Cancer Immunity (23 July 2010) Vol. 10, p. 6
Submitted: 28 May 2010. Accepted: 8 July 2010.

CANCER IMMUNITY
Article

BORIS/CTCFL expression is insufficient for cancer-germline antigen gene expression and DNA hypomethylation in ovarian cell lines

Anna Woloszynska-Read[1], Smitha R. James[1], Chajoun Song[2], Boquan Jin[2], Kunle Odunsi[3] and Adam R. Karpf[1]

[1]*Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY, 14263, USA*
[2]*Department of Immunology, Fourth Military Medical University, Xi'an 710032, China*
[3]*Department of Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY, 14263, USA*

Communicated by: LJ Old

Expression of the cancer-germline (CG) (or cancer-testis) antigen gene BORIS/CTCFL has been proposed to mediate activation of CG antigen genes in cancer. Consistent with this idea, we have observed that BORIS is frequently expressed in ovarian cancer, often in conjunction with other CG genes. Here we assessed the role of BORIS in CG antigen gene regulation and DNA methylation using normal and cancerous ovarian cell lines, and the CG genes *MAGE-A1*, *NY-ESO-1*, and *XAGE-1* as models. Adenoviral vectored BORIS was expressed at robust levels and exhibited predominant nuclear localization in ovarian cells. However, BORIS expression in immortalized ovarian surface epithelial cells or ovarian cancer cell lines did not induce CG antigen gene expression or lead to CG antigen promoter DNA hypomethylation. BORIS overexpression also did not alter global DNA methylation, as assessed by genomic 5-methyl-deoxycytidine levels and *LINE-1* methylation. We used decitabine to further assess the role of BORIS in CG gene activation and found that decitabine treatment induced BORIS and other CG genes with similar kinetics, suggesting that BORIS induction does not account for the induction of other CG genes by decitabine in ovarian cancer cells. In agreement, siRNA knockdown of *BORIS* did not block decitabine-mediated induction of CG genes or DNA hypomethylation in ovarian cancer cells treated with this agent. We conclude that BORIS is insufficient for CG antigen gene expression and DNA hypomethylation in ovarian cell lines, and that additional factors are likely required for CG antigen expression in ovarian cancer.

Keywords: human, ovarian cancer, BORIS, CT antigens, gene expression, DNA methylation

Introduction

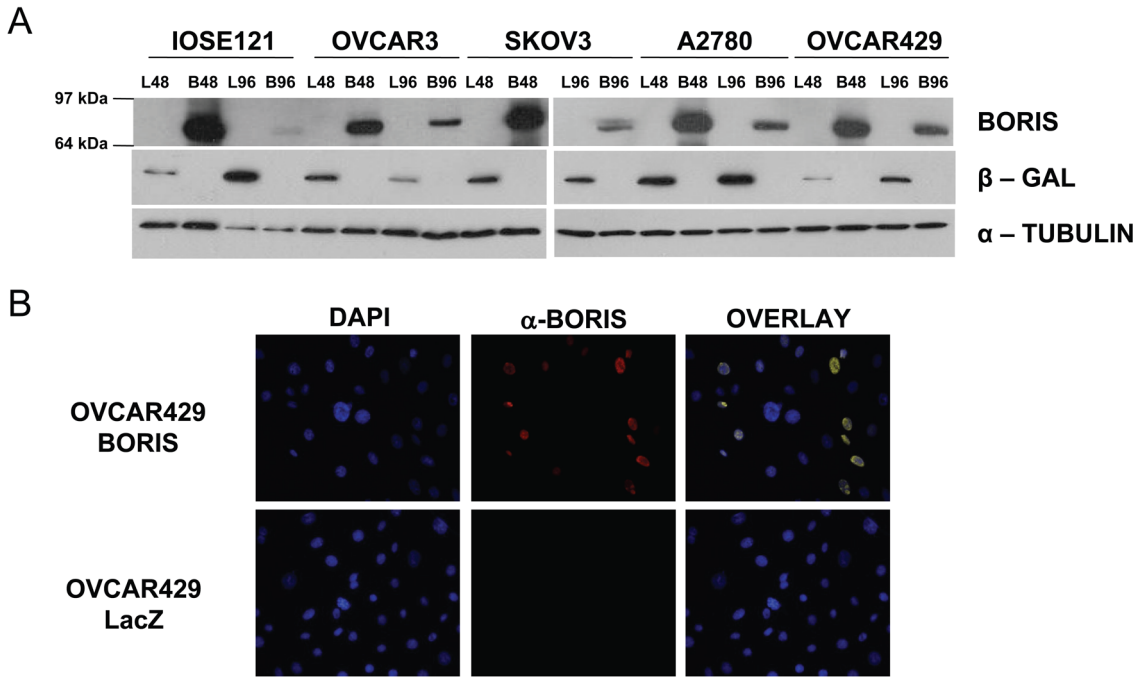
Cancer-germline (CG) (also known as cancer-testis or CT) antigen genes are infrequently expressed in normal adult somatic tissues but are expressed in germ cells, trophoblast, and cancer (1, 2). The inherent immunogenicity and tumor specificity of these genes have made them attractive cancer vaccine targets (1). When expressed in germ cells, CG antigens do not elicit immune responses due to the lack of HLA expression; however, when CG antigens are expressed in tumors they can elicit spontaneous immune responses in cancer patients (1). Recent clinical studies suggest that CG antigen-targeted vaccines are safe and promote immunological responses (3-5), encouraging continued clinical investigation of this approach.

