2

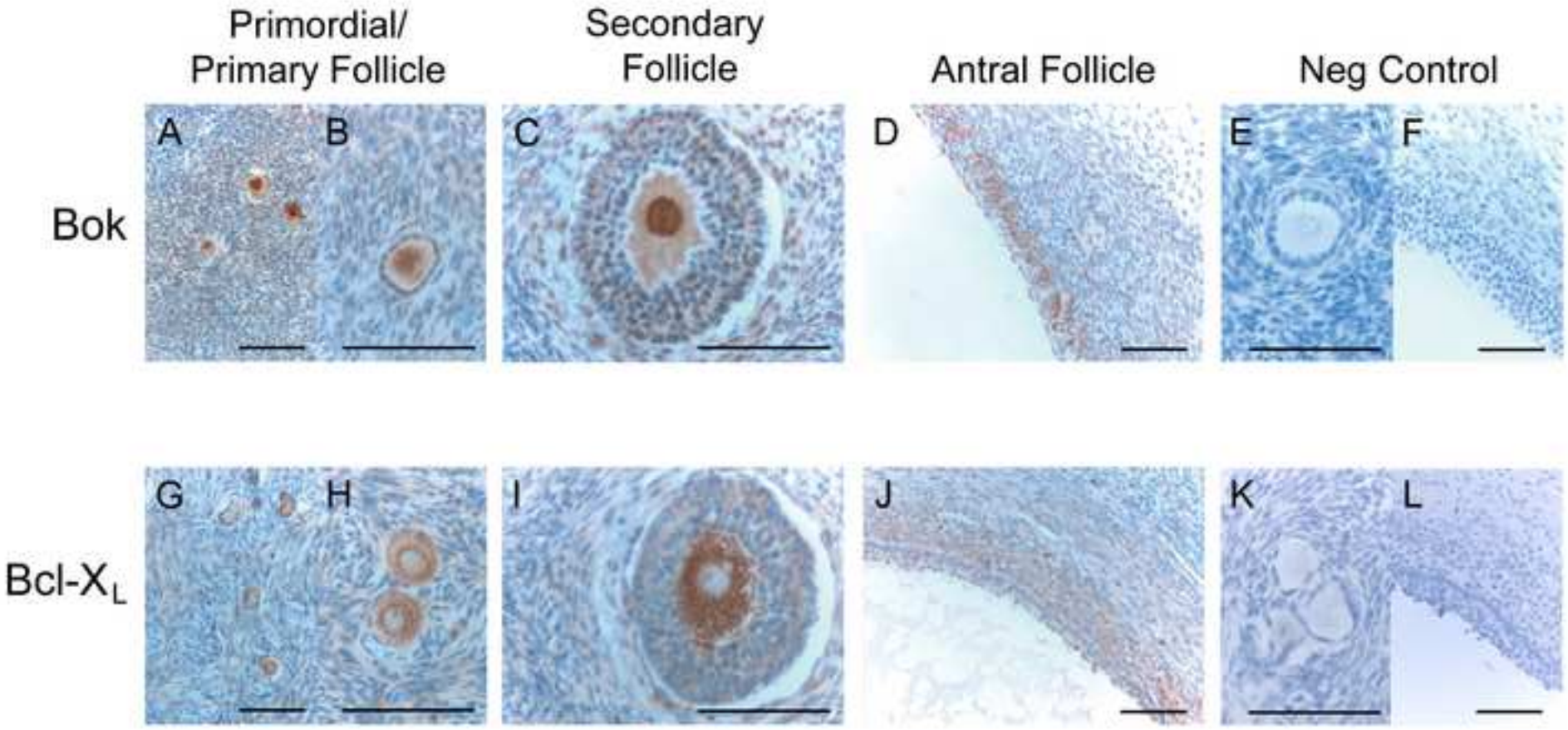
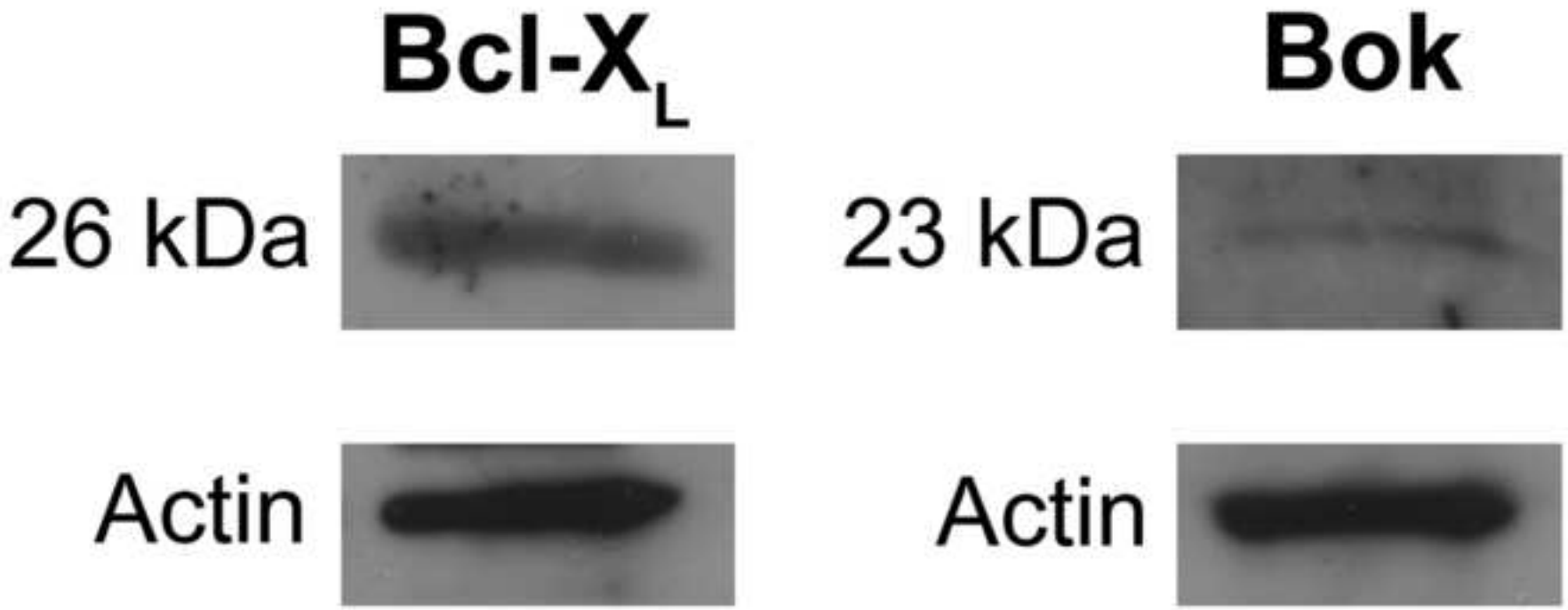

