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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This current proposal is based on previous publications that (1) the proapoptotic Bcl-2 family member Bok, is capable of exerting growth inhibition on cancer cells growing in tissue culture, and (2) the apoptotic activity of Bok is not inhibited by the anti-apoptotic molecules Bcl-2 and $Bclx_L$. To further investigate the anti-tumor ativity of Bok on breast cancer and to develop a Bok gene therapy for breast cancer, we have proposed three specific aims to accomplish our objectives.

Specific Aim 1: To confirm hBok-mediated transformation suppression in breast cancer cells.

Specific Aim 2: To investigate the mechanisms of hBok-induced apoptosis.

Specific Aim 3: To develop a tumor specific promoter using hTERT promoter driven hBok to examine the preclinical effect of hBok for breast cancer gene therapy.

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Table of Contents

INTRODUCTION

Defects in the processes regulating apoptosis will prolong cell growth and result in carcinogenesis (*1*). Central to the process of apoptosis is the activation of caspases (*2, 3*). Members of the Bcl-2 family, play a pivotal role in regulating apoptosis by controlling the mitochondrial changes associated with the release of cytochrome C (*4*). Bax and Bak function as the major pro-apoptotic molecules at the mitochondria (*5, 6*) molecules (*7-9*), while Bcl-2 and Bcl-x_L function as the major anti-apoptotic molecules at the mitochondris (*10-12*) Recently the apoptotic functions of Bax and Bak were associated with the endoplasmic reticulum inducing release of calcium (*13*). There is at present no evidence linking nuclear localization of any member of the Bcl-2 family with their anti- or pro-apoptotic function. Rat Bok (rBok) is a pro-apoptotic member of the Bcl-2 family that is similar in structure to Bax and Bak, (*14*) (*15*) (*16*). However, unlike Bax and Bak, rBok does not form heterodimers with the antiapoptotic Bcl-2 or Bcl-x_L and in addition, Bcl-2 does not suppress the killing ability of rat rBok (*14*) suggesting that Bok might have a unique role in the apoptotic cascade. The human homolog of rBok, hBok, differs from rBok, by only nine amino acid residues but we show that its apoptotic activity is similar to rBok,. The BH3 domain of hBok contains the highly conserved amino acid residues present in all anti- and pro-apoptotic members of the Bcl-2 family (*17-19*). However the BH3 domain of hBok contains a short leucine rich stretch of amino acids representative of a nuclear export signal (NES)(*20*) not seen in the BH3 domain of any other member of the Bcl-2 family. We detected hBok in the nucleus of cells and successfully increased its nuclear concentration with leptomycin B, an inhibitor of Crm1. Crm1 mediates the export of leucine rich NES-containing proteins (*21*). Mutating the

NES of Bok resulted in an increased nuclear concentration of the protein accompanied with a more potent pro-apoptotic function. Utilizing the apoptotic property of mutant Bok we show that it inhibited survival of breast cancer cells to a greater degree than wildtype. These results suggest that hBok might function at the level of the nucleus, suggesting a unique mechanism of action. In our investigation of this mechanism we have shown that hBok-induced apoptosis can be partially inhibited by both caspase 8 and 3 inhibitors suggesting that hBok-induced apoptosis might require the extrinsic apoptotic pathway

BODY:

A. SPECIFIC AIMS: (NO CHANGES)

Specific Aim 1: To confirm hBok-mediated transformation suppression in breast cancer cells.

Specific Aim 2: To investigate the mechanisms of hBok-induced apoptosis.

Specific Aim 3: To develop a tumor specific promoter using hTERT promoter driven hBok to examine the preclinical effect of hBok for breast cancer gene therapy.

B. STUDIES AND RESULTS

Specific Aim 1: To confirm hBok-mediated transformation suppression in breast cancer cells.

We have shown that hBok can induce apoptosis inhibit proliferation of breast cancer cells in tissue culture (Appendix Figure 1A,B and C) To further investigate the anti tumor effect of hBok we set out to establish hBok stable transfectants in known metastatic human breast cancer cell lines MCF-7 MDA-MB435 and MDA-MD 231. Our initial

attempt in obtaining positive stable transfectants using the tet-inducible system failed. We are at present in the process of setting up the Ecdysone-Inducible Mammalian Expression System (Invitrogen) in a second attempt to generate stable transfectants

Specific Aim 2: To investigate the mechanisms of hBok-induced apoptosis.

Analysis of the BH3 domain of hBok revealed a short leucine rich stretch of amino acids representative of a NES(Appendix Fig 2A). Expanding our study on this observation we detected hBok in the nuclear and cytoplasmic fractions of transformed HEK293T cells (Appendix Fig 2B). In addition, we detected hBok in the nucleus of MDA-MD 231 cells by Immunostaining. As indicated by the arrow we show hBok as a nuclear protein independent from its location within the endoplasmic reticulum (Appendix Fig 2C). Treatment of the cells with leptomycin B resulted in an increase nuclear concentration of hBok (see arrow Appendix Fig 3A) suggesting that nuclear translocation of hBok is perhaps a regulated process. To determine if nuclear localization of hBok has functional relevance we substituted ^{72}LRL^{74} within the putative nuclear export sequence with alanine. We observed that the ^{72}AAA^{74} mutant of hBok was present in the nucleus at a higher concentration than wildtype hBok (see arrow Appendix Fig 3A), and in addition, was more apoptotic than wildtype (Appendix Fig 3B). These results indicate that hBok might function at the level of the nucleus, making it a novel member of the proapoptotic Bcl-2 family, and suggests a unique mechanism of action associated with this protein. We are at present attempting to further understand this mechanism of Bok-induced apoptosis. As a preliminary observation we observe that the apoptotic activity of hBok can be partly inhibited the the caspase 8-inhibitor Z-IETD-FMK (data not shown).

Specific Aim 3: To develop a tumor specific promoter using hTERT promoter driven hBok to examine the preclinical effect of hBok for breast cancer gene therapy.

The proposed experiments of this aim will be addressed in the coming year.

KEY RESEARCH ACCOMPLISHMENTS

- hBok reduces the proloferation of the breast cancer cells MDA-MB-231, MDA-MD 435 and MCF-7 in tissue culture.
- Demonstrated that hBok is a pro-apoptotic Bcl-2 member that translocates to the nucleus.
- Generated a NES mutant by substituting leucine residues within the putative NES with alanine and showed that this mutant had a stronger apoptotic activity than wildtype

REPORTBLE OUTCOMES

We are in the process of submitting a manuscript entitled "Nuclear translocation of the pro-apoptotic Bcl-2 Family member Bok induces apotosis". A draft of the manuscript is attached.

CONCLUSIONS:

In this report we demonstrated that CMV-hBok expression reduces the cellular proliferation of breast cancer cells in-vitro. In addition, we identified a putative NES within the BH3 domain of hBok. A similar sequence is not present in the BH3 domain of any other member of the Bcl-2 family. We identified hBok in the nucleus of cells and

showed that nuclear localization of hBox is a regulated process since inhibition of the exportin CRM1 by leptomycin B resulted in an increase in the concentration of hBok. We substituted two leucine residues within this NES with alanine and showed that in addition to sequestering hBok in the nucleus, we were able to enhance the apoptotic potential of the protein. Finally, it has been shown that the apoptotic activity of hBok is not inhibited by either Bcl-2 or Bcl-x_L. Overexpression of Bcl-2 has been associated with many tumors including breast tumors (*22*) It is also known that overexpression of Bcl-2 renders tumor cells refractory to chemotherapy and radiation.(*23*) (*24, 25*). We propose that successful targeting of the NES mutant of hBok to breast cancer might lead to beneficial outcomes in the treatment of this disease.

