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Apoptosis in Bcl2l13 Epididymal Cells of Mice

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Abstract

Apoptosis, i.e., controlled cell death, occurs in response to many different environmental stimuli and it plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms. The apoptotic cascade that occurs within the cell in response to these cues, leads to morphological and biochemical changes that trigger the death of the cell. Bcl2 (B-cell lymphoma 2), encoded in humans by the BCL2 gene, is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis), by either inducing (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis and thus represents a crucial part in the cascade of cell death. We analyzed the effect of BCL2l13 in murine knockout (k.o.) following apoptosis. These mice show a phenotype in the epididymis; A gland that is prerequisite to guarantee fertility. By inducing apoptosis and thereafter observing the cell death, the results did not indicate a significant difference between k.o. and wild type suggesting BCL2l13 having less of importance in the BCL2 protein family.

Keywords: Apoptosis; Epididymal; Transgenic animals

Introduction

The definition of apoptosis is programmed cell death which is required for instance during embryologic development to separate fingers and toes, to determine the immune repertoire and terminating immune response and to avoid DNA damaged cell progressing into a tumor, as one of the mechanism of tumor development. Consequently, a disturbed regulation of apoptosis is involved in a large number of pathologies such as cancer, autoimmunity and degenerative disorders. The link between apoptosis and cancer emerged when BCL2 (B-Cell lymphoma 2) protein, the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death [1,2].

As cells may undergo apoptosis, during which they shrink and bleb, undergoing chromatin condensation and internucleosomal DNA cleavage, before being tidily packed into vesicles that are rapidly engulfed by macrophages. The main enzymes that induce cell death are caspases; aspartate-directed cysteine proteases. This protein family was initially discovered in the nematode *Caenorhabditis elegans*. The worm protein CED-9 is the functional homologue of BCL2 and regulates the activation of caspases [3] (Figure 1).

The BCL2 protein family functions as a switch between "life and death". This switch operates through interactions of proteins within three subfamilies. Whereas pro-survival subfamilies (i.e., BCL2) protect cells from cytotoxic conditions, other subfamilies, such as Bax-like proteins promote cell death. Members of this subfamily (Bak, Bax and Bok) are very similar to BCL2 sequence, particularly in three conserved "BCL2 Homologues" region (BH1, BH2 and BH3). The other proapoptotic subfamily, the "BH3-only-proteins" with all its members is not related to the structure of the BCL2 proteins, apart the BH3 domain, which is essential for its killing function. It seems that BH3 acts as a damage sensor and straight antagonist of BCL2 as well as other pro-survival proteins. Once activated, the proapoptotic proteins act further downstream irreversibly [1,2].

In this study we analyzed epithelial cells taken from murine epididymis; a male genital organ which is indispensable for sperm maturation. We were particularly interested in the epididymis of the Bcl2l13 k.o. mouse, because the epididymis displayed a particular phenotype. It showed as an only organ, a macroscopic, positive x-gal staining in the epididymis suggesting a functional and maybe morphological difference in comparison with the wild type when

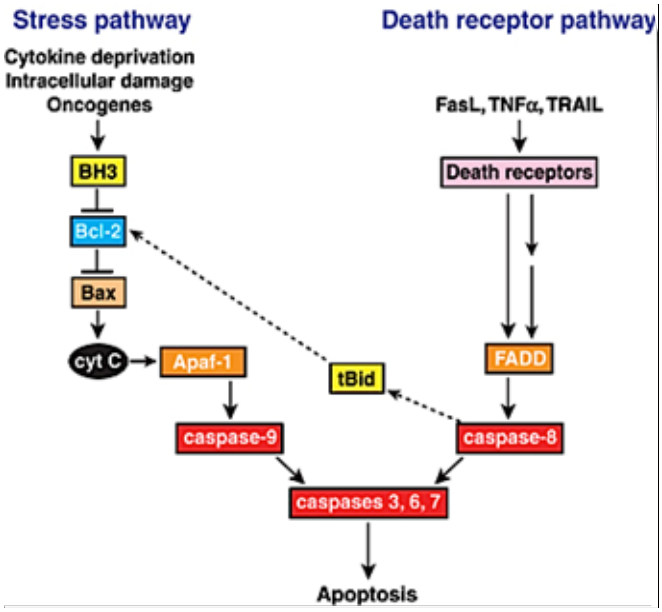

Figure 1: The stress pathway is triggered by different inter- and intracellular signals and reactions. Proapoptotic factors, such as Bak, Bax or Bok release cytochrome c from damaged mitochondrial membrane, which activates the apoptotic protease activating factor 1 (Apaf-1) and induces the production of caspase 9. This intrinsic pathway, is primarily regulated by the BCL-2 proteins. The extrinsic pathway is induced by ligands of the Tumor Necrosis Factor (TNF) family when they bind on the "death receptor". This pathway activates caspase-8 trough adaptor proteins that include Fas-associated death domain protein (FADD). Once activated, caspase-9 or -8 activates further caspases (i.e. caspases-3, 6 and 7), which provoke cellular destruction by cleaving several hundred cellular proteins. The two pathways are largely independent, in certain cell types (e.g. hepatocytes) however, the two pathways interact via the pro-apoptotic factor tBid [1].

