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**NOTE**

Due to unavoidable personnel changes, progress during Year 2 of this project has been limited. Dr. Joanna Mroczkowska, the post-doctoral research associate who performed the majority of the cellular and molecular studies during Year 1, obtained a staff position at the new UC Merced campus shortly before the Year 1 report was completed. After a long search, we are pleased to report that we have hired a talented new post-doctoral research associate, Dr. Curt Hines, to continue the studies initiated by Dr. Mroczkowska. Dr. Hines, who has extensive experience in telomerase and breast cancer biology, completed his Ph.D. at the U. of New Mexico in November, 2005, and joined us in December. We anticipate that with the 100% effort of Dr. Hines, we will be able to resume the good productivity that was exhibited during Year 1, and that we will be able to address all of the objectives stated within the initial Statement of Work.

**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.

**BODY**

**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. We have recently purchased a newly developed anti-BORIS antibody from a commercial vendor, and will test its utility for immunoblotting and immunohistochemical assays.

**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. We have collected protein and RNA samples for assessment of: 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*.

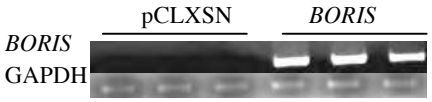


Figure 1. Expression of *BORIS* mRNA in 184 HMEC transduced with control vector (pCLXSN) or *BORIS* as determined by semi-quantitative RT-PCR. Expression of the housekeeping gene, GAPDH, was assayed as a control.

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.

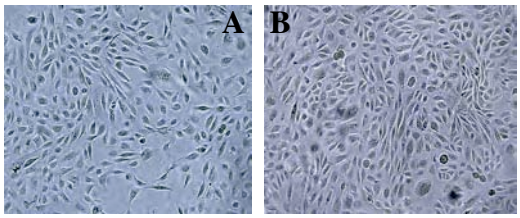


Figure 2. Morphology of post-selection 184 HMEC infected with (A) control, or (B) *BORIS* virus.

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.

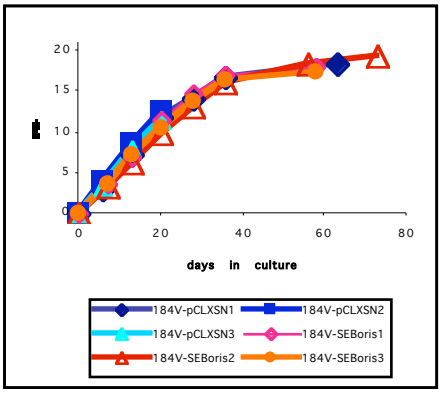


Figure 3. Growth rates of post-selection 184 HMEC infected with control (blue) or *BORIS* (red) containing viruses at passage 8 and grown until most cells stopped dividing.

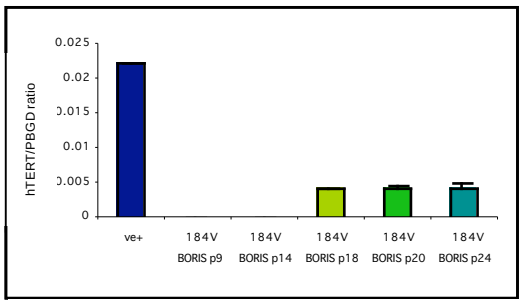


Figure 4. The levels of hTERT transcripts in 184 cells transduced with *BORIS* were analyzed by quantitative RT-PCR. The results were normalized to the expression of porfobilinogen deaminase – a constitutively expressed "housekeeping" gene.

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 δ-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)**.

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 agonescence 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 agonescence 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