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Figure Legends

Figure1 Determination of the Apoptotic Potential of hBok. **a.** HEK293T cells ($1x10^5$ cells/chamber) growing in two chambered slides were transfected with pcDNA3 (vector) and pcDNA3 expressing either rBok or hBok. Apoptotic cells (indicated by an arrow) were identified by TUNEL assay. **b.** HEK293T cells ($1x10^6$ cells/100 mm plate) transiently transfected with pcDNA3 (vector) and pcDNA3 expressing either rBok or hBok. Apoptosis was determined by DNA fragmentation analysis. **c.** Breast cancer cell lines MDA-MB 231, MDA-MB 435 and MCF-7 were transiently co-transfected with either pcDNA3 (control) and pLuc, or phBok and pLuc. Cell viability was determined by the luciferase reporter assay.

Figure. 2. Nuclear Translocation of hBok **a.** Open reading frame of hBok highlighting the putative nuclear export sequence (LLRLGDELE) within the BH3 domain. **b.**

Western Blot analysis of nuclear and cytoplasmic fractions of transfected HEK293T cells with antibodies against flag, PARP and a-tubulin **c.** MDA-MB 231 cells in a 4 chambered slide ($3x10^5$ cells / chamber) were transfected with Flag-tagged hBok (0.6 mg) The immunofluorescence staining shows flag-tagged hBok the nucleus and the endoplasmic reticulum and identified hBok in the nucleus(see arrow).

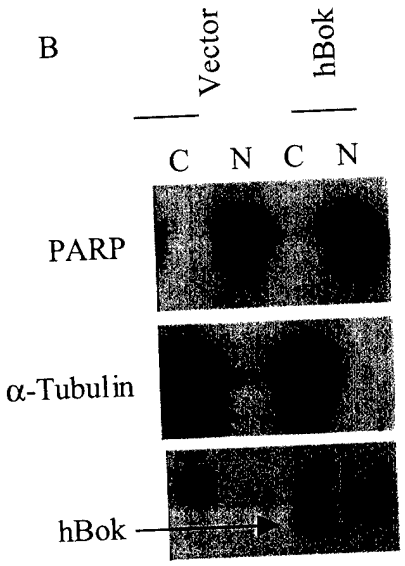
Figure. 3. Apoptotic activity of hBok influenced by nuclear sequestration. **a.** MDA-MB 231 cells in a 4 chambered slide ($3x10^5$ cells / chamber) were transfected with Flag-tagged hBok (0.6 mg). One set of transfected cells were exposed to LMB (20 ng / ml) for 6 h. The immunofluorescence staining shows an increase of nuclear hBok following treatment with LMB. In addition, MDA-MB 231 cells in a 4 chambered slide ($3x10^5$ cells / chamber) were transfected with either Flag-tagged hBok (0.6 mg) or the NES mutant of hBok (0.6 mg). The immunofluorescence staining shows a stronger staining Bok in the nucleus following treatment with LMB or mutation of the NES sequence. b. HEK293T cells were transiently co-transfected with either pcDNA3 (control) and pLuc , phBok and pLuc. or NES mutant phBok and pLuc. Cell viability was determined by the luciferase reporter assay.

Figure 1

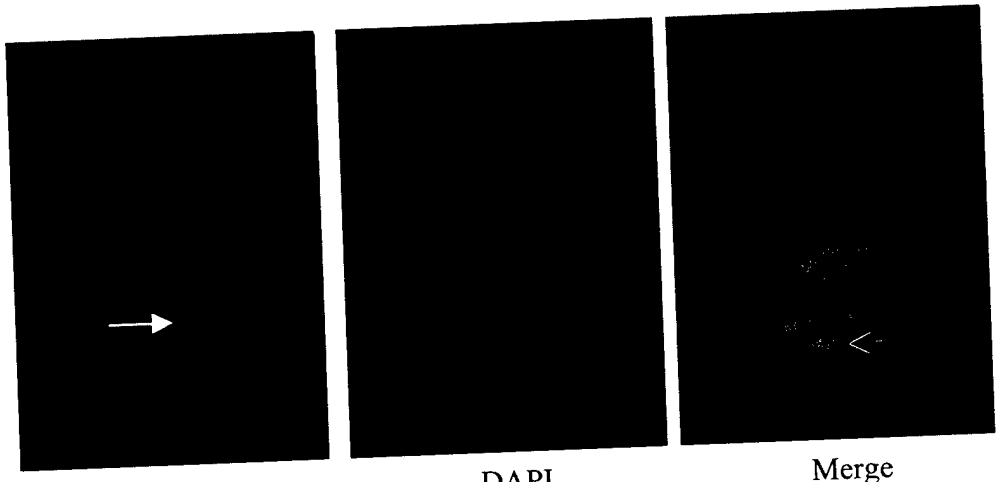
A
pcDNA3
prBok
phBok
Apoptotic Cells (%)
35
30
25
20
15
10
5
0
pcDNA3
rBok
hBok
B
rBok
hBok
C
% Viability of Control
125
100
75
50
25
0
Control
hBok
MDA-MB 231
MDA-MB 435
MCF-7

