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<b>13. ABSTRACT (Maximum 200 Words)</b> We have shown by transient expression of hBok that this member of the Bcl-2 pro-apoptotic family is unique since its translocation to the nucleus is important for protein to induced apoptosis. Concern were raised since our observation did not apply to endogenous Bok. We are now in a position to detect endogenous Bok by western Blot analysis and have shown that endogenous Bok is present in the cytoplasm and nucleus of HeLa cells but only in the nucleus of MDA-MB-231 cells suggesting that the intracellular localization of Bok is cell type specific. Bax is translocated from the cytoplasm to the mitochondria upon the induction of apoptosis by the kinase inhibitor staurosporine. We therefore treated HeLa cells with staurosporine to determine if Bok translocation from the cytoplasm to the nucleus is induced by staurosporine. We show that unlik Bax, cytoplasmic Bok is modified by a non-phosphorylation event prior to being translocated to the nucleus. We are currently in the processes of confirming if this modification is required for the overall mechanism of Bok-induced apoptosis or is a staurosporin dependent event. In addition, we hope to identify the type of modification observed in Bok and determine its significance in Bok-induced apoptosis.				
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## 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<sub>L</sub> function as the major anti-apoptotic molecules at the mitochondria (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<sub>L</sub> 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.

The work described in my first report was submitted to Molecular Cell. We received a letter dated October 2003 from the editor that although the work was

interesting it lacked endogenous observations to support our in-vitro study. Since at the time of our study no antibody was available we decided to have an antibody commercially made for us. However, this antibody which we received in December of 2003 did not work. Subsequently the company Cell Signaling came out with a Bok antibody at the end of December 2003 and we successfully used this antibody in our Western Blot Analysis for endogenous Bok. Thus the only work that we were able to carry out on this project has been during the last two months. We detected endogenous Bok in both the cytoplasm and nucleus of HeLa cells under normal growth conditions. On the other hand endogenous Bok was only detected in the nucleus of MDA-MB-231 cells suggesting that the intracellular distribution of Bok under normal growth conditions is cell type specific. In comparison, Bax was only observed in the cytoplasm of these cells agreeing with the published literature. A time course analysis of HeLa cells treated with Staurosporin indicated that upon the induction of apoptosis by this agent cytoplasmic Bok was modified prior to being translocated into the nucleus. Preliminary evidence suggests that this modification is not due to phosphorylation. This recent work supports our in-vitro studies suggesting that Bok is a unique member of the Bcl-2 family carrying out its apoptotic within the nucleus of cells.

**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.

As described in the previous report the remaining objective of this aim was to establish hBok stable transfectants in known metastatic human breast cancer cell lines MCF-7 MDA-MB435 and MDA-MD 231 utilizing the Ecdysone-Inducible Mammalian Expression System (Invitrogen). Once again we were unsuccessful in isolating positive clones.

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

As described in the previous report the BH3 domain of hBok revealed a short leucine rich stretch of amino acids representative of a NES. In support of our in-vitro studies we have detected endogenous Bok in the nucleus of both HeLa and MDA-MB-231 cells under normal growth conditions (Appendix Fig 1A). This observation is unique to Bok since under the same experimental conditions Bax was only detected in the cytoplasm. As a first step to understanding the mechanism of Bok induced apoptosis, we treated HeLA cells with the kinase inhibitor Staurosporin (1 $\mu$ M) and harvested cells at the indicated times (Appendix Fig 1B). Our results suggest that the cytoplasmic pool of Bok undergoes a modification prior to being translocated to the nucleus. Since Staurosporin is a kinase inhibitor we predict that the modification of Bok is not a phosphorylation event. This we confirmed by pre-treating the cells with either a MAPkinase inhibitor, or PI3Kinase inhibitor. In addition the modification was not affected when the cell lysate

were treated with Alkaline phosphatase (Appendix Fig 1C). These results support our in-vitro data submitted in the previous report that Bok 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 determine how Bok is modified and understand the significance of this modification in the mechanism of Bok-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.