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apoptosis is induced. Female mutants did not have a phenotype but showed a decreased number of pups (2-4 per gestation instead of 6-8).

Materials and Methods

Transgenic animals

The mouse genotypes; BCL2l13$^{+/-}$ were obtained from Sanger Institute Gene Trap (Hinxton, Cambridge, UK) and maintained at the biomedical department of the University of Basel. Homozygote mice were obtained by crossing the heterozygote mice and confirmed by genotyping of the tail. The gestational age of the embryos was determined as E0.5 on the day the vaginal mucus was observed. Animal tissues were obtained post-mortem after euthanasia done according to the regulations of the Swiss Veterinary Association.

Gentotyping

Genomic DNA: The primers used for the PCR were ml13-3' and ml13-5', each with 0.5 µM. The PCR-mix supplied by Qiagen© contains a PCR buffer $MgCl_2$ 25 mM, dNTP mix 10 mM and polymerase (HotStar©) 5 U/µl. For the first 15 min the PCR ran in 94°C and thereafter 30 cycles with the following temperatures 94°C for 20 s, 56°C for 30 s and 72°C for 2 min. The genotype was analyzed by gel-electrophoresis performed with 2% agarose gel. The agarose gel was prepared from TBE solution; a mix of tris base, boric acid and 0.5 M EDTA. The analysis of the reaction continued by observation of the image under UV-light.

Gene expression/RT-PCR: Qiagen© (Sigma© Switzerland) provided all of the solutions needed for the RNA extraction. The protocol for RNA-extraction is described in the RNeasy© handbook, as well provided by Qiagen©. Tissue samples of different organs were homogenized in a QIAshredder©, 600 µl of RLT buffer was added and this mixture was centrifuged for two minutes, following which one volume of 70% ethanol was added and 700 µl of this lysate was transferred to RNeasy© spin column. After having been centrifuged for 15 s the flow-through was discarded and 700 µl of RW1 buffer was added, gently mixed and centrifuged for another 15 s. Again the flow-through was discarded, RPE buffer was added and the mixture was centrifuged for 15 s. Finally 30 µl of RNase-free water was added to the column and centrifuged one last time for two minutes. The concentration of the collected mRNA was measured by spectroscopy using Nanodrop©.

The cDNA was synthesized with the SuperScript™ (Sigma© Switzerland) First-Strand Synthesis System obtained from the RT-PCR kit. The components for the first mixture were 10 mM dNTP, Oligo(dT) 0.5 µg/µl, 5 µg of extracted RNA and DEPC-treated water; the total volume of the mixture being 10 µl. This mixture was then incubated for five minutes in 65°C and thereafter placed on ice for one minute. In the meanwhile the mastermix for the RT-PCR was prepared containing 10x RT buffer, 25 mM MgCl2, 0.1 M DTT and RNaseOUT™. Nine microliters of the mastermix was added to each sample of the first mixture and incubated at 42°C for two minutes. Following this one microliter of enzyme SuperScript™ II RT was added to each sample and incubated at 42°C for further 50 min followed by 15 min at 70°C. The samples were placed on ice and the amplification of the cDNA was performed as described in genomic DNA above.

Epididymal cell culture

General reagents (Sigma© Switzerland):

- Principal cell medium (PCM) was used for culture procedures and this was prepared as follows: Dulbecco's minimum essential medium (DMEM) supplemented with 200 nM hydrocortisone, 1 mM sodium pyruvate, 100 nM insulin, 5 µg/ml transferrin, 1 µg/ml retinol, 10% new borne calf serum, 1 µM androstenedione and 100 µg/ml of an antibiotic mixture (penicillin, streptomycin and gentamycin).
- Serum free medium (SFM) was used for enzymatic digestion. It is prepared as PCM however without new borne calf serum and androstenedione and supplemented with collagenase Type II (Sigma) from Clostridium histolyticum and hyaluronidase.
- DMEM was supplemented with Cyproterone acetate (CPA) in order to obtain a 12 mM CPA solution.