Transcriptional silencing of CG antigen genes in normal somatic tissues is attributed to promoter DNA hypermethylation, while activation of these genes in cancer closely correlates with promoter DNA hypomethylation (1, 6). In agreement, CG antigen gene expression is induced by treatment with hypomethylating agents such as decitabine (DAC) and 5-azacytidine (7-9). Other epigenetic mechanisms, including histone modifications and methylated DNA-binding proteins (MBDs) also regulate CG antigen gene expression (10-14). While it is apparent that DNA hypomethylation plays a crucial role in CG antigen gene induction, it may not be sufficient to promote CG antigen gene expression in cancer in all circumstances (15, 16). Other factors, including sequence-specific transcription factors and global epigenomic regulators, may also promote CG antigen expression (15, 17, 18).

One example of a global epigenomic regulator of CG antigen genes is a recently-identified male germline-specific protein, Brother of the Regulator of Imprinted Sites (BORIS), which is expressed in several types of human cancers including epithelial ovarian cancer (EOC) (1, 17, 19-22). The expression pattern and chromosomal localization of BORIS indicates that it is an autosomal CG antigen gene (20, 23). BORIS, which is also known as CTCF-like protein or CTCFL, is a paralog of CTCF (CCCTC-binding factor), a transcription factor that plays a key role in chromatin insulation and genomic imprinting (20, 23). In addition, BORIS was recently shown to play a role in murine spermatogenesis (24). BORIS and CTCF differ in their N- and C-termini, but share a central zinc finger-binding domain capable of binding a similar spectrum of DNA sequences (20). Interestingly, BORIS and CTCF may have reciprocal expression patterns, as spermatocytes positive for BORIS are negative for CTCF (20, 23).

BORIS expression has been implicated in the activation of CG antigen genes (19, 21). In lung cancer cells, occupancy of the CG antigen gene *NY-ESO-1* promoter by CTCF was associated with gene silencing, while BORIS occupancy coincided with gene activation (19). In primary human fibroblasts, exogenously expressed BORIS led to de-repression of previously silent CG antigen genes including *MAGE-A1* (21). BORIS overexpression was also found to alter the DNA methylation state of specific regions of the *MAGE-A1* promoter (21). However, in later studies BORIS overexpression in melanoma cell lines did not

Figure 1



Ectopic BORIS expression in ovarian cell lines. (A) Western blot analysis of BORIS and β-galactosidase (LacZ) expression in the indicated cell lines at 48 and 96 hours post-transduction. The approximate position of the molecular weight markers on the BORIS Western blot is shown. α-Tubulin was used as a loading control. L48 and L96; LacZ recombinant adenovirus infection at 48 and 96 hours, respectively. B48 and B96; BORIS recombinant adenovirus infection at 48 and 96 hours, respectively. (B) IF staining of BORIS in OVCAR429 cells 48 hours post-infection. DAPI staining was used as a nuclear staining control. Overlay indicates computational merging of BORIS expression and DAPI staining.

activate MAGE-A1 expression or induce promoter hypomethylation (25). In addition, melanoma tissue samples were found to express MAGE-A1 but not BORIS, calling into question the general role of BORIS in *MAGE-A1* gene expression (25). More recently, glioma stem cells were observed to frequently express CG antigen genes in the absence of BORIS expression (26). Other data have questioned the frequency with which BORIS is expressed in breast cancer, presenting data discordant with an earlier report (21, 27). Based on these conflicting findings, it is clear that additional studies are required to clarify the role of BORIS in promoting CG antigen gene expression.