Primordial/
Primary Follicle
Secondary
Follicle
Antral Follicle
Neg Control
Bok
Bcl-X$_L$
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3

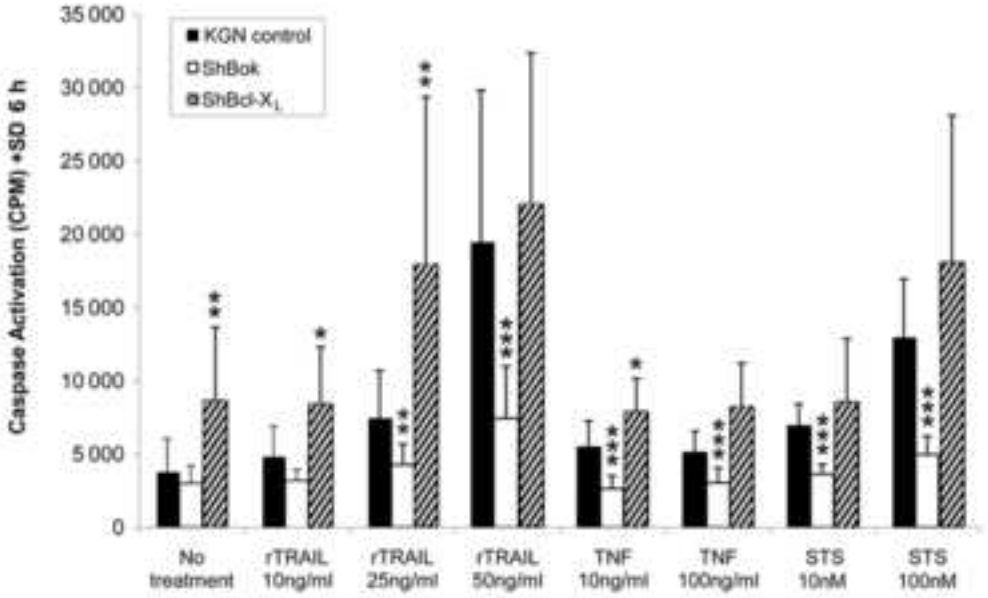


A

Blot Intensity	ShBok	KGN control
Bok	832	2029
β-Tubulin	7377	7067
Bok adjusted to β-tubulin / KGN control	0,39	1,00

Blot Intensity	ShBd-X$_L$	KGN control
Bd-X$_L$	1894	7089
β-Tubulin	6849	6538
Bd-X$_L$ adjusted to β-tubulin / KGN control	0,34	1,00

B



C