Extracellular matrix (ECM) and culture plate: Cultrex© (R&D Systems) diluted by 1:80 was used as ECM. The major compounds of the solution are laminin, collagen IV, entacin and heparin sulfate proteoglycan. Inserts with micropores (pore size of 0.4 µm) for a 12-well plate were impregnated with Cultrex© and incubated for 30 min at 37°C, at this temperature the solution polymerizes to a thin layer of ECM.

Enzymatic digestion cell culture and apoptosis induction: Byers et al. [4] first described this method for preparation and culturing in 1985. The epididymides of five wild type and five BCL2l13$^{+/-}$ mice, sacrificed by cervical dislocation, were the source for the cell cultures. After the epididymis was removed, minced into 2 mm fragments and washed in PBS, the tissue was suspended in serum free medium containing 360 U/ml collagenase and incubated at 37°C for 60 min under continuous shaking. Thereafter the non-epithelial cells were decanted and a second enzyme treatment with 360 U/ml collagenase and 70 U/ml hyaluronidase was performed for further 60 min in 37°C under continuous shaking. After the enzymatic digestion, tissue fragments were centrifuged at 800 g_{max} for 5 min, washed with PBS and plated in the insert of a 12-well plate having 400 µl of the cell suspension and 500 µl of PCM per insert. The plate was incubated at 34°C. After 24 h the cells were transferred to freshly ECM-coated inserts of a 6-well plate. In order to induce apoptosis [5] CPA was added in different concentrations (30, 60, 120 and 240 µM); the control samples instead had PCM.

Identification of androgen-receptor (AR) in cell culture: After 48 h, the cells in culture were trypsinized and collected as a cell pellet. The further steps to extract and quantify the RNA as well as the synthesis of the first strand cDNA have been described previously. As described by Tachibana et al. in 2000 [6], 0.5 µM sense primer CCATCCAAGACCTATCGSGG (position 75-94) and anti-sense primer TGAGTCATCCTGATCTGGAG (position 424-443) were used for the amplification of cDNA. Following this, we performed a gel-electrophoresis with 2% agarose in TBE solution.

Cell count and cell viability assessments: The cells were counted after 24, 48, 72 and 96 h after culturing. Prior to the counting the cells in the inserts were trypsinized with 800 µl 0.5% trypsin-EDTA, following immediate addition of 200 µl calf serum in order to antagonize the trypsin. For quantifying the cell number 100 µl cell suspension was pipetted into a holder with one milliliter standard CASY™ buffer. The count was performed with CASY™, an electric field multi-channel cell counting system. The principle of the distinction between viable and dead cells is based on the integrity of the plasma membrane; living cells have an intact plasma membrane whereas death cells have a broken one. When a cell is exposed to a low voltage field, the electric current cannot pass through the intact membrane, which functions as an electric isolator, if the cell is viable. On the contrary in an injured cell, as the cellular membrane is broken hence pores on the membrane, the electric current can go through (Figures 2 and 3).

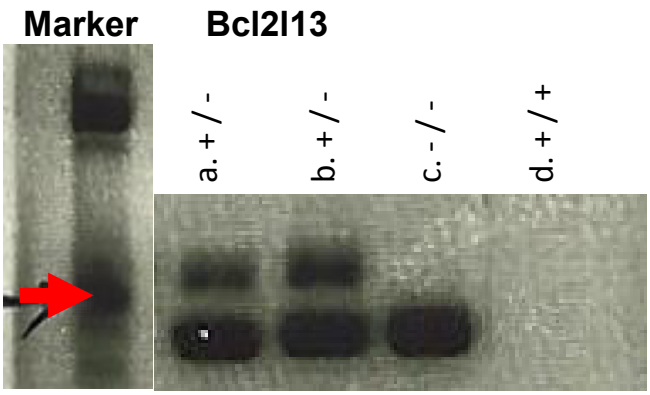

Figure 2: Genotyping.
For the marker we used a 100 bp (0.5 mg/lane) ladder. The arrow marks the 600 bp band
a. Bcl2l13 heterozygote
b. Bcl2l13 heterozygote
c. Bcl2l13 homozygote
d. Wild type