Figure 2

A ^{69}AVLLRLGDELEQIR^{82}



C



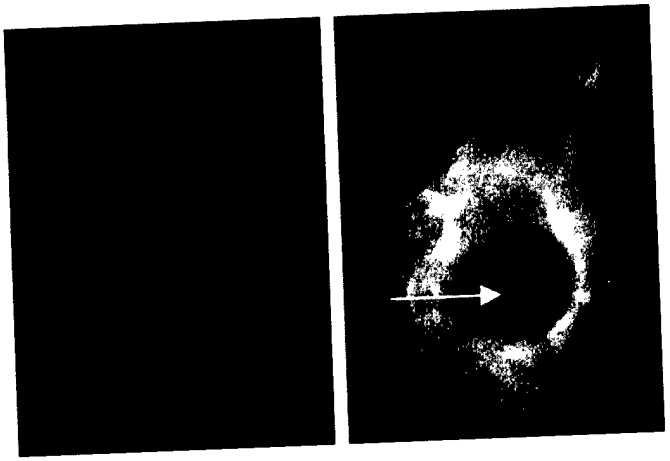
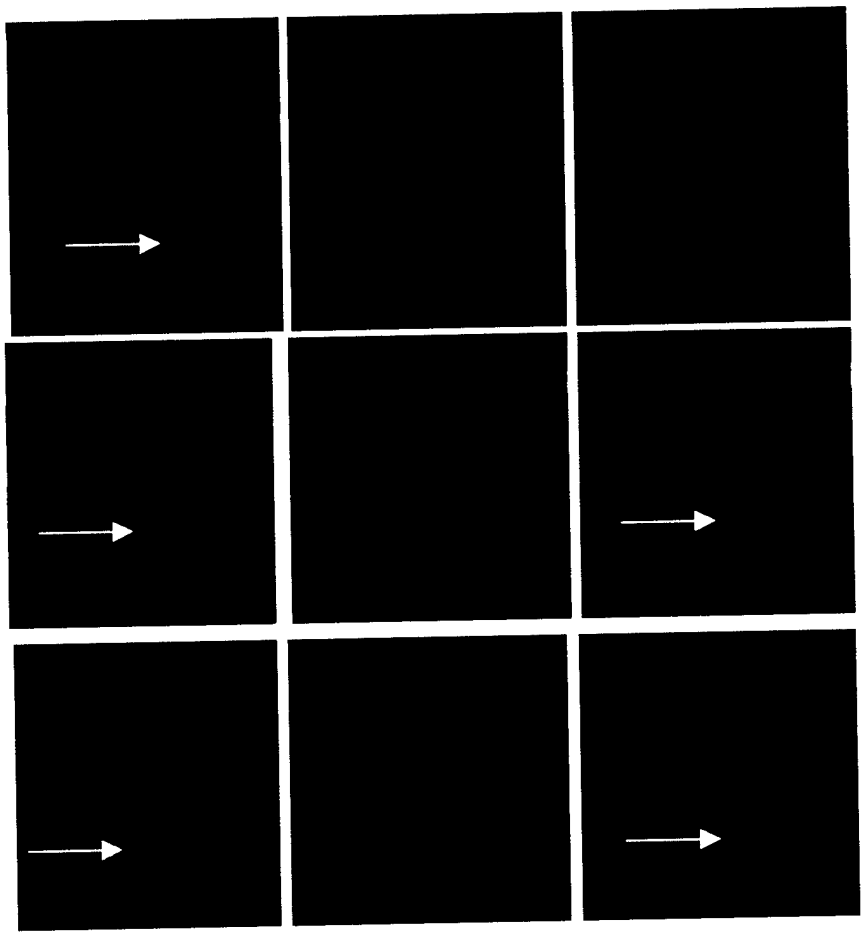
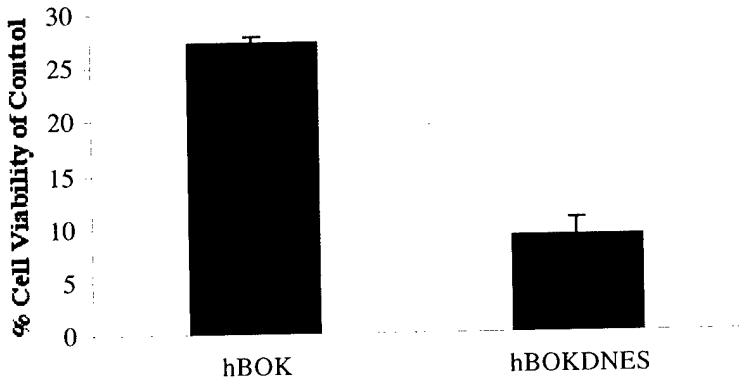


Figure 3

A


(-) LMB
(+) LMB (20 ng ml-1)
Mutant hBok
hBok
DAPI
Merge

B


% Cell Viability of Control
30
25
20
15
10
5
0
hBOK
hBOKDNES

Appendix 1.0

Manuscript in preparation

Nuclear translocation of the pro-apoptotic Bcl-2 Family member Bok induces apotosis

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Bcl-2 and Bcl-X_L inhibit the apoptotic activities of Bax or Bak but not Bok. This difference could be attributed to the ability of Bok to translocate into the nucleus. Treatment of cells with leptomycin B or mutating the NES of Bok sequestered the protein in the nucleus. The NES mutant demonstrated a stronger apoptotic activity than wildtype in selected cancer cells revealing a functional relevance to its nuclear translocation. The apoptotic activities of both wildtype and mutant Bok were not inhibited by Bcl-2 indicating a unique mechanism of action. We predict a therapeutic potential to mutant Bok.

Central to apoptosis is the activation of caspases [1,2]. One of the apoptotic pathways activating caspases is the intrinsic apoptotic pathway which induces changes in mitochondrial membrane permeability and the release of mitochondrial proteins such as cytochrome C [3] which binds to the adaptor protein Apaf-1 and in the presence of dATP activates pro-caspase 9. Activated caspase 9 activates caspase3 leading to apoptosis [4].

The Bcl-2 family of proteins are key regulators of the intrinsic pathway. Members either promote cells survival (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1), or cell death Bax, and Bak (the Bcl-2 homology domains (BH1-3) group) or Bad, Bid, Bik, Bim, Puma, Noxa and others (the BH3 domain only group). The BH3 domain only group serve as sensors of the apoptotic signals [5-7]. Bcl-2, Bcl-x_L, Bax and Bak contain a stretch of hydrophobic amino acids at their COOH termini that serves to anchor these proteins into organelle membranes [8,9]. Predominantly cytosolic in healthy cells, Bax translocates to the mitochondria in response to an apoptotic stimulus where it promotes cell death by altering the permeability of the mitochondrial membrane [10,11]. Bax and Bak demonstrate functional redundancy at the mitochondria since one has to be functional for the apoptosis related mitochondrial changes to occur [12-14]. Recently, the apoptotic functions of Bax and Bak were associated with the endoplasmic reticulum inducing release of calcium [15]. Although detected in the membranes of the endoplasmic reticulum, mitochondria and nucleus [8,9,16], the major function of Bcl-2 and Bcl-x_L is at the mitochondrial membrane where they oppose the mitochondrial changes initiated by Bax or Bak.

Rat Bok, a little studied pro-apoptotic member of the Bcl-2 family is similar to Bax and Bak comprising BH1, BH2 BH3 domains and a COOH-terminal transmembrane region [17,18]. However, Bok fails to form heterodimers with Bcl-2 or Bcl-x_L and its apoptotic activity is not antagonized by Bcl-2 [17] suggesting, that Bok might have a unique role in the apoptotic cascade. The human homolog of rBok, hBok, differs from rBok, by only nine amino acid residues but we show that its

apoptotic activity is similar to rBok,. The BH3 domain of hBok contains the highly conserved amino acid residues present in all anti- and pro-apoptotic members of the BCL family [19-21], but unique to the Bok BH3 domains is a short leucine rich stretch of amino acids representative of a nuclear export signal (NES)[22]. A consensus for the NES has been defined [23] but it is now becoming clear that the NES are quite divergent [24]. Expanding our study on this observation we detected Bok in the nucleus of cells and successfully increased its nuclear concentration with leptomycin B, an inhibitor of Crm1. Crm1 mediates the export of leucine rich NES-containing proteins[25]. Mutating the NES of Bok resulted in an increased nuclear concentration of the protein and a more potent pro-apoptotic function. Utilizing the apoptotic property of mutant Bok we show that it inhibited survival of breast cancer cells. These results suggest that hBok might function at the level of the nucleus, making it a novel member of the proapoptotic Bcl-2 family, and suggest a unique mechanism of action associated with this protein.

Results

Human Bok induces apoptois.

The human homolog of the rBok gene has 95% identity to rBok. To investigate if the apoptotic activity of hBok is similar to rBok the pcDNA3 empty-vector and vector-expressing either hBok or rBok were transiently transfected into human epithelial kidney (HEK) 293T cells. Cells were harvested 24 h after transfection, and Bok-induced apoptosis determined by TUNEL assay (Fig 1a) and DNA Fragmentation Analysis (Fig. 1b). Thirty percent of HEK293T cells transfected with hBok underwent apoptosis, while 20% of rBok transfectants and 5% of vector transfectants

underwent apoptosis. These observation indicate that the minor difference in amino acid residues present in hBok had no significant influence on its apoptotic activity.

