Award Number: W81XWH-04-1-0283

TITLE: Functional Analysis of BORIS, a Novel DNA Binding Protein

PRINCIPAL INVESTIGATOR: Paul Yaswen, Ph.D.

CONTRACTING ORGANIZATION: Lawrence Berkeley National Laboratory
Berkeley, California 94720

REPORT DATE: March 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Functional Analysis of BORIS, a Novel DNA Binding Protein

Paul Yaswen, Ph.D.

Lawrence Berkeley National Laboratory
Berkeley, California 94720
E-Mail: p-yaswen@lbl.gov

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Original contains color plates; All DTIC reproductions will be in black and white.

Approved for Public Release; Distribution Unlimited

BORIS (Brother of the Regulator of Imprinted Sites) is a paralogue of the gene encoding CTCF, a multifunctional DNA binding protein that utilizes different sets of zinc fingers to mediate distinct gene regulatory functions, including those involved in cell growth regulation. Unlike CTCF, the expression of BORIS is normally restricted to specific cells in testes (the only cells where CTCF is not expressed), where it may play a role in reprogramming the methylation pattern of male germ line DNA. To define the possible consequences of aberrant BORIS expression in human breast cancers, we are using a well-characterized human mammary epithelial cell (HMEC) culture model. Our preliminary results suggest that BORIS expression alone is not an efficient immortalizing factor, but that under certain conditions it may cooperate with other changes (e.g. p53 inactivation) to destabilize the genomes of the cells in which it is aberrantly expressed. Since BORIS-eGFP protein sometimes co-localizes with centrosomes, it is possible that BORIS expression can cause genomic instability through aberrant affects on centrosome duplication during the cell cycle. BORIS expression may also cause genomic instability through its significant effects on the regulation of several key early growth response genes.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover</td>
<td>1</td>
</tr>
<tr>
<td>SF 298</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>5</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>8</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>9</td>
</tr>
<tr>
<td>Conclusions</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>9</td>
</tr>
</tbody>
</table>
INTRODUCTION

Very recently, a new gene has been mapped to the 20q13.2 region that is commonly amplified in cancers of the breast and other tissues. This gene, **BORIS** (Brother of the Regulator of Imprinted Sites), is a paralogue of the gene encoding CTCF, a multifunctional DNA binding protein that utilizes different sets of zinc fingers to mediate distinct functions in regulation of gene expression (reviewed in (1)). These functions include context-dependent promoter repression or activation, creation of modular hormone-responsive gene silencers, and formation of enhancer blocking elements (insulators). Several lines of evidence suggest that ubiquitously expressed CTCF is a critical determinant of cell growth regulation.

Unlike CTCF, which is expressed ubiquitously, the expression of BORIS is normally restricted to specific cells in testes (the only cells where CTCF is not expressed), where it may play a role in reprogramming the methylation pattern of male germ line DNA (2). BORIS encodes an 11-zinc finger domain functionally equivalent to that in CTCF, while being completely divergent at the amino- and carboxy-termini – domains that have recently been shown to be critical for CTCF’s insulator function (3). Based on the finding that BORIS maps to the 20q13.2 region frequently altered in many malignancies, various cancer cell lines were screened for BORIS expression. BORIS transcripts were found in substantial proportions of a wide variety of tumor cell types (Loukinov et al., manuscript in prep.). Analyses of 17 breast tumor cell lines and of 148 randomly selected primary breast cancer samples (obtained from A. Lindblom, Karolinska Hospital, Sweden) without CTCF mutations (A. Lindblom, unpublished) demonstrated frequencies of BORIS expression of 80 and 88%, respectively. In contrast, using an extremely sensitive 2 step multiplex RT-PCR method, the same team was unable to detect BORIS mRNA in any normal mammary tissues, or other normal somatic tissues (data not shown). Based on these findings, and results indicating that BORIS protein can compete for CTCF binding sites in the H19 imprinting control region, the globin FII insulator, and the c-MYC promoter, we hypothesize that aberrant expression of BORIS may interfere with certain CTCF functions, thereby promoting cancer progression.