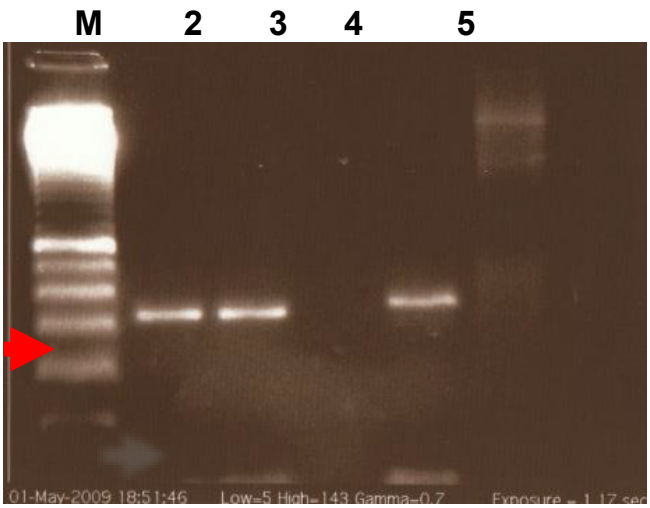

Figure 3: Androgen receptor in epididymal cell culture.
Demonstration of AR transcript by RT–PCR in epididymus and testis cell cultures. RNA of tissues from three male mice were mixed and used for RT–PCR. Amplified products were electrophoresed on 2% agarose gel. In non-RT controls, RNA of epididymus was PCR-amplified without RT
Lane 1 (M): Size marker; lane 2 epididymus; lane 3 epididymus; lane 4 Non RT control; lane 5 testis.The arrow marks the 600 bp band. The expected size of the AR is 374 bp

Results

Cell culture microscopy

The cell clumps from epididymal tissue composed mainly of epithelial cells, sperm cells and fibroblasts form a monolayer on the insert (approximately 10^5 cells/well). Different cell sizes could be explained by their different region of origin, the cells originating from the head of epididymis are larger than from the body part of the epididymis (Figures 4c and 4d).

12 h after culturing the first pictures were taken and thereafter 24 h later the first cell count was performed. The 24 h are needed for the cells to become detached from the clumps and spread through the well in order to form a monolayer (Figures 4a-4d) [7,8].

The cells were counted (CASY® Cell Counter) after 24 h without CPA and then 24, 48 and 72 h after incubation with CPA 10 µM. The CPA concentration was suggested by previous experiments of apoptosis in liver cells [9]. After 24 h CPA was added and we compared the number of cells in the Bcl2l13 k.o. culture versus wild type culture. CPA is an androgen receptor antagonist which leads to block the androgen supply and induces consequently apoptosis in epididymal cells. The principal medium contained the androgenic substance androstenedione (1 µM).

The diameter of an epididymal cell in a mouse is on average of 7 to 12 µm, depending from which region the cells originate. Thus we concentrate on the cells in that size range and the general viability of the cells. Under 6 µm of cell size we only found cell debris respectively dead cells and since for sizes over 12 µm single cells can be excluded, we conclude that the single cell clumps were counted as one with a larger diameter.

The difference between the k.o and the wild type cells after the first 48 h of incubation with 10 µM CPA was not remarkable, however there was a difference in the viability after 72 h; the k.o. cells had a 20% higher viability than the wild type cells (Figure 8). Cells, which were not treated with CPA, served as control. After 72 h the cell viability amounted on average 55% in both cell lines. The cells could be kept in culture for maximum of 5 days thereafter they expired (Figures 9-12).

Discussion

The protocol used for this project was developed for rat epididymis [4] whereas we applied it to mice. After some procedural modifications, using testosterone instead of androstenedione, decreasing the incubation temperature to 34°C and coating the wells with collagene type IV, we obtained a satisfiable setting in order to keep the cells in culture for up to 5 days. As the results show there is barely any difference between k.o. and wild type cells following the first 48 h of incubation, however there is a remarkable difference after 72 h.

The Bcl2l13 k.o. cells have in average a 20% higher viability compared to the wild type. What is remarkable is that the control cells which were not treated with CPA show a similar trend after the first 48 h; however there is a major decrease of viability after 72 h without a difference between wild type and Bcl2l13 k.o. cells. However, in order to confirm this conclusion, the experiment would be needed to be repeated (at least 3 valuable experiments) and the mean values compared in statistical tests. In our study, the cell cultures consists of both fibroblasts and epididymal cells. Thus we only quantified the total number of dead cells.