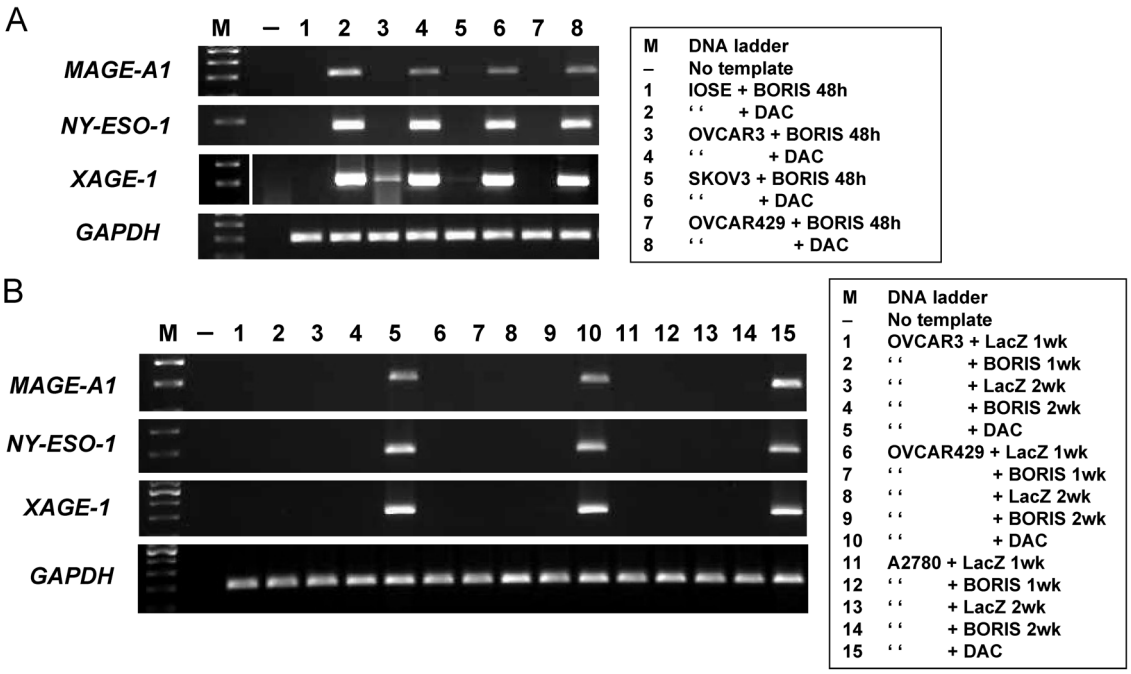
Recently, we have reported that BORIS is frequently expressed in ovarian cancer and have observed that the expression of BORIS coincides with expression of other CG genes in this malignancy [(22) and Woloszynska-Read *et al.*, manuscript in preparation]. Similar coordinate expression of BORIS and other CG antigen genes has been observed in head and neck, lung, and prostate cancer (28, 29). Taken together, these observations spurred us to examine the functional role of BORIS in the activation of CG antigen genes, using cultured ovarian cells. We also sought to determine whether BORIS overexpression altered DNA methylation, either specifically at CG antigen gene promoters or globally. Finally, we investigated whether BORIS plays a functional role in the activation of CG genes elicited by pharmacological inhibition of DNA methylation with decitabine.

Results

Exogenous BORIS expression in ovarian cells

We have previously shown that immortalized human ovarian surface epithelial cells (IOSE121) and four ovarian cancer cell lines (OVCAR3, SKOV3, A2780, and OVCAR429) do not express BORIS or other CG antigen genes at significant levels (22, 30). However, decitabine treatment induces CG antigen gene expression in all five cell lines, indicating that DNA methylation actively represses these genes (22, 30). To determine whether BORIS expression is capable of activating CG antigen genes in these cell lines, we overexpressed BORIS using an adenoviral approach. Western blot analyses revealed high levels of BORIS protein expression upon infection with recombinant BORIS virus, at both 48 and 96 hours post-infection (Figure 1A). Overexpressed BORIS ran at or near the expected molecular weight of 75.7 kDa (Figure 1A). Although BORIS expression appeared to be reduced at 96 hours (Figure 1A), we observed no obvious phenotypic differences (e.g. altered cell growth or death) in cells overexpressing BORIS (data not shown). To determine whether BORIS protein localizes to the nucleus, consistent with its role as a transcription factor, we conducted immunofluorescence (IF) analysis in OVCAR429 cells (Figure 1B). BORIS IF analysis was performed using two different clones of BORIS-specific mouse monoclonal antibodies to confirm BORIS antibody specificity (data not shown) (31). As shown in Figure 1B, BORIS showed predominantly nuclear localization, as indicated by co-localization with DAPI nuclear staining. Nuclear BORIS expression is in agreement with its putative role as a transcriptional regulator.