To define the possible consequences of aberrant BORIS expression that may promote cancer progression in the human breast, we are using a well-characterized human mammary epithelial cell (HMEC) culture model (reviewed in (4)). HMEC cultured from normal breast tissue display a finite life span, low or undetectable telomerase activity, and decreasing telomere length with passage (5). HMEC can spontaneously overcome a first RB-mediated, non-telomere length dependent proliferative arrest (stasis), associated with down-regulation of p16 expression (6). The resultant p53(+), p16(-) post-selection HMEC cease net proliferation when their mean terminal restriction fragment (TRF) length is ~5 kb. As cells approach this second proliferative barrier, telomere dysfunction is evidenced by the presence of widespread chromosomal aberrations, particularly telomeric fusions, and mitotic failures (7). In the p53(+) cultures, most cells remain viably arrested at all phases of the cell cycle, a growth arrest termed agonescence (7). When p53 is inactivated, populations display the massive cell death typical of crisis (J. Garbe et al., submitted). Rare p53(+) and p53(-) immortal HMEC lines have been obtained following exposure to chemical carcinogens, over-expression of c-myc or ZNF217 oncogenes, and/or a dominant negative p53 genetic suppressor element, GSE22 (8-10). Surprisingly, the newly immortal p53(+) lines initially show very low or undetectable telomerase activity and continue to divide with increasingly shortened mean TRF lengths. When the mean TRF length gets extremely short (<3 kb), growth becomes slow and heterogeneous. An extended process, termed conversion, ensues, during which telomerase activity and growth capacity gradually increase (11). In contrast, newly immortal p53(-) lines quickly display telomerase activity (10). Our studies indicate that overcoming telomerase repression and telomere dysfunction are rate-limiting factors in the malignant transformation of cultured HMEC. Once these barriers have been overcome, the resulting immortal lines can be induced to become growth factor- and anchorage-independent, as well as tumorigenic, by introduction of a variety of well-characterized oncogenes.
Task 1: Perform correlative studies of endogenous BORIS expression and DNA binding activity in HMEC at specific stages of immortal transformation. As no reliably specific antibody is yet available to analyze the levels of BORIS protein, we have used RT-PCR to analyze BORIS mRNA levels instead. Our preliminary analysis of BORIS expression in HMEC indicates that this gene is not expressed in finite lifespan cultures derived from normal tissues, but that it is expressed in some cultures immortalized after exposure to a known chemical carcinogen or to specific oncogenes (data not shown). This data suggests that activation of BORIS expression may be a cause or a consequence of changes accompanying immortalization.

Task 2: Determine whether exogenous BORIS expression influences properties associated with conversion of p53(+) immortal HMEC. To address this problem, we introduced retroviruses containing BORIS cDNA or a control eGFP reporter gene into growing cultures of conditionally immortal 184A1 HMEC prior to conversion. Cell cultures were treated with selection agent G418. No significant differences in proliferation rate were noted between cells expressing the exogenously introduced BORIS gene and cells expressing the exogenously introduced eGFP reporter gene alone. During the long process of conversion, we have been collecting protein and RNA samples, which will be analyzed later. The following criteria will be assessed at different time-points following initial infection: a) BORIS expression, b) hTERT expression, c) telomerase activity, d) p57 expression, and e) c-MYC expression. Gene expression will be analyzed by quantitative RT-PCR, and where possible, immunoblotting.

Task 3: Determine whether exogenous BORIS expression extends the proliferative potential of, or immortalizes finite lifespan HMEC when expressed alone or in combination with oncogenes c-MYC or ZNF217, or a dominant negative p53 genetic suppressor element. We tested the oncogenic properties of BORIS in HMEC directly by transducing the gene alone or in combination with additional changes that may act cooperatively with it. We transduced growing cultures of post-selection 184 HMEC with BORIS or a control vector. To monitor BORIS expression, we used a vector that contains an eGFP reporter gene linked to the BORIS gene by an internal ribosome entry site (IRES). The IRES sequence allows the translation of two proteins from a single transcript. Since BORIS is located 5' to the eGFP coding region, all cells expressing eGFP green fluorescence are expected to also express high levels of BORIS protein. In each experiment, 3 plates of each condition were infected and monitored independently to control for jackpot effects and overgrowth by rare variants within a population. As shown in Fig.1, all three cell populations infected with the BORIS virus expressed very high levels of the transcript. In contrast, cells infected with the control vector did not show any expression of BORIS.

The transduced cells were monitored for population doubling times between subcultures, and morphological signs of agonescence or crisis. We did not observe any significant differences in cell morphology (Fig.2) or in growth rate (Fig. 3) initially after the infections. None of the cells transduced with BORIS showed detectable levels of hTERT prior to agonescence (Fig. 4). However, one (SEBORIS2) of the three cell cultures infected with the BORIS virus yielded a single clone that overcame agonescence and became immortal. Cells originating from this clone retained functional p53 and gradually reactivated hTERT expression (Fig 4). We
performed similar experiments in parallel, using finite lifespan pre-
stasis HMEC and human fibroblasts derived from the same
specimen. None of the cells transduced with BORIS immortalized in
these latter experiments.