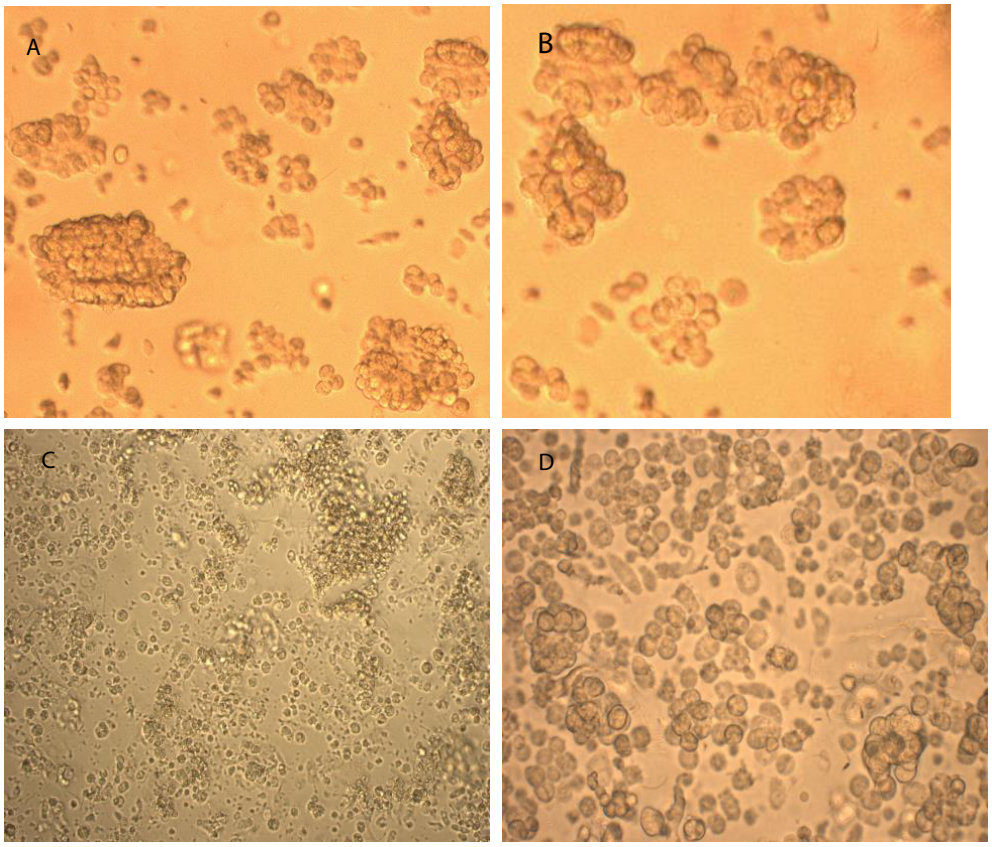

Figure 4: Cell culture microscopy.

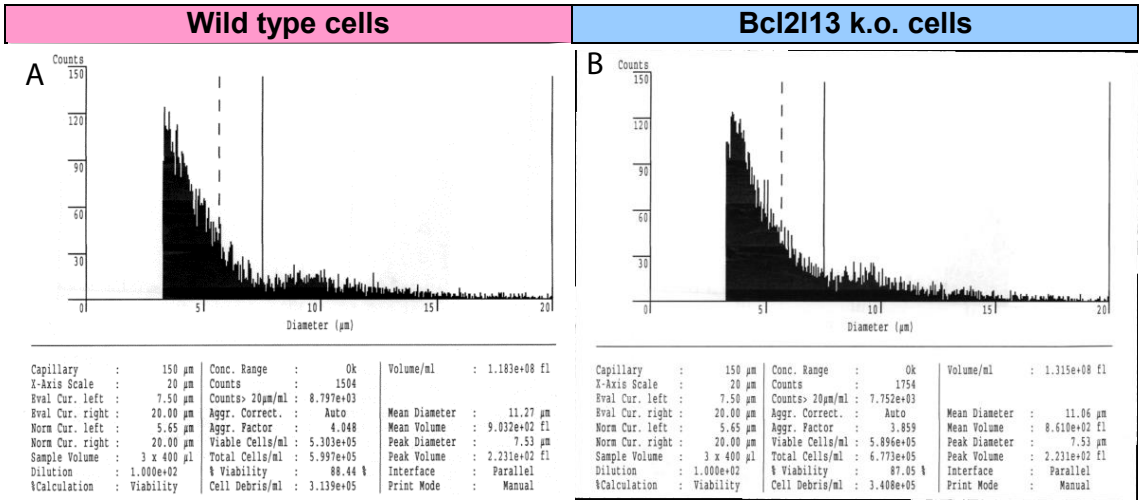

Figure 5: Cell viability.
a: Wild type cells: 24 h after culturing. No CPA added yet. The viability at that point is 88.4% (5.3 x 10^5 cells).
b: Bcl2l13 k.o. cells: 24 h after culturing. No CPA added yet. The viability at that point is 87 % (5.9 x 10^5 cells).