Figure 2



BORIS expression does not induce CG antigen gene expression in ovarian cell lines. (A) BORIS recombinant adenovirus was used to infect the indicated ovarian cell lines for 48 hours, and RNA was harvested to measure *MAGE-A1*, *NY-ESO-1*, *XAGE-1*, and *GAPDH* (control) expression by RT-PCR. Cells were treated with 1.0 μM decitabine (DAC) for 96 hours as a positive control for CG antigen gene induction. Sample key is shown at right. (B) LacZ or BORIS recombinant adenovirus was used to infect the indicated ovarian cell lines for 1 or 2 weeks, and RNA was harvested to measure *MAGE-A1*, *NY-ESO-1*, *XAGE-1*, and *GAPDH* (control) expression by RT-PCR. Cells were treated with 1.0 μM decitabine (DAC) for 96 hours as a positive control for CG antigen gene induction. Sample key is shown at right.

Exogenous BORIS expression does not activate CG antigen gene expression or cause CG antigen promoter hypomethylation in ovarian cells

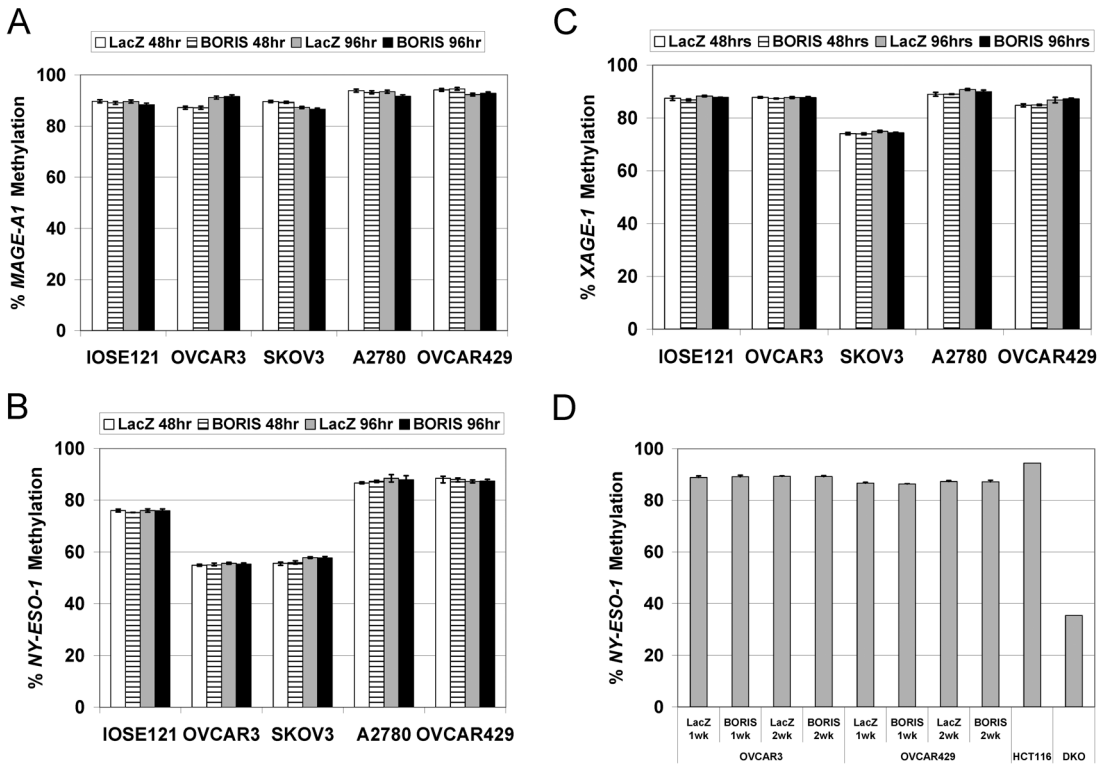
To determine whether BORIS expression activates CG antigen gene expression in ovarian cells, we analyzed the expression of the prototype CG genes *MAGE-A1*, *NY-ESO-1*, and *XAGE-1* by RT-PCR, following BORIS overexpression. Decitabine (DAC) treatment of these cell lines served as a positive control for CG antigen gene induction. At 48 hours post-infection, BORIS expression did not induce the expression of CG antigen genes in either IOSE121 cells or ovarian cancer cell lines, while DAC treatment induced CG gene expression, as expected (Figure 2A). Identical results were seen at 96 hours post-infection (data not shown). The small amount of *XAGE-1* PCR product seen in OVCAR3 cells infected with recombinant BORIS adenovirus at 48 hours was not due to BORIS overexpression, as similar levels of PCR product were observed in the LacZ control (Figure 2A and data not shown). We also examined the effect of BORIS overexpression on CG antigen gene expression at extended times post-infection of 1 and 2 weeks. At these time points, CG antigen genes remained repressed (Figure 2B). All end-point RT-PCR results for CG genes were confirmed using quantitative real-time RT-PCR, which gave identical results (data not shown).