Additional gene transfer experiments were performed using a
second retroviral construct in which the BORIS gene was expressed
as a fusion protein with eGFP at its C-terminus to directly track
protein expression in real time. Cells transduced with this construct
exhibited green fluorescence in the nuclei, as expected, whereas
cells transduced with the control virus (containing the eGFP gene
alone) showed fluorescent signal predominantly in the cytoplasms.
Despite the high levels of BORIS-eGFP expressed by the cells in
this experiment, no immortalization was observed.

I. Additional gene transfer experiments were performed using a
second retroviral construct in which the BORIS gene was expressed
as a fusion protein with eGFP at its C-terminus to directly track
protein expression in real time. Cells transduced with this construct
exhibited green fluorescence in the nuclei, as expected, whereas
cells transduced with the control virus (containing the eGFP gene
alone) showed fluorescent signal predominantly in the cytoplasms.
Despite the high levels of BORIS-eGFP expressed by the cells in
this experiment, no immortalization was observed.

Z

Additional gene transfer experiments were performed using a
second retroviral construct in which the BORIS gene was expressed
as a fusion protein with eGFP at its C-terminus to directly track
protein expression in real time. Cells transduced with this construct
exhibited green fluorescence in the nuclei, as expected, whereas
cells transduced with the control virus (containing the eGFP gene
alone) showed fluorescent signal predominantly in the cytoplasms.

Figure 4. The levels of hTERT transcripts in
184 cells transduced with BORIS were
analyzed by quantitative RT-PCR. The
results were normalized to the expression of
porfobilinogen deaminase – a constitutively
expressed “housekeeping” gene.

Altogether, the above results suggest that
BORIS alone is not an efficient immortalizing
factor, but that under certain conditions it can
cooperate with other unknown changes to
immortalize normal finite lifespan cells.
Aberrant BORIS expression may cooperate
with other defects to enable cells to overcome
agonesence and express telomerase. As one
test of this hypothesis, we transduced growing
cultures of post-selection HMEC with BORIS
and/or the dominant negative p53 genetic
suppressor element, GSE22. In this
experiment, 3 plates of each condition were
infected and monitored independently to
control for jackpot effects and overgrowth by
rare variants within a population. During the
experiment, cells were monitored for
morphological changes and population
doubling times between subcultures, as well
as signs of agonesence or crisis. In this
experiment, we did not observe that cells
infected with both BORIS and GSE22 had any
advantage over the other cells in terms of
growth rate. However, these cells
demonstrated significant aberrations in
cytokinesis, which were not detected in cells
transduced with GSE22 alone (Fig.5). p53 is
known to play a role in control of cell division
check points, as well as in processes controlling centrosome maturation and amplification. It is possible that
lack of functional p53 in concert with BORIS over-expression can affect centrosome duplication during the cell
cycle, leading to hyper-amplification. Centrosome amplification (the presence of more than two centrosomes at
mitosis) is characteristic of many human cancers. Extra centrosomes can cause the assembly of multipolar
spindles, which unequally distribute chromosomes to daughter cells resulting in genetic imbalances. The cells of
most late-stage human cancers are aneuploid, genomically unstable and show high incidence of centrosome
amplification. Genomic instability is thought to be a major driving force in multiple-step carcinogenesis. We
hypothesize that BORIS can be one of the proteins involved in control of centrosome duplication during the cell
cycle. This hypothesis is currently being tested in our laboratory. Our preliminary data indicate that, in addition
to their nuclear localization, BORIS-eGFP fusion proteins sometimes co-localize with γ-tubulin, a specific
marker of centrosomes (Fig.6A). HMEC cultures transfected with BORIS displayed higher percentages of cells
with > 2 centrosomes than cultures transfected with control or GSE22 vectors alone, and these differences
persisted with passage (Figs.6B&7).
Fig. 6 A) Co-localization of BORIS-GFP and γ-tubulin in 184-GSE22 cells transfected with BORIS-GFP; B) Cells displaying abnormal numbers of γ-tubulin positive centrosomes in cultures transfected with GSE22 and BORIS (184-GSE-BORIS), transfected with BORIS alone (184-BORIS), or immortalized after p53 inactivation (184AA2). A representative cell displaying 2 centrosomes in a control culture (184) is shown for comparison. Arrows indicate cells with ≥3 centrosomes.

Task 4: Determine whether exogenous BORIS expression influences additional phenotypic properties of normal or immortalized HMEC.