To determine if hBok could effectively kill proliferating breast cancer we performed the luciferase reporter assay on the breast cancer cell lines MCF-7, MDA-MB 231 and MDA-MB 435 that had been transfected with the pcDNA3 empty-vector and vector-expressing hBok. We observed that hBok induced cell death in the breast cancer cell lines (Fig. 2a), and inhibited their rate of proliferation (Fig. 2b). It has been shown that the apoptotic activity of rBok is not antagonized Bcl-2. We observed that hBok-induced apoptotis of the breast cancer cell lines studied was also not influenced by endogenous levels of Bcl-2 (Fig 2c). We conclude that the observed tumor suppressor effect in the breast cancer cells was specific to hBok, since transfection of the vector had minimal effect on cell survival, and hBok expression was only detected in the pcDNA3 vector-expressing hBok and not from the empty-vector (Fig.2d)

hBok translocates to the nucleus.

A putative nuclear export sequence (NES) ^{69}AVLLRLGDELEM^{78} is present within the BH3 domain of hBok (Fig 3a). To investigate if hBok is translocated to the nucleus both nuclear and cytoplasmic fractions obtained from HEK293T cells transfected with pcDNA3 empty-vector or vector-expressing Flag-tagged hBok were examined by Western Blot (Fig 3b). We observed hBok in both the nuclear and cytoplasmic fractions of cells transfected with Flag-tagged hBok but not with the vector. A band was also detected only in the cytoplasmic fraction of both samples. This band is larger in size than hBok and is most likely the result of a non-specific interaction. To rule out the possibility that nuclear hBok could have come from the endopasmic reticulum, indirect immunofluorescent staining of transfected MDA-MB-231 was carried out. Although we observed a

co-localization of hBok with the endoplasmic reticulum, we did notice a significant nuclear staining of hBok distinct from the endoplasmic reticulum (Fig. 3c). We conclude that hBok translocates to the nucleus of the cells tested.

The export of nuclear proteins that contain classical leucine-rich NES s is mediated by the transporter protein CRM1. CRM1-mediated export of proteins can be inhibited by Leptomycin B (LMB), an unsaturated, branched-chain fatty acid identified as a specific inhibitor of CRM1. To investigate if nuclear export of hBok is a regulated process, indirect immunofluorescent staining was carried out on transfected MDA-MB-231 cells grown in either the presence or absence of LMB. We observed that LMB could sequester hBok in the nucleus (Fig 4a). Although we observed Flag-tagged hBok in the nucleus of untreated cells there was a distinct increase in the concentration of nuclear hBok following exposure to LMB (Fig 4b). We conclude that the transport of hBok across the nuclear membrane is a regulated process.

To investigated if there is a functional relevance associated with the nuclear translocation of hBok we generated an NES mutant in which the presumably critical leucine residues within the putative NES sequence (72 Leu and 74 Leu) were substituted with alanine. Indirect immunofluorescent staining of MDA-MB-231 cells transfected with either wildtype hBok or the NES mutant clearly demonstrated that mutating the NES of hBok resulted in a significant sequestration of this protein in the nucleus (Fig 5a +5b). A luciferase viability assay carried out on HEK293T cells transfected with the pcDNA3 empty-vector or vector-expressing either wild type or mutant hBok indicated that sequestering hBok in the nucleus greatly enhanced its killing potential (Fig 5c). The cell-killing effect of both the NES mutant and wild type hBok was specific because transfection of the vector had minimal effect on cell survival (data not shown). The increased potency of mutant hBok is attributed to its ability to induce apoptosis at a rapid rate. FACS analysis

of transfected HEK293T cells indicated that mutant hBok induced apoptosis to a greater extent than wildtype as early as 6 h after transfection (fig 5d).

To further demonstrate the difference in apoptotic activity between wild type and NES mutant hBok, we cloned their cDNA s into the pADTrack vector in order to independently express the GFP and the hBok proteins from the same vector (Fig.6). We tested the apoptotic potential of the NES mutant in breast cancer cell lines MDA-MB-231 and MDA-MB-435 together with the Chinese Hamster Ovary (CHO) cells. Our results clearly show once again that the NES mutant of hBok demonstrates a higher apoptotic activity

Discussion

The BCL-2 family of anti-and proapoptotic members play a critical role in regulating apoptosis. As a result it has been proposed in the recent review by Cory and Adams [29] that the antiapoptotic members could function as oncogenes, while the proapoptotic members could function as tumor suppressors. It has been well documented in transgenic mouse models that overexpression of BCL-2 contributes to the onset of B-lymphoid tumors [30,31], breast tumors [32] and pancreatic cell tumors [33]. Overexpression of Bcl-2 also renders tumor cells refractory to chemotherapy and radiation [34 35,36]. In an attempt to identify a tumor suppressor that could be successfully utilized in most tumors, including those that have high expression Bcl-2, we selected the little-studied proapoptotic Bcl-2 family member hBok. This choice is of clinical significance since Bcl-2, an inhibitor of apoptosis, is overexpressed in many different human tumors [37-40], and the activity of hBok is not antagonised by Bcl-2[17]

Human Bok, is similar in its apoptotic activity and response to Bcl-2 to rBok. (Fig 1 and Fig 2c). Human Bok , Bax and Bak belong to the subgroup of proapoptotic channel-forming Bcl-2

proteins containing BH1, BH2 BH3 and a COOH-terminal transmembrane region [17,18,41]. However, unlike Bax and Bak, hBok does not form heterodimers with the antiapoptotic molecules Bcl-2 or Bcl-x_L [17]. This raises the possibility that despite hBok being structurally similar to Bax and Bak, it may differ functionally from members of this group. The hydrophobic COOH termini of Bax and Bak serve to anchor these proteins into organelle membranes. Bax is predominantly in a cytosolic latent form in healthy cells and translocates to the mitochondria in response to an apoptotic stimulus where it anchors into the outer mitochondrial membrane promoting cell death by altering the permeability of the mitochondrial pores resulting in the disruption of the mitochondrial membrane barrier [10,11]. It was recently demonstrated that in addition to their effect on the mitochondrial membrane Bax and Bak operate at the level of the endoplasmic reticulum and inducing ER-release of calcium [15]. No published work to date has associated nuclear localization or attributed a biological function with nuclear localization to member of the Bcl-2 family.

The BH3 domain of most pro- and anti-apoptotic members of the Bcl-2 family including hBok contain highly conserved leucine and aspartic acid amino residues [19-21]. However, situated within the BH3 domain of hBok is a short leucine rich stretch of amino acids not seen in the BH3 domains of any other member of this family. The most prevalent nuclear NES found consist of such a short stretch of leucine rich amino acids in which the leucine residues are critical for function [22]. Although a consensus for leucine-rich NES has been defined [23] it is now becoming clear that the leucine rich NES are quite divergent [24]. We detected hBok in the nucleus (Fig 3c) of cells suggesting that, unlike other members of the Bcl-2 family, nuclear localization might be important for its function. To address this possibility, we mutated the NES of hBok and showed that not only did this result in sequestration of hBok in the nucleus (fig 5a), it also enhanced the apoptotic activity of this protein (Fig 5c , Fig 5d and Fig 6). Since the exportin Crm1 mediates the export of leucine rich NES-containing proteins [25] we used leptomycin B, an inhibitor of Crm1 function, and showed

that inhibition of CRM1 activity resulted in an accumulation of nuclear hBok (Fig 4a). These results suggest that nuclear transport of hBok is most likely a regulated process and nuclear localization might be important for its apoptotic activity. This observation differed from earlier studies in which deletions within the BH3 domain of the protein failed to alter its apoptotic activity [42]. A possible reason for this difference is that these mutations did not alter the putative NES within the BH3 domain. In a third mutant, the leucine residues within the NES was replaced with glutamic acid residues. This mutant too, failed to demonstrate an alteration in apoptotic activity, raising the possibility that replacing the critical residues of the NES with alanine rather than glutamic acid might result in enhanced apoptotic activity. The observation that nuclear localization is important for hBok apoptotic activity is novel, and it is the first time that a biological function has been associated with the nuclear localization of a member of the Bax family of proapoptotic Bcl-2 proteins.