The data presented above suggest that BORIS overexpression may not affect CG antigen promoter methylation. However, it is possible that BORIS could partially influence the DNA methylation state of CG antigen gene promoters, but not to an extent required to induce CG gene expression. To test this, we utilized quantitative sodium bisulfite pyrosequencing to determine the DNA methylation state of the *MAGE-A1*, *NY-ESO-1*, and *XAGE-1* promoters in ovarian cell lines following BORIS overexpression. Notably, we observed no significant changes in the promoter methylation levels of CG antigen genes upon BORIS overexpression in both IOSE121 and ovarian cancer cell lines (Figure 3, panels A-C). In addition, at extended times of BORIS overexpression of 1 or 2 weeks, CG antigen promoter methylation remained unaltered in ovarian cancer cell lines (Figure 3D and data not shown). These data suggest that BORIS expression is insufficient for DNA hypomethylation of CG antigen gene promoters.

Exogenous BORIS expression does not alter global DNA methylation in ovarian cells

BORIS expression during male germ cell differentiation correlates with the resetting of DNA methylation marks, and spermatocytes positive for BORIS show reduced or absent 5-methyl-cytosine (5mC) signal (20). While BORIS expression did not affect CG antigen promoter methylation as described above, it remained plausible that BORIS expression could impact global DNA methylation, which has not been investigated previously. To test this, we used two quantitative measures of global DNA methylation: (i) a liquid chromatography-mass spectrometry (LC-MS) assay to measure total 5-methyl-2'-deoxycytidine (5mdC) in genomic DNA (32), and (ii) a pyrosequencing assay for the *LINE-1* repetitive DNA element (30). Overall, we did not detect significant changes in 5mdC in ovarian cells transduced with BORIS, with the exception of a small increase in SKOV3 cells (Figure 4A). In

Figure 3



BORIS overexpression does not alter CG antigen promoter methylation levels in ovarian cell lines. LacZ or BORIS recombinant adenovirus was used to infect the indicated ovarian cell lines for 48 or 96 hours, and genomic DNA was harvested to measure (A) *MAGE-A1*, (B) *NY-ESO-1*, and (C) *XAGE-1* promoter methylation. The data shown represent the average methylation level of five CpG sites for *MAGE-A1*, fifteen CpG sites for *NY-ESO-1*, and ten CpG sites for *XAGE-1*. As an internal control, pyrosequencing of human testis DNA showed significant hypomethylation of each gene (23.9%, 24.9%, and 21.8% methylation, respectively). (D) LacZ or BORIS was expressed in OVCAR3 and OVCAR429 cells as described above, and genomic DNA was harvested at 1 and 2 weeks post-infection and used to measure *NY-ESO-1* promoter methylation by pyrosequencing. The human colorectal cancer cell lines HCT116 and DKO served as internal positive and negative controls for *NY-ESO-1* methylation, respectively. Error bars indicate ±1 SD.

agreement, *LINE-1* did not show significant methylation changes upon BORIS overexpression in ovarian cell lines (Figure 4B). As expected, internal controls (cell lines treated with decitabine) showed both reduced 5mdC levels and *LINE-1* methylation (Figure 4). Taken together, the data indicate that BORIS overexpression does not significantly alter global DNA methylation in ovarian cells.

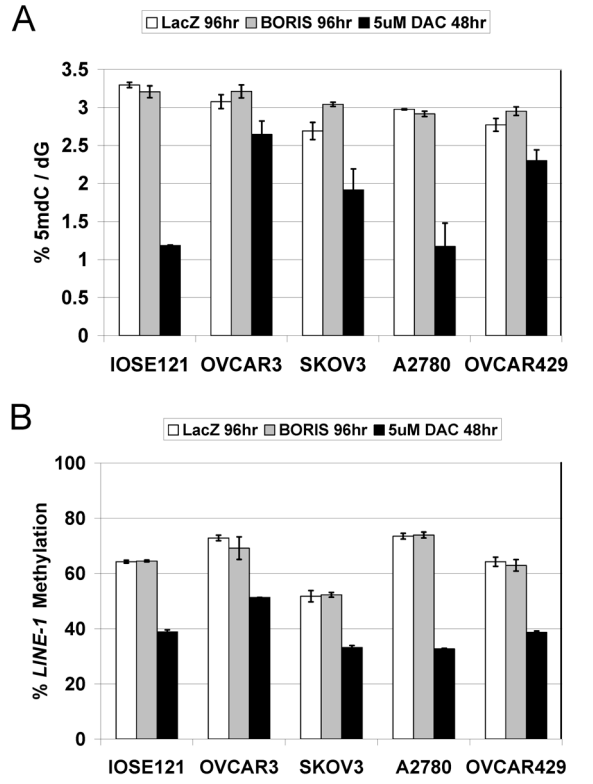
The role of BORIS in decitabine-mediated CG antigen gene induction in ovarian cells