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

#### **KEY RESEARCH ACCOMPLISHMENTS**

- The Pro-apoptotic BCL-2 member Bok is present in both the cytoplasm and nucleus of cells growing under normal conditions.
- Induction of apoptosis by the kinase inhibitor Staurosporin results in the modification of cytoplasmic Bok prior to its translocation to the nucleus. The modification of Bok is not due to phosphorylation

#### **REPORTABLE OUTCOMES**

We are in the process of addressing the reviewers comments before re-submitting our manuscript entitled “ Nuclear translocation of the pro-apoptotic Bcl-2 Family member Bok induces apoptosis”. A draft of the initial manuscript was submitted in the previous report attached.

## CONCLUSIONS:

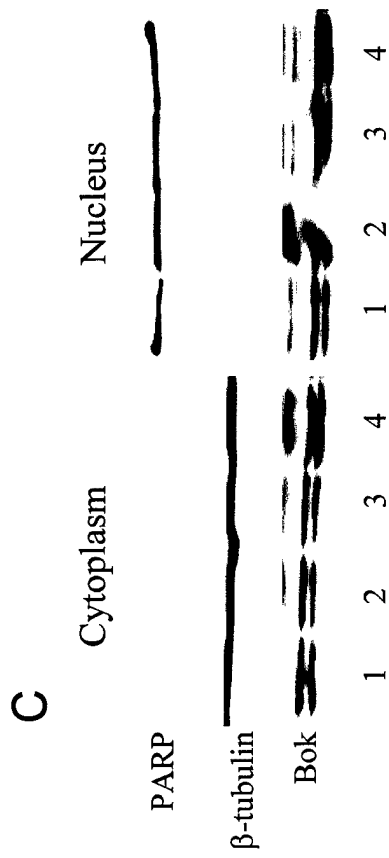
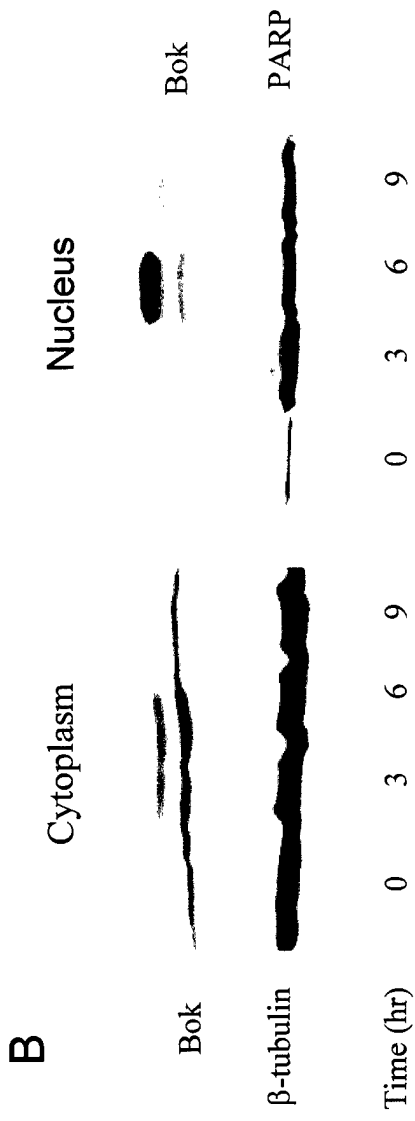
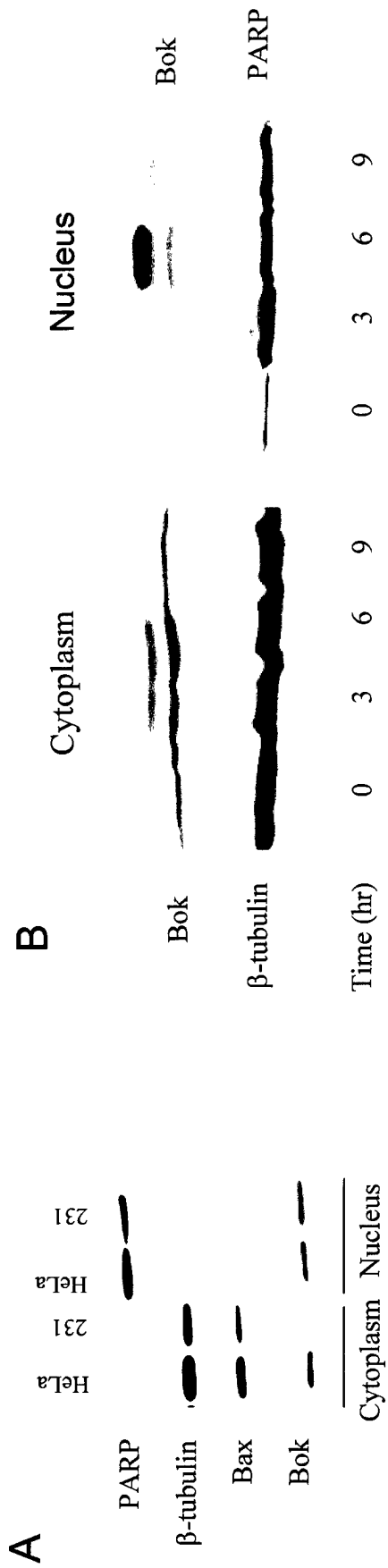
In this report we confirm our in-vitro studies that endogenous Bok is located both in the cytoplasm and nucleus of HeLa cells. In addition we show that Bok is modified in the cytoplasm prior to being translocated to the nucleus following the induction of apoptosis by the kinase inhibitor Staurosporin. We show that this modification is not a phosphorylation event.. Finally, it has been shown that the apoptotic activity of hBok is not inhibited by either Bcl-2 or Bcl-x<sub>L</sub>. Overexpression of Bcl-2 has been associated with many tumors including breast tumors (21) It is also known that overexpression of Bcl-2 renders tumor cells refractory to chemotherapy and radiation.(22) (23, 24). We propose that a good understanding of the mechanism of Bok-induced apoptosis together with 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 LEGEND