In conclusion, we predict that nuclear translocation of hBok is important for its apoptotic activity. We are optimistic that this nuclear function of hBok adds a new link to the already complex mechanisms by which members of the BCl-2 family regulate apoptosis. It is tempting at this time to speculate that hBok-induced apoptosis might be Caspase 2 dependent since this caspase has been identified as a nuclear protein with the ability to trigger mitochondrial dysfunction and cytochrome C release from the nucleus [43]. Although it is tempting to link caspase 2 activation to a hBok-induced apoptotic pathway we caution that further studies of the role of nuclear localization on the Bok-induced apoptotic pathway are warranted and may lead to the identification of an unknown pathway of programmed cell death. The n-terminal end of hBok contains multiple kinase recognition motifs, which raises the possibility that phosphorylation may play an important role in the regulation of hBok function. It is known that phosphorylation influences the function of both anti- and proapoptotic members of the Bcl-2 family, such as Bcl-2, Bad and Bik [44-48 49]. We

substituted the 21S amino acid residue of the putative MAP kinase motif and the 23T amino acid residue of the putative protein kinase C substrate motifs with either alanine or glutamic acid and tested the apoptotic activity of these mutants in tumor cells growing in tissue culture. The alanine mutants did demonstrate a greater killing ability than wild type (data not shown). We are currently continuing our study to further understand the significance of phosphorylation on the regulation of Bok-induced apoptosis.

Methods

Cell lines and culture conditions. HEK293T cells and the human breast cancer cell lines MCF-7, MDA-MB435 and MDA-MB231 were grown in Dulbecco s modified Eagle s medium/F12 medium (DMEM/F12) (GIBCO Laboratories, Grand Island, NY, USA), supplemented with 10% fetal calf serum and penicillin/streptomycin. All cells were incubated in a humidified chamber set at 37°C; the air contained 5% CO2.

Plasmid construction. To generate the NES mutant of hBok, specific alanine mutations were constructed by site-directed mutagenesis using the hBok cDNA template that was cloned into the pcDNA3 mammalian cell expression vector (Invitrogen). The selection primer 5 GGGACTCTGGGGATCCAAATGACCGACCAAGCG 3 was designed such that the BstB1 site in the pcDNA3 vector was replaced with BamH1. The mutagenic primer designed was; 5 GCGCGGTGCTGGCGGCGGCGGGCGATGAGCTG 3 , replacing ^{74}LRL^{76} of the putative NES within the BH3 domain with alanine. The nucleotide sequence was identified and the presence of mutations confirmed by DNA sequencing. For the expression of 5 Flag-tagged fusions of wild type and NES mutant hBok, polymerase chain reaction (PCR)-generated fragments of the different hBok cDNAs were subcloned into the pCMV-Tag 2-5 mammalian expression vector (Stratagene). The

authenticity of wild type and mutant Flag-tagged NES mutant hBok constructs was confirmed by nucleotide sequencing. To clone wild type and NES mutant hBok cDNA s into the AdTrack vector Kpn1/Xba1 Bok fragments were cloned into the corresponding sites in the AdTrack vector. The correct orientation and positioning of the hBok fragments in the AdTrack vector were confirmed by nucleotide sequencing.

Transfection. All DNA transfections were carried out using the liposome delivery system Cells to be transfected were grown overnight in Dulbecco s modified Eagle s medium/Haris F-12 medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were incubated with the plasmid/liposome complex in a serum- free medium (optimum) for 2, hours followed by replacement with Dulbecco s modified Eagle s medium/Haris F-12 medium and continued incubation at 37°C for 24 h or times indicated in figure legends.

Immunoflorescence. Cells were seeded onto four chambered slides at a concentration between 3 $x10^4$ to $5x10^4$ cells per chamber and transfected with the control pCMViTag, pCMV-FLAG-hBok or pCMV-FLAG NES mutant hBok. At respective time points (3, 6 and 9 hrs), cells were washed three times with ice-cold PBS for 5 min per wash, fixed with 4% paraformaldehyde for 15 min and permeabilized on ice with 0.2% Triton X-100 for 5 min. Permeabilized cells were washed in PBS- and treated with 0.1% normal goat serum for 30 min (to minimize non-specific adsorption of antibodies) and incubated for 1h with polyclonal rabbit anti-FLAG (Sigma, St. Louis, USA) and Calreticulin (Santa Cruz Biotecnology, Inc, USA). They were then incubated for 1h with goat anti-rabbit TexasRed secondary antibody (Vector, USA). Stained cells were examined under deconvolution microscopy (Zeiss AxioPlan2, Germany). The green and red fluorescence of FITC

and TexaRed were visualized and images were captured by use of a Zeiss AxioPlan2 equipped with a HAMAMATSU digital camera.

When treatment with LMB was performed the cells were incubated at 37°C for 6 h in Dulbecco s modified Eagle s medium/Haris F-12 medium containing 20 ng ml^{-1} LMB.

DNA Fragmentation by Agarose gel analysis. The method described by Herrmann et al was followed [26].

Colony-forming assay. Breast cancer cells were transfected with the control vector pcDNA3, phBok or pNES mutant hBok using liposome. Approximately 2 h after transfection, the cells were washed with phosphate-buffered saline, cultured in fresh medium for 24 h and then split 1:20. The cells were then grown in a selection medium containing G418 (Genetisin; Life Technologies, Inc.) at 500 μg ml^{-1} for 2 weeks. G418-resistant colonies were stained with crystal violet / ethanol (1% / 20%) and counted.

Nuclear Fractionation. HEK 293T cells transfected with the mammalian expression vector pCMV-tag and the vector expressing Flag-tagged hBok were harvested 24 h after transfection and the cytoplasm and nuclear fractions obtained as previously described [27]

TUNEL assay. Apoptotic cells were identified using the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay previously described [28]. Briefly, HEK293T cells were seeded in a two chambered slide ($1x10^5$ cells/chamber) a day before transfection. Cells were transfected with 1μg DNA using liposome as a gene delivery vehicle. Twenty four hours after transfection, cells were washed in cold phosphate-buffered saline and fixed in 4% paraformaldhyde,

and the TUNEL assay was performed. Percentage apoptosis was determined by counting the number of apopotic cells and dividing by the total number of cells in the field. The data were compiled from a combination of three independent experiments.