It was previously reported that decitabine treatment induces *BORIS* expression in normal human dermal fibroblasts prior to the induction of *MAGE-A1*, leading to the suggestion that BORIS may mediate the induction of other CG antigen genes by this agent (21). To test this scenario in ovarian cells, we treated OVCAR429 cells with 1 μM decitabine and collected RNA over a time course of 8, 12, 24, and 48 hours to use for gene expression analysis of *BORIS*, *MAGE-A1*, *NY-ESO-1*, and *XAGE-1* (after normalization to *GAPDH*). Of these transcripts, only *MAGE-A1* was induced at 8 hours, while *MAGE-A1*, *BORIS*, and *NY-ESO-1* showed similar levels of induction at 24 hours post-treatment (Figure 5A). All four genes were induced by 48 hours post-treatment (Figure 5A). These data are inconsistent with a general role for BORIS in decitabine-mediated CG antigen gene induction.

We additionally examined the kinetics of CG antigen promoter DNA hypomethylation using pyrosequencing. All four CG antigen gene promoters showed similar kinetics of DNA hypomethylation, but there were small differences in the magnitude of DNA hypomethylation at each region (Figure 5B). It is interesting to note that *MAGE-A1* expression was induced prior to detection of hypomethylation of its promoter (Figure 5, panels A and B). These data suggest either that additional effects of decitabine contribute to the early activation of *MAGE-A1*, or alternatively that a very small number of hypomethylated alleles can significantly contribute to *MAGE-A1* induction. We additionally determined the kinetics of decitabine-mediated induction and hypomethylation of *BORIS* and other CG genes in additional cancer cell lines and observed similar results (data not shown). Taken together, these data suggest that decitabine-mediated induction of CG antigen genes is not dependent on BORIS.

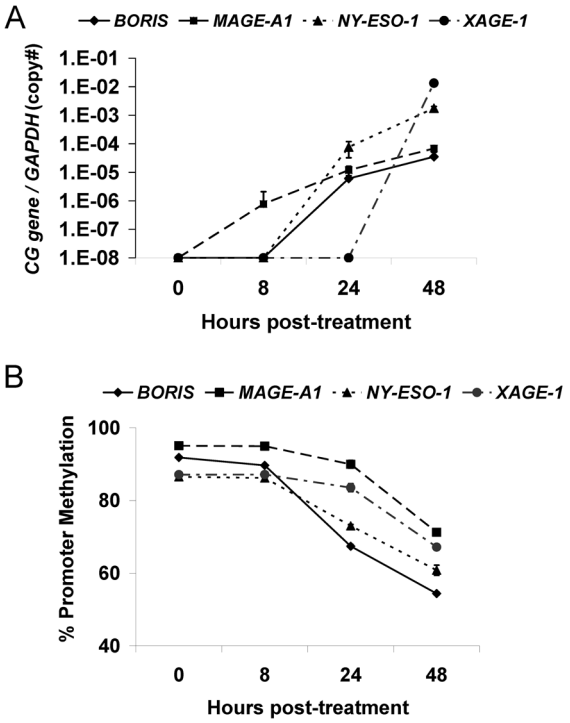
As an additional test of the role of BORIS in decitabine-mediated CG antigen gene activation, we used BORIS-specific siRNAs to knockdown BORIS expression in decitabine-treated OVCAR3 cells. OVCAR3 cells were utilized as they displayed greater siRNA transfection efficiency and more robust BORIS knockdown relative to other ovarian cell lines (data not shown). The BORIS monoclonal antibodies available to us were unable to detect endogenous BORIS protein in ovarian cell lines (data