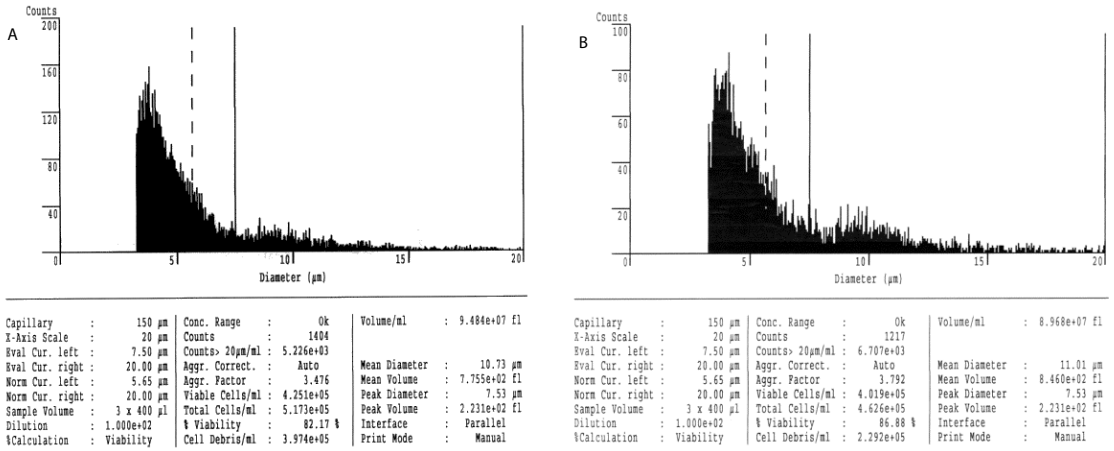

Figure 6: a 24 h after adding 10 µM CPA the cells were counted again. The viability, 82 %, has hardly decreased (4.3 x 10^5 cells).
b: 24 h after adding 10 µM CPA the cells were counted again. The viability, 87 %, has hardly decreased (4 x 10^5 cells).

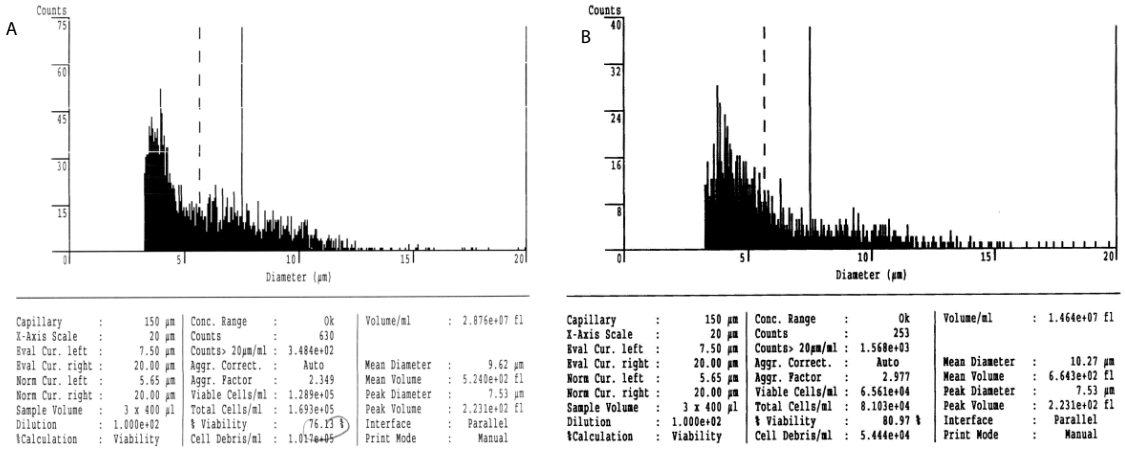

Figure 7 a: 48h after incubation with 10 µM CPA the cell viability decreased to 76.2% (1.3 x 10^5 cells).
7b: The viability decreased to 81% (6.6 x 10^4 cells).

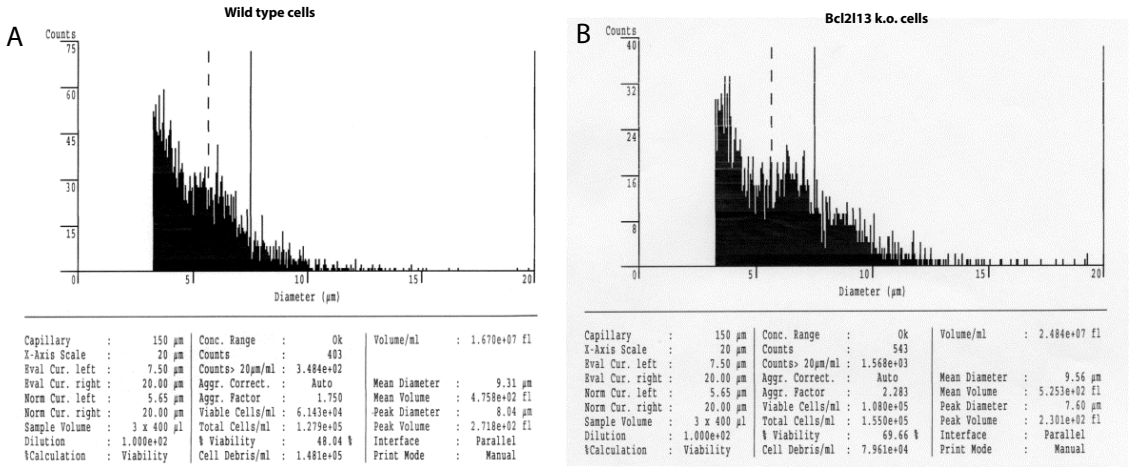

Figure 8a: 72 h after incubation with 10 µM CPA the viability reached 48% (6.1 x 10^4 cells).
b: The viability in the Bcl2l13 k.o. cells reached 70% (10^5 cells) after 72 h.