FACS Analysis HEK 293T cells were seeded in a six well plate ($1x10^5$ cells per well) a day before transfection. Cells were transfected with 2 μg DNA using liposome as a gene delivery vehicle. Adherent and floating cells were harvested by trypsinization washed twice in phosphate-buffered saline and resuspended in 420 μl 1x PBS. 980 μl cold 100% ethanol was added dropwise into each tube while vortexing at slow speed. The ethanol fixed cells were stored at —20C until needed. Fixed cells were centrifuged between 7000 and 8000 rpm for 5 minutes and the pellet resuspended in 500 μL of PBS/RNAse (final concentration 0.1 mg ml^{-1}). The cells were incubated at 37°C for 15 minutes and then mixed with 500 μl of PBS/PI (final concentration 25 μg ml^{-1}). Cells were analyzed by flow cytometry

Cell viability assays. The luciferase-based in-vitro cell viability assay was performed. Briefly, HEK293T and the breast cancer cells MCF-7, MDA-MB 435 and MDA-MB231 were co-transfected with either wild type or NES mutant hBok together with 1/10 fractions of the indicator plasmid cytomegalovirus promoter-luciferase expressing vector, using liposome as the gene-delivery vehicle. Inclusion of a ten fold excess of the hBok expression vector as compared with the pluc reporter plasmid ensures that most of the pluc-expressing cells also express hBok. Twenty four hours after transfection, cells were lysed, and the luciferase activity determined. Standard deviations were calculated from three independent experiments. Cell viability was also analyzed by transfecting the cells with the wild type or mutant hBok cloned into the AdTrack vector so that both the hBok construct and green fluorescent protein (GFP) could be expressed independently from the

same expression vector. Twenty four hours after transfection, the cells were washed and the percentage of GFP expressing cells from the population of cells in each field determined by microscopic analysis. The percentage of cell viability was normalized by using the number of green cells in the population transfected, with only the vector (100%) as the base line.

Western blot analysis. Proteins were extracted from the cells with protein lysis buffer (50 mM Tris (pH 8.0), 150 mM Nacl, 0.1% sodium dodecyl sulphate [SDS], 0.5% sodium deoxycholate, and 1% NP40). Proteins (50 μg per sample) in SDS-loading buffer (100 mM Tris [pH6.8], 200 mm dithiothreitol, 4% SDS, 20% glycerol and 0.2% bromophenol blue) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Immun-Blot PVDF Membrane (Bio-Rad, Hercules,CA). The membrane was blocked with 5% dry milk and 0.1% Tween 20 (U.S. Biochemical, Cleveland, OH) in phosphate-buffered saline followed by an incubation with Anti-Flag M5 monoclonal antibody (Sigma) and then with horseradish peroxidase-conjugated secondary antibody according to the manufacturer s instructions. The immunoblots were visualized by an enhanced chemiluminescence (ECL) kit obtained from Amersham Pharmacia Biotech.

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Figure1. Determination of the Apoptotic Potential of hBok in HEK 293T Cells. **a.** HEK293T cells ($1x10^5$ cells/chamber) growing in two chambered slides were transfected with pcDNA3 (vector) and pcDNA3 expressing either rBok or hBok. Apoptotic cells (indicated by an arrow) were identified by TUNEL assay. **b.** HEK293T cells ($1x10^6$ cells/100 mm plate) transiently transfected with pcDNA3 (vector) and pcDNA3 expressing either rBok or hBok. Apoptosis was determined by DNA fragmentation analysis.

Figure 2. Potential of hBok to kill Breast Cancer Cells. **a.** Breast cancer cell lines MDA-MB 231, MDA-MB 435 and MCF-7 were transiently co-transfected with either pcDNA3 (control) and pLuc, or phBok and pLuc. Cell viability was determined by the luciferase reporter assay. **b.** Cells were

transfected with vector and vector-expressing hBok. Forty eight hours after transfection, cells were seeded at a 1/20 dilution in a selection medium containing G418 (Genetisin) at 500 μg / mL for 2 weeks. G418-resistant colonies were stained with crystal violet / ethanol (1% / 20%) and counted. **c.** Western Blot analysis was performed on total cell lysates of the breast cancer cell with antibodies against Bcl-2 and Mcl-1. **d.** Western Blot analysis was performed on total cell lysates of transfected HEK293T cells with antibodies against Flag.

Figure. 3. Nuclear Translocation of hBok **a.** Open reading frame of hBok highlighting the putative nuclear export sequence (LLRLGDELE) within the BH3 domain. **b.** Western Blot analysis of nuclear and cytoplasmic fractions of transfected HEK293T cells with antibodies against flag, PARP and α-tubulin **c.** MDA-MB 231 cells in a 4 chambered slide ($3x10^5$ cells / chamber) were transfected with Flag-tagged hBok (0.6 μg). The immunofluorescence staining shows the endoplasmic reticulum, flag-tagged hBok and the nucleus and identified hBok in the nucleus.

Figure. 4. Leptomycin B sequesters hBok in the nucleus. **a.** MDA-MB 231 cells in a 4 chambered slide ($3x10^5$ cells / chamber) were transfected with Flag-tagged hBok (0.6 μg) and harvested 6h after transfection. One set of transfected cells were exposed to LMB (20 ng / ml) for 6 h. The immunofluorescence staining shows an increase of nuclear hBok following treatment with LMB. **b.** Quantitative analysis of the nuclear concentration of hBok. + weak staining , ++ strong staining

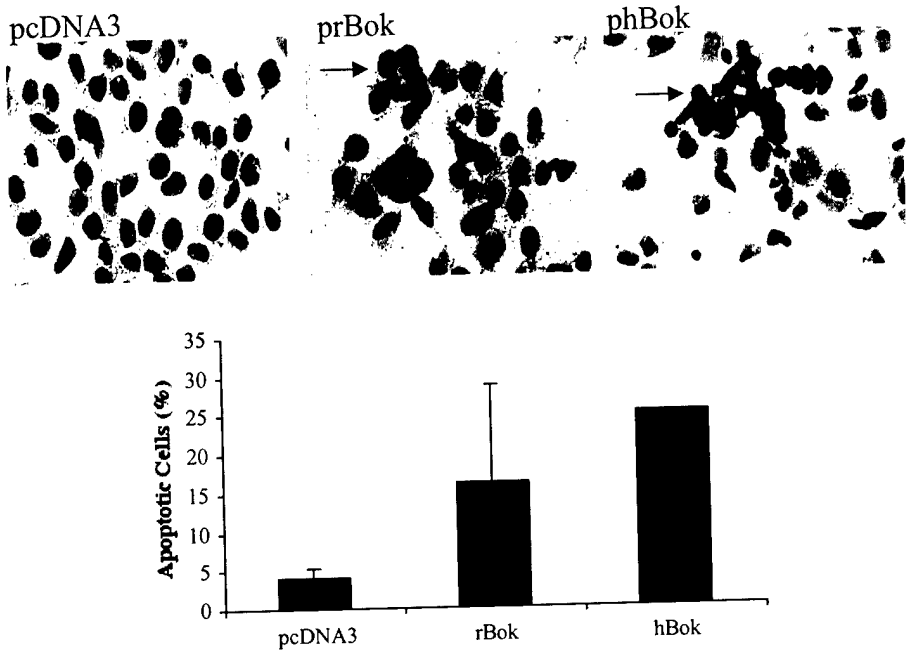
Figure. 5. Mutation of the putative NES of hBok sequesters the protein in the nucleus and enhances its killing potential. **a.** MDA-MB 231 cells in a 4 chambered slide ($3x10^5$ cells / chamber) were transfected with either Flag-tagged hBok (0.6 μg) or the NES mutant of hBok (0.6 μg) and harvested 6 h after transfection. The immunofluorescence staining shows a stronger staining for flag-tagged NES mutant hBok compared to wildtype. **b.** Quantitative analysis of the nuclear

concentration of wildtype and NES mutant hBok. + weak staining , ++ strong staining. **c.** HEK293T cells were transiently co-transfected with either pcDNA3 (control) and pLuc , phBok and pLuc. or NES mutant phBok and pLuc. Cell viability was determined by the luciferase reporter assay. **d.** HEK293T cells ($1x10^6$ cells/100 mm plate) transiently transfected with pcDNA3 (vector) and pcDNA3 expressing either hBok or NES mutant hBok. Apoptosis was determined by FACS analysis.6h after transfection.