A) Anchorage dependence: Anchorage-independent growth (AIG) is often exhibited by cells derived from human breast tumors or by cells that have been tumorigenically transformed in vitro. To determine if BORIS is able to promote AIG we suspended retrovirally transduced immortal HMEC in 1.5% methylcellulose in growth medium. The BORIS expression level in cells used in the experiment was determined by semi-quantitative RT-PCR prior to plating. The cells were fed weekly and, at 4 weeks post-plating, the plates were visually inspected for the presence of cell colonies displaying AIG. No such colonies were detected in cultures of immortal HMEC over-expressing BORIS (data not shown), suggesting that BORIS alone is not sufficient to confer this phenotype.

B) Gene expression: To determine whether BORIS directly or indirectly influences cellular transcription, we have performed expression microarray experiments using a new state-of-the-art Affymetrix HTA GeneChip system recently purchased by LBNL’s Life Sciences Division. This high throughput facility presently employs U133A 2.0 chips of reduced feature size in a 96-well format. We transduced 184 HMEC with either BORIS or control retroviruses and harvested RNA after a brief selection. Microarray analysis was performed using duplicate samples, and the resulting data were analyzed using commercial GeneTraffic software (Stratagene). Genes which showed consistent differences of > 2 fold compared with controls were selected for further study. PCR primers were designed for several of these genes, and altered regulation was confirmed by semi-quantitative RT-PCR (Fig.8). Transient transfections were performed in 184 HMEC with increasing amounts of the BORIS-IRES-eGFP or control plasmids, and the harvested RNA subjected to quantitative RT-PCR to further document the dependence of selected gene transcripts on BORIS expression. The example shown in Fig.9 shows the direct correspondence between amount of BORIS plasmid transfected and ATF3 transcripts expressed. ATF3 is
of particular interest because it is a member of the CREB protein family of transcription factors. In addition to ATF3, a number of early growth response genes, including the oncogenes Fos and Jun, appear to be significantly upregulated by BORIS expression. Also of interest is the up-regulation of ZNF165, which like BORIS itself, has been reported to be a cancer-testis antigen.

**Figure 9.** Transient transfections with increasing concentrations of BORIS-IRES-eGFP plasmid (0.5, 1, and 2 μg/60 mm dish) and empty vector at the same concentrations confirm that BORIS regulates ATF3 expression. The cells were harvested 24 hrs. after transfection with the indicated plasmids, and the levels of ATF3 mRNA were quantified by quantitative RT-PCR. The levels were then normalized using the levels of CTCF transcripts for normalization.

**Task 5:** Determine whether interference with endogenous BORIS expression blocks growth and/or telomerase expression in immortalized HMEC. BORIS expression may or may not be continuously required for maintenance of an altered phenotype in transformed cells. To address this problem we transduced immortal HMEC expressing high levels of endogenous BORIS with retroviral constructs encoding gene-specific short interfering RNAs (siRNAs) to suppress BORIS expression. To date, it has not proved possible to predict which gene-specific sequences work best as siRNAs, so we used constructs containing sequences from different regions of the BORIS gene: siBORIS772 and siBORIS1594. After antibiotic selection, BORIS mRNA levels in siRNA-retrovirus infected cell lines were determined by semi-quantitative RT-PCR (Fig. 10). Cells transduced with siBORIS772 virus showed about 50% reduction of BORIS level as compared to parental cells, and are currently being used in ongoing experiments.

**KEY RESEARCH ACCOMPLISHMENTS**

* Our results suggest that BORIS alone is not an efficient immortalizing factor.

* Cells infected with both BORIS and GSE22 (a dominant negative p53 genetic suppressor element) demonstrated significant aberrations in cytokinesis, which were not detected in cells transduced with GSE22 alone.

* In addition to their nuclear localization, BORIS-eGFP fusion proteins sometimes co-localize with γ-tubulin, a specific marker of centrosomes.
HMEC cultures transfected with BORIS displayed higher percentages of cells with > 2 centrosomes than cultures transfected with control or GSE22 vectors alone, and these differences persisted with passage.

Microarray and RT-PCR analyses have identified several key early growth response genes as well as a cancer-testis antigen gene whose regulation is altered by BORIS expression.

**REPORTABLE OUTCOMES**

None to date

**CONCLUSIONS**

Our results suggest that BORIS alone is not an efficient immortalizing factor, but that under certain conditions it may cooperate with other changes (e.g. p53 inactivation) to destabilize the genomes of the cells in which it is aberrantly expressed. Since BORIS-eGFP protein sometimes co-localizes with centrosomes, it is possible that BORIS expression can cause genomic instability through aberrant affects on centrosome duplication during the cell cycle. BORIS expression may also cause genomic instability through its significant effects on the regulation of several key early growth response genes.

**REFERENCES**