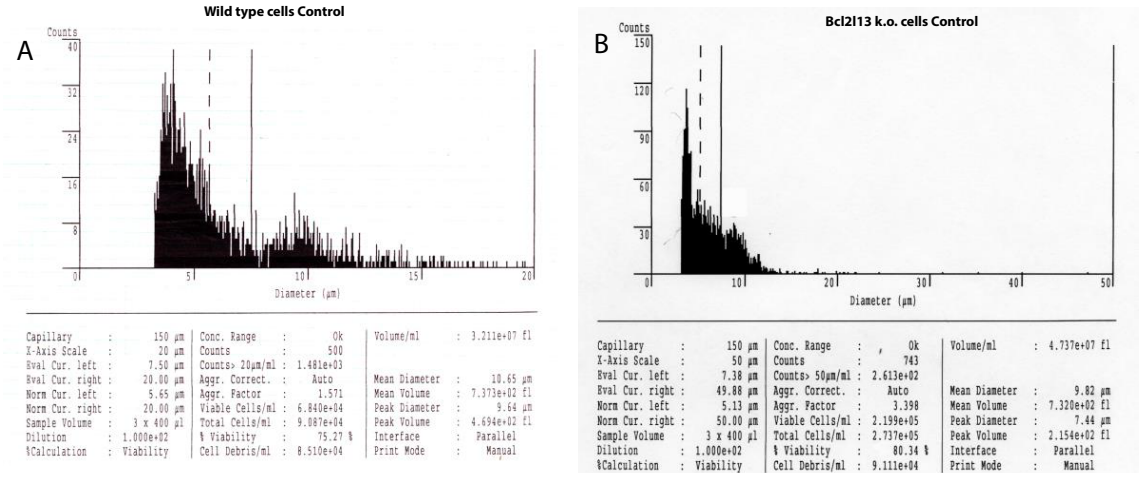

Figure 9a: 0 h culturing without CPA.

b: 0 h culturing without CPA.

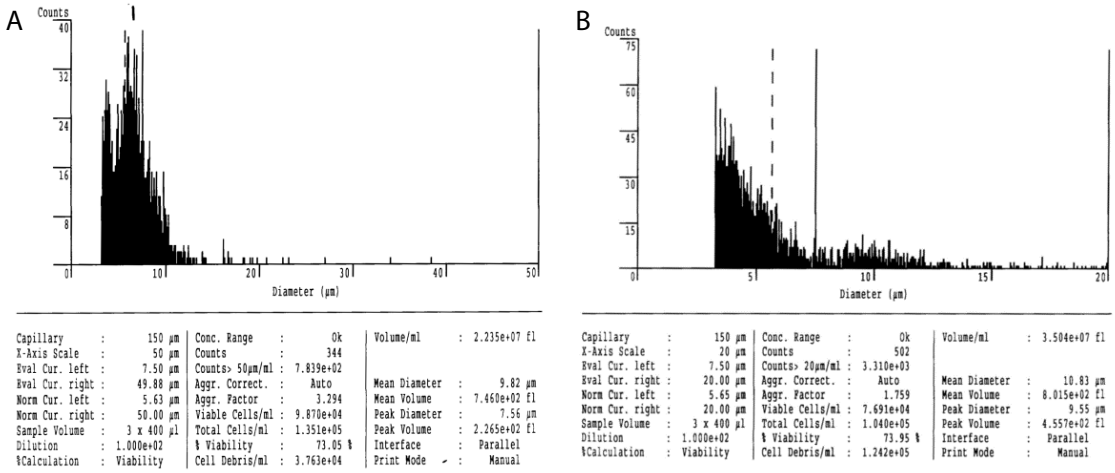

Figure 10a: 24 h culturing without CPA.
b: 24 h culturing without CPA.