Figure.6. The NES mutant of hBok inhibits cell proliferation to a greater extent than wild type. MDA-MB 435, MDA-MB 231 and CHO cells ($1x10^6$ cells / plate) were transfected with 10 μg AdTrack or vector expressing either wildtype or mutant hBok such that both hBok and GFP could be independently expressed from the same expression vector. The percentage of GFP-expressing cells were determined by fluorescent microscopy from three separate fields to determine the percentage of cell death in each population.

Figure 1
G. Bartholomeusz et al.

A



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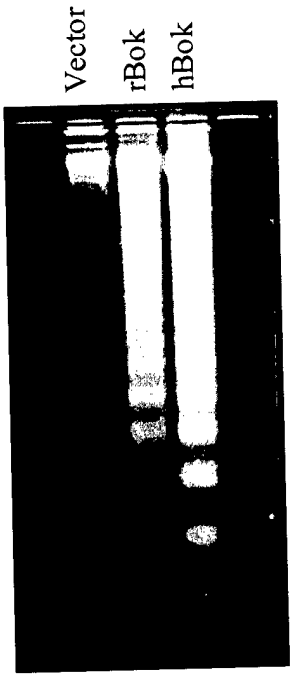


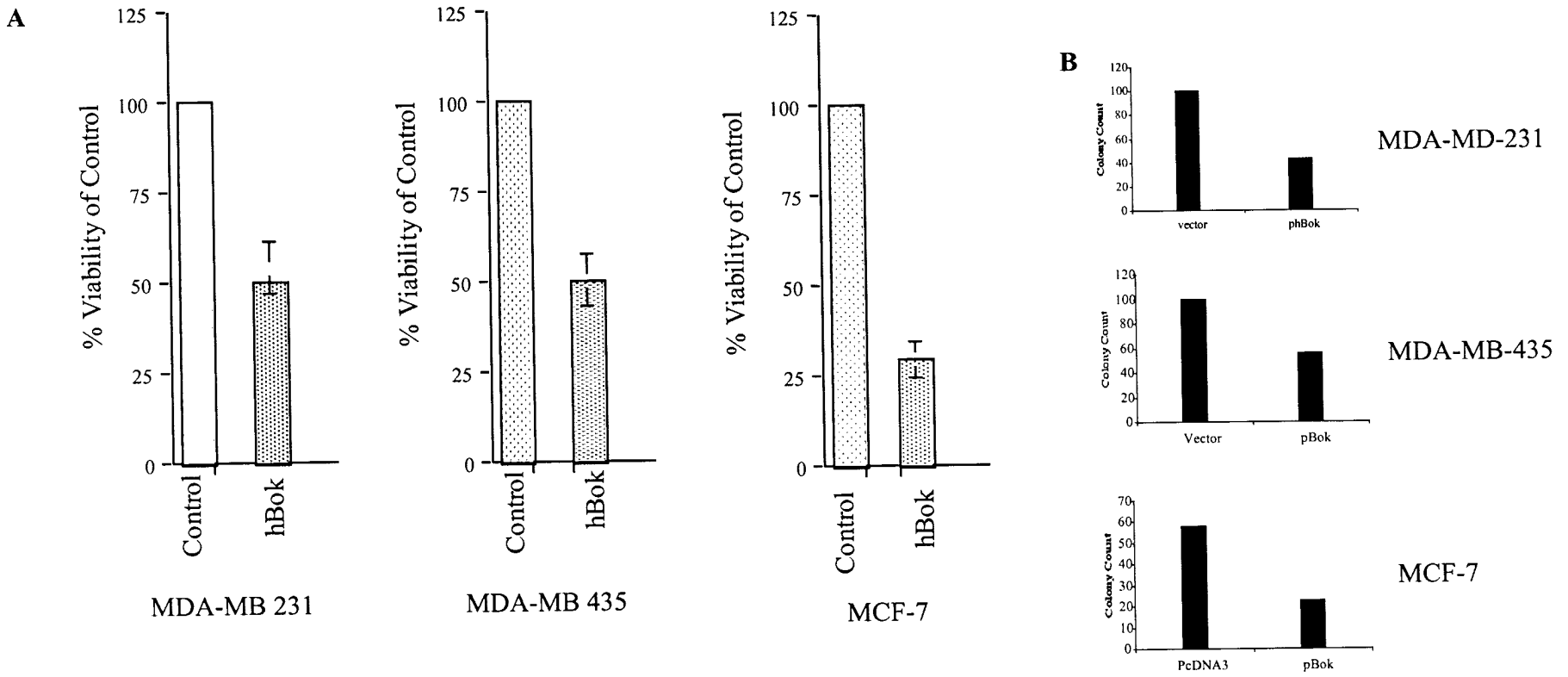
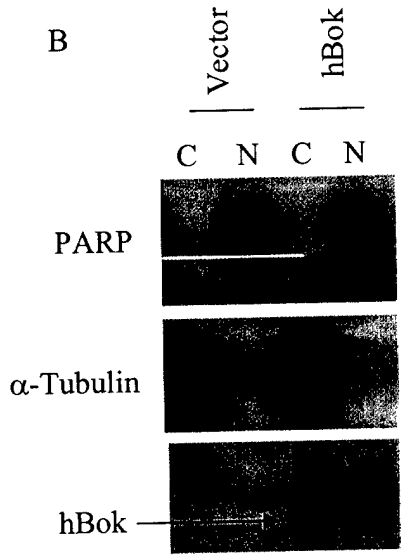


Figure 2
G. Bartholomeusz et al.

Figure 3
G. Bartholomeusz et al.

A

1....MEVLRRSSVFAAEIMDAFDRSPTDKELVAQAKALGREYVH...40
41...HARLLRAGLSWSAPERAAPVPGRLAEVC**AVLLRLGDELE**M 80
81...IRPSVYRNVARQLHISLQSEPVVTDAFLAVAGHIFSAGITWG...122
123.. KVVSLYAVAAGLAVDCVRQAQPAMVHALVDCLGVFERKT.. 159
160.. LATWLRRRGGWTDVLKCVVSTDPGLRSHWLVAALCSFGRF 200
201 LKAAFFVLLPER 213