Figure 4



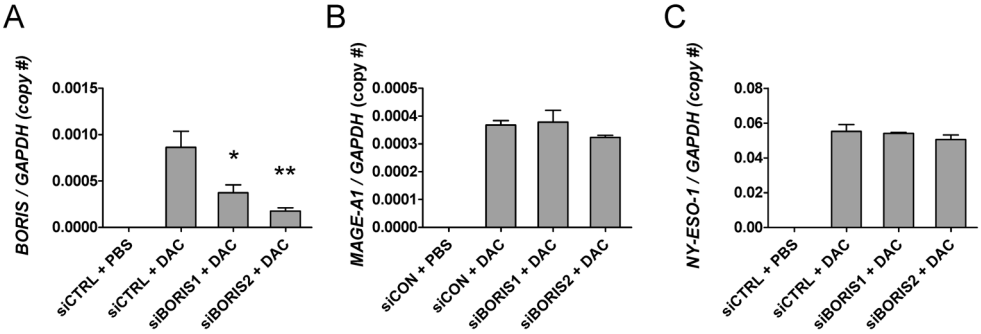
BORIS expression does not alter global DNA methylation in ovarian cell lines. LacZ or BORIS recombinant adenovirus was used to infect the indicated ovarian cell lines, and genomic DNA was harvested at 96 hours post-infection. As a control, cells were treated with 5 μM decitabine (DAC) for 48 hours. (A) 5mdC levels were measured using LC-MS. (B) *LINE-1* DNA methylation was measured using bisulfite pyrosequencing. The data shown represent the average methylation level of 3 CpG sites. Error bars indicate ±1 SD.

Figure 5



Kinetics of CG antigen gene induction and promoter DNA hypomethylation following decitabine treatment. OVCAR429 cells were treated with 1.0 μM decitabine and cell extracts were harvested at the indicated time points post-treatment. (A) qRT-PCR was used to measure CG antigen gene expression. (B) Sodium bisulfite pyrosequencing was used to measure CG antigen gene promoter methylation.

Figure 6



Effect of BORIS siRNA knockdown on decitabine-mediated CG antigen gene induction in OVCAR3 cells. Two different BORIS-targeting siRNAs, or a control siRNA, were transfected into OVCAR3 cells at a concentration of 50 nM. 24 hours post-transfection, cells were treated with 2.0 μM decitabine (DAC). 48 hours later, RNA extracts were harvested and used for qRT-PCR analysis of (A) *BORIS*, (B) *MAGE-A1*, and (C) *NY-ESO-1* mRNA expression. Error bars indicate ±1 SEM. Student's *t*-test *P* values: *, $P < 0.05$; **, $P < 0.01$.

not shown), so we assessed BORIS knockdown using quantitative real-time reverse transcriptase PCR (qRT-PCR). BORIS or control siRNAs were administered 24 hours prior to decitabine treatment, which was then conducted for an additional 48 hours. Figure 6A shows that two different BORIS-targeting siRNAs significantly diminished *BORIS* mRNA expression post-decitabine treatment, as compared to control siRNA. In contrast, decitabine-mediated induction of

MAGE-A1 and *NY-ESO-1* were not significantly affected by BORIS knockdown (Figure 6, panels B and C). Moreover, BORIS knockdown did not alter the extent of decitabine-mediated CG antigen gene promoter methylation or global DNA methylation (data not shown). These data reinforce the notion that BORIS is not required for decitabine-mediated induction of CG antigen genes in ovarian cells.

Discussion

While promoter DNA hypomethylation is a key mechanism leading to activation of CG antigen genes in human cancer, the mechanism underlying this process has not been resolved (1, 15, 33). Thus a report that ectopic expression of BORIS led to CG antigen gene expression in cultured normal human dermal fibroblasts, coincident with methylation changes at the *MAGE-A1* promoter, was of great interest (21). These data provoked us to assess the functional role of BORIS in CG antigen gene induction using *in vitro* ovarian cell models. Ectopic overexpression of BORIS in ovarian cells did not induce CG antigen gene expression, nor did it affect the methylation of CG antigen gene promoters. These observations held true for both ovarian cancer cell lines as well as an immortalized surface ovarian epithelial cell line. In contrast to BORIS overexpression, decitabine treatment induced CG gene expression in ovarian cell lines, demonstrating that these cells contain the transcriptional machinery needed to drive CG antigen gene expression, if epigenetic repression is relieved. The lack of an effect of BORIS expression on CG gene promoter methylation, and its coincidence with CG gene repression, is consistent with a strict dependence of CG gene expression on promoter DNA hypomethylation (15).