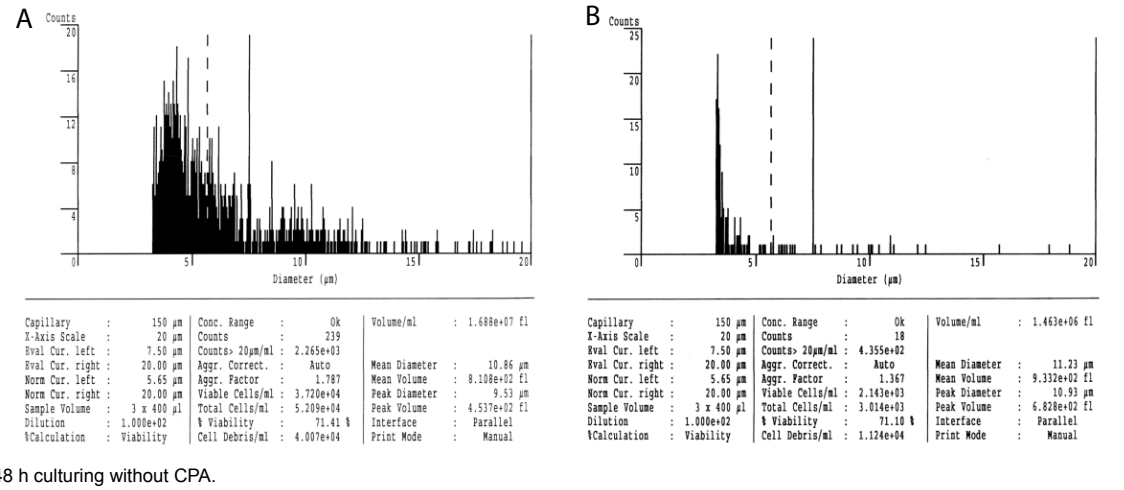

Figure 11a: 48 h culturing without CPA.
b: 48 h culturing without CPA

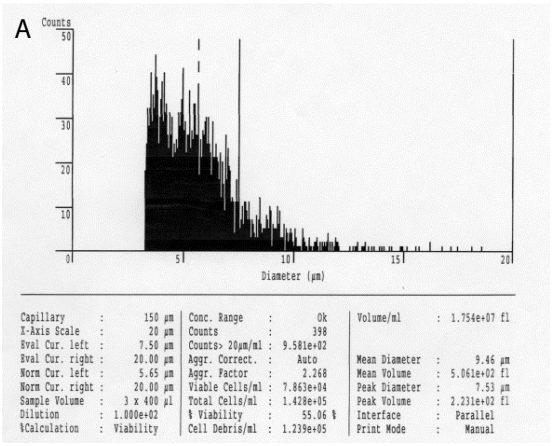

Figure 12a: 72 h culturing without CPA.
b: 72 h culturing without CPA.

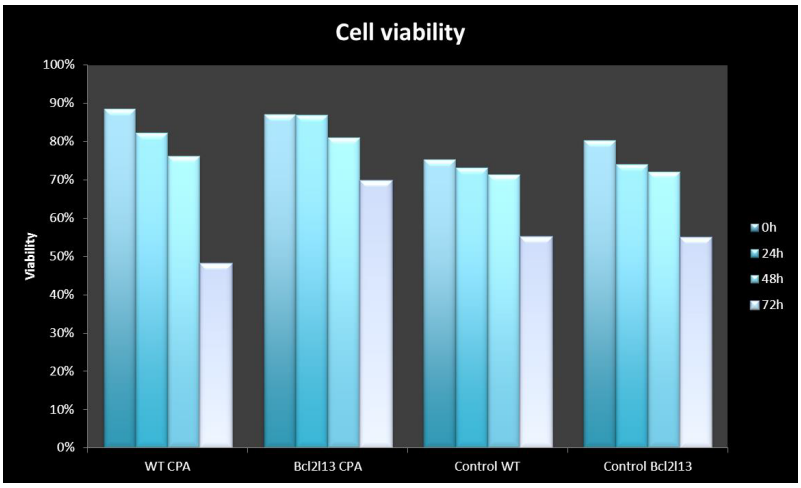

Figure 13: Cell viability.

Conclusion

What we can conclude from these results however is that the epididymal tissue of Bcl2l13-k.o. compared to the wild type appears to be more resistant towards apoptosis. There is nonetheless no evidence of epithelial cells being the only cells involved in this phenomenon, presumably fibroblasts were contributory as well.

We conclude that the single knock out of Bcl2l13 contributes to the apoptosis resistance however; its mutation might be redundant. This could be expected by observing the phenotype. Would the mutation of Bcl2l13 have serious consequences, the function of the organ would be affected compromising fertility, which we did not observe. On the other hand, considering that apoptosis has a crucial function in a living organism it makes sense from the evolutionary point of view to have various proteins helping to regulate this process, so that if one is impaired the consequences would not be drastic. Nevertheless there are BCL2 mutations in human with a crucial regulatory defect of apoptosis and these having often neoplastic consequences, i.e., lymphoma.

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