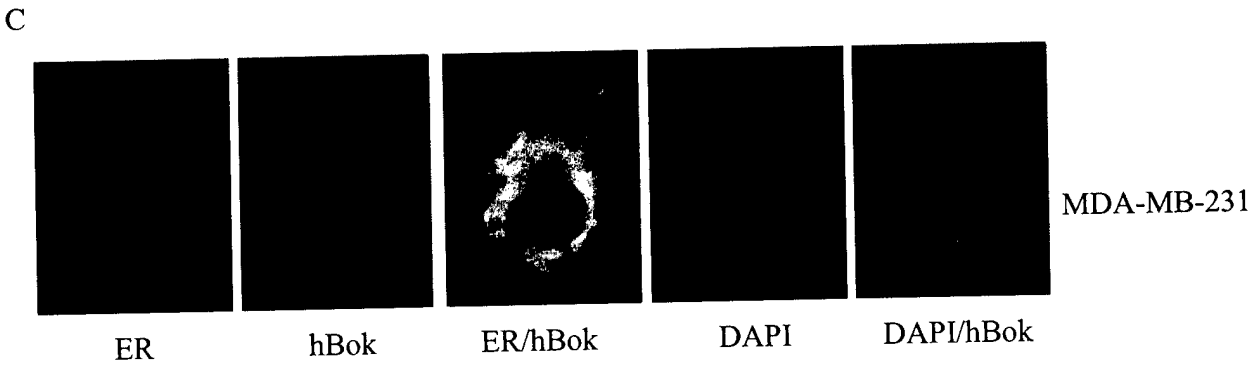
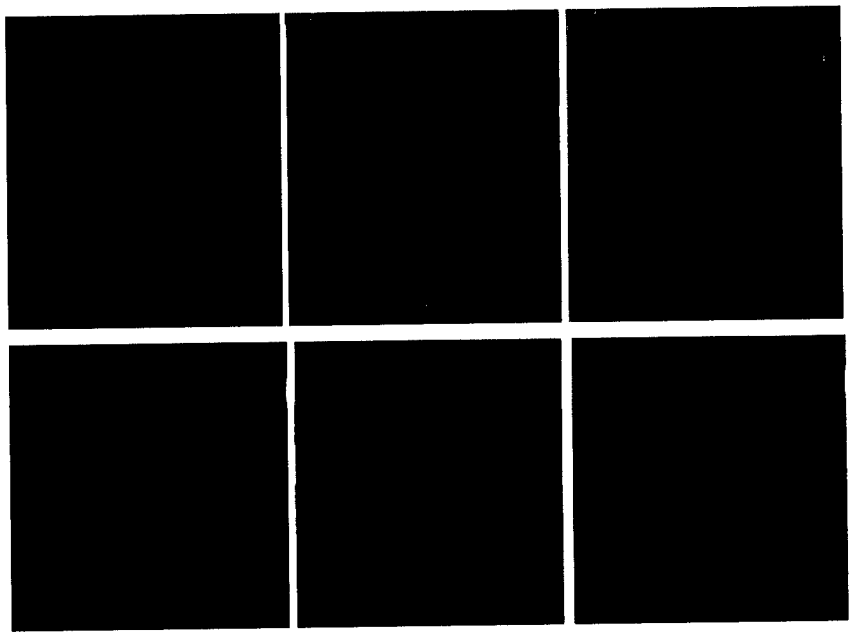


Figure 4
G. Bartholomeusz et al.

A



(-) LMB

(+) LMB (20 ng ml^{-1})

DAPI TX RED Merge

B

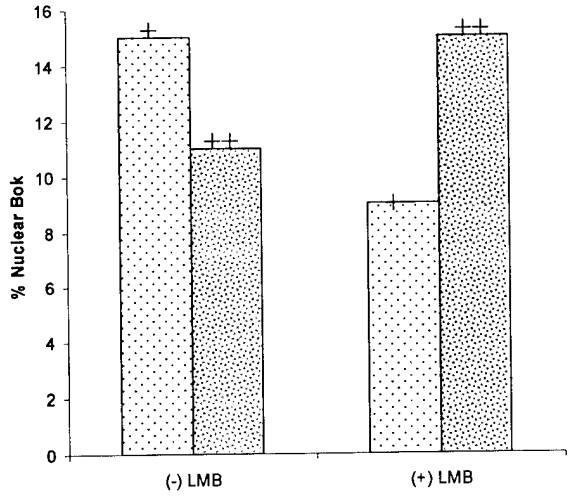


Figure 5
G. Bartholomeusz et al.

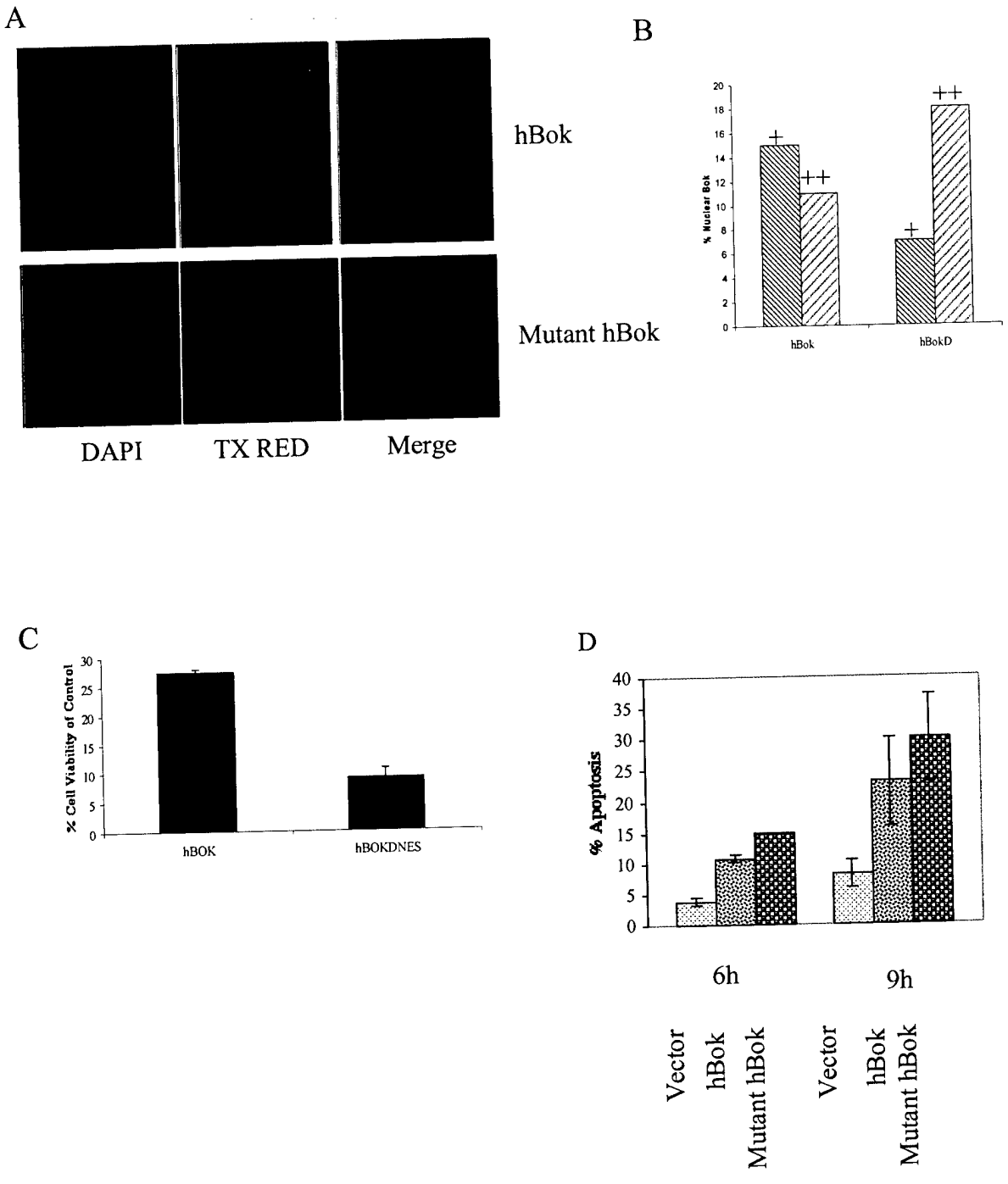


Figure 6
G. Bartholomeusz et al.