Consistent with our data, previous work showed that ectopic expression of BORIS using a plasmid vector failed to induce *MAGE-A1* expression in melanoma cell lines (25). In addition, it was recently demonstrated that BORIS knockout does not prevent hypomethylation of its gene target *Gal3st1* in murine spermatocytes (24). However, a recent study reported that overexpression of BORIS using a plasmid vector led to upregulation of CG antigen genes and an increase in hypomethylated CG antigen promoter alleles in an immortalized oral keratinocyte cell line (28). Interestingly, oral keratinocytes responded distinctly to ectopic BORIS expression depending on the level of overexpression, with greater effects on cell proliferation at lower levels of overexpression (28). Thus it is possible that titrating the expression of BORIS in ovarian cells could result in effects distinct from that which we report. More generally, the contradictory findings on the effects of BORIS overexpression in different cell types may be explained if the response to BORIS expression is cell context dependent. For example, other transcriptional or epigenomic regulatory factors may work in concert with BORIS to drive CG gene expression and these factors may be limiting in specific cell types (18).

In addition to its potential role in CG antigen gene regulation, it was postulated that BORIS expression in cancer may disrupt methylation boundaries imposed by CTCF and that this could allow the aberrant methylation patterns to spread, leading to hypomethylation of normally methylated DNA (34). This mechanism would presumably be anticipated to affect DNA methylation globally. We addressed this question using ovarian cells by measuring the impact of BORIS overexpression on global DNA methylation parameters. BORIS expression did not impact either total genomic 5mdC levels or methylation of the common repetitive element *LINE-1*. Moreover, knockdown of BORIS in the context of decitabine treatment did not alter the ability of the drug to cause global DNA hypomethylation (data not shown). Therefore, our *in vitro* data do not support a functional connection between BORIS expression and global DNA hypomethylation. However, our findings do not exclude the possibility that in the *in vivo* setting BORIS expression may either be associated with and/or contribute to the global DNA hypomethylation phenotype.

An observation made in a previous study was that decitabine treatment led to the induction of *BORIS* at an earlier time point (8 hours) than it did *MAGE-A1* (48 hours) (21). This led to the intriguing proposal that BORIS induction may functionally contribute to decitabine-mediated induction of *MAGE-A1* and other CG genes (21). Our data are not in agreement with this model. In ovarian cancer cells, decitabine treatment induced BORIS expression coincident with other CG antigen genes, and the kinetics of promoter hypomethylation of BORIS and other CG antigen genes was also very similar. Moreover, siRNA knockdown of BORIS did not significantly impact decitabine-mediated CG antigen gene induction or promoter DNA hypomethylation.

The current study may not be fully explanatory for understanding the functional contribution of BORIS to CG antigen gene regulation in ovarian cancer as it utilized *in vitro* model systems. We have previously shown that BORIS is relatively frequently expressed in ovarian cancer, and that its expression is closely associated with epigenetic activation of the gene by promoter DNA hypomethylation (22). Our unpublished data in ovarian cancer, as well as observations from other malignancies, suggest that CG antigen genes, including BORIS, are coordinately upregulated, in conjunction with promoter DNA hypomethylation, in human cancer (1, 15, 28, 29). In the future, it will be constructive to utilize *in vivo* tumor model systems to continue to dissect the potential role of BORIS in CG antigen gene regulation.

Abbreviations

CG, cancer-germline; DAC, decitabine (5-aza-2'-deoxycytidine); 5mdC, 5-methyl-2'-deoxycytidine; qRT-PCR, quantitative real-time reverse transcriptase PCR

Acknowledgements

We thank Dr. Nelly Auersperg and the Canadian Ovarian Tissue Bank for IOSE121 cells, Dr. Ivan Still of Roswell Park Cancer Institute for ovarian cancer cell lines, and Dr. Bert Vogelstein of Johns Hopkins University School of Medicine for the HCT116 and DKO cell lines. This work was supported by NIH RO1CA11674 (ARK), a Cancer Vaccine Collaborative Grant from the Cancer Research Institute/Ludwig Institute for Cancer Research (KO), the Ovarian Cancer Research Fund (KO and ARK), and NIH CA16056 (to Roswell Park Cancer Institute). AWR was supported in part by NIH training grant 5T32CA009072.

References

1. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005; **5:** 615-625. (PMID: 16034368)

2. Koslowski M, Bell C, Seitz G, Lehr HA, Roemer K, Müntefering H, Huber C, Sahin U, Türeci O. Frequent nonrandom activation of

germ-line genes in human cancer. *Cancer Res* 2004; **64:** 5988-5993. (PMID: 15342378)

3. Odunsi K, Qian F, Matsuzaki J, Mhawech-Fauceglia P, Andrews C, Hoffman EW, Pan L, Ritter G, Villella J, Thomas B