Figure 1 Nuclear Translocation of endogenous Bok . **a.** Cytoplasmic and nuclear fractions of HeLa and MDA-MB-231 cells ( $1 \times 10^6$  cells/100 mm dish) grown in DMEM-F12 medium with 10% FCS were separated on a 13.5% SDS PAGE transferred to a PVDF membrane and blotted with antibodies against Bax, Bok, PARP and  $\beta$ -tubulin. **b.** HeLa cells ( $1 \times 10^6$  cells/100 mm plate) were incubated in DMEM-F12 medium containing 10% FCS for 24 hr. Cells were treated with  $1 \mu\text{M}$  Staurosporin and harvested at the indicated times. Cytoplasmic and nuclear fractions were transferred onto a PVDF as described in (1a) were blotted with the anti-bok antibody. **c.** HeLa cells were cultured as described in 1B. All cells were treated with  $1 \mu\text{M}$  staurosporin for 6hr. However, two sets of cells were pre-treated with either the MAP kinase inhibitor PD8059 (20mM) or the PI3-Kinase inhibitor LY294002 (20mM) for 1 hr prior to the addition of staurosporin. Cells not treated with the inhibitors were used as a control and for the treatment with alkaline phosphatase(30 U) at  $37^\circ\text{C}$  for 2hrs.



1. Lysate of He La cells treated with STS (1μM)
2. Lysate incubated with 30 U alkaline phosphatase
3. Lysate of cells pre-treated with PD98059 (20μM)
4. Lysate of cells pre-treated with LY294002 (20μM)