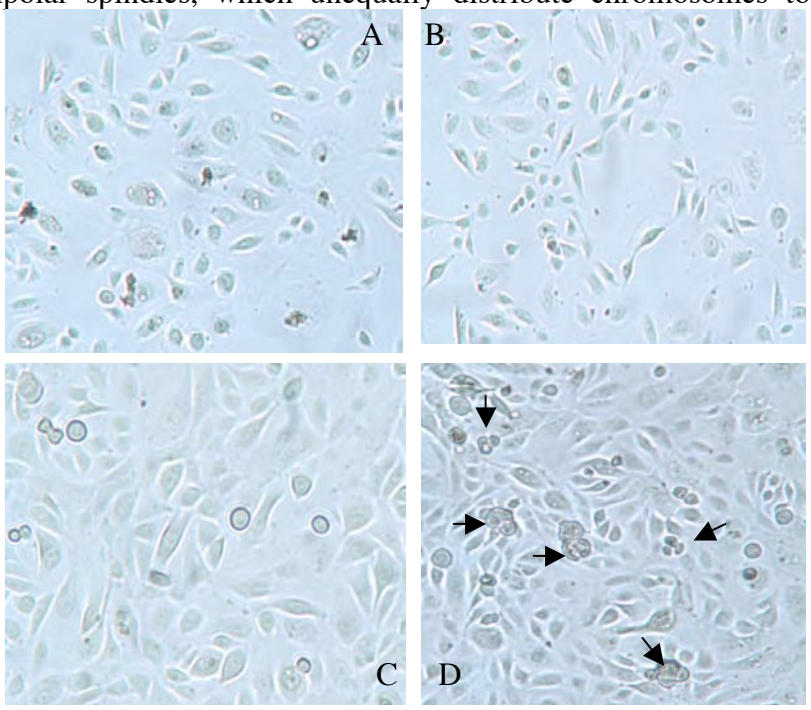


Figure 5. Morphology of 184 cells infected with: (A) *BORIS*/pBABE control; (B) pCLXSN control/pBABE control; (C) pCLXSN control/GSE22 (D) *BORIS*/GSE22. The arrows in panel D point to cells undergoing aberrant cytokinesis.

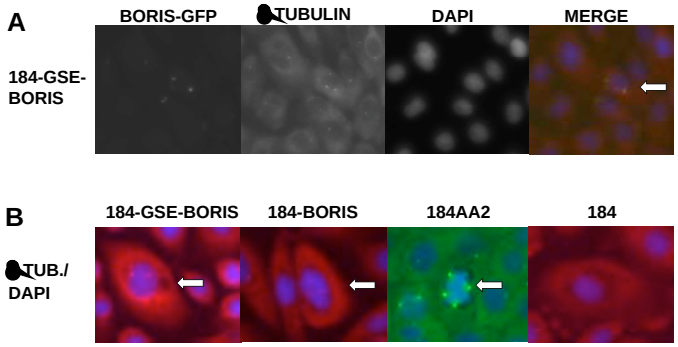


Fig. 6 A) Co-localization of *BORIS*-GFP and δ-tubulin in 184-GSE22 cells transfected with *BORIS*-GFP; B) Cells displaying abnormal numbers of δ-tubulin positive centrosomes in cultures transfected with GSE22 and *BORIS* (184-GSE-*BORIS*), transfected with *BORIS* alone (184-*BORIS*), or immortalized 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.

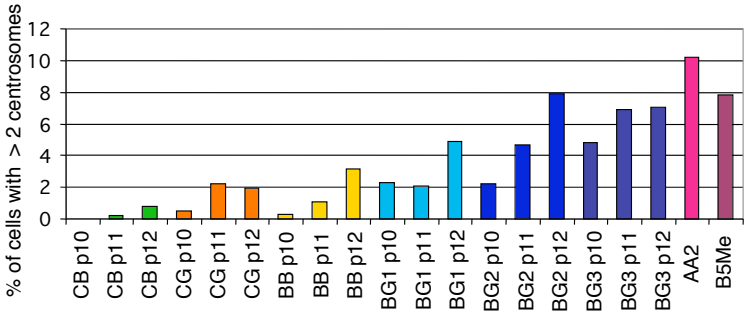


Figure 7. Quantitation of percentages of cells with > 2 centrosomes in 184 cells infected with: pCLXSN control/pBABE control (CB); pCLXSN control/GSE22 (CG); *BORIS*/pBABE (BB); *BORIS*/GSE22 (BG) retroviruses and assayed at passages (p) 10-12. Immortally transformed HMEC lines AA2 and B5Me were assayed for comparison.

**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


| GENE | EGR2 | ZNF165 | ID2 | ATF3 | FOSB | | GSTE1 | DOCK4 |
|---|---|---|---|---|---|---|---|---|
| BORIS | 0.236 | 0.972 | 1.17 | 0.27 | 1.9 | | 0.02 | 0.28 |
| pCLXSN | 0.046 | 0.484 | 0.56 | 0.01 | 0.29 | | 0.28 | 0.56 |

Figure 8. Semi-quantitative RT-PCR results for genes that showed differential expression in 184 HMEC transduced with *BORIS* vs. control retroviruses.

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.

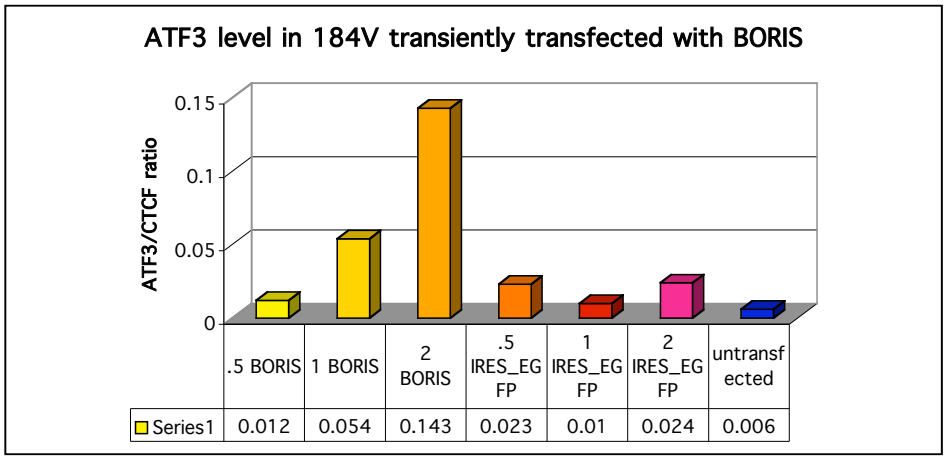


Figure 9. Transient transfections with increasing concentrations of *BORIS*-IRES-eGFP plasmid (0.5, 1, and 2 $\mu$g/60 mm dish) and empty vector at the same concentrations confirm that *BORIS* regulates ATF3 expression. The cells were harvested 24 hrs. after transfection with the indicated plasmids, 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 hairpin RNAs (shRNAs) to suppress *BORIS* expression. To date, it has not proved possible to predict which gene-specific sequences work best as shRNAs, so we used constructs containing sequences from different regions of the *BORIS* gene: si*BORIS*772 and si*BORIS*1594. After antibiotic selection, *BORIS* mRNA levels in shRNA-retrovirus infected cell lines were determined by semi-quantitative RT-PCR **(Fig. 10)**. Cells transduced with si*BORIS*772 virus showed about 50% reduction of *BORIS* level as compared to parental cells, and are currently being used in ongoing experiments.

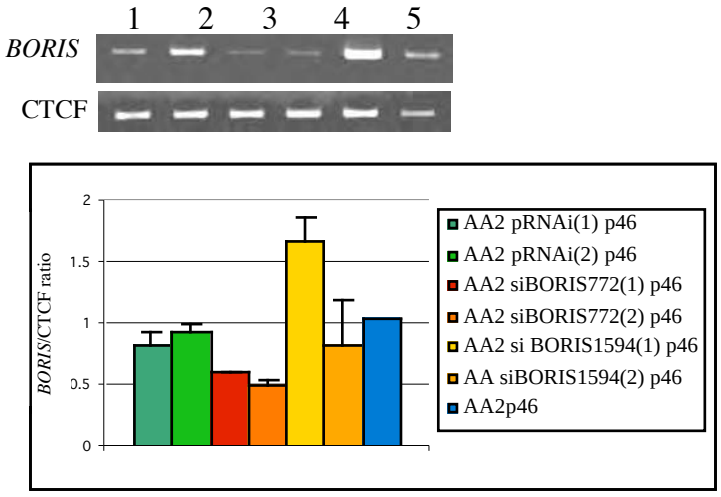


Figure 10. *BORIS* mRNA expression in 184AA2 HMEC was quantitated after transduction with *BORIS*-specific shRNA retroviruses. The semi-quantitative RT-PCR results were normalized to the levels of CTCF transcripts in the same cells. pRNAi is a non-specific control.

## 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 δ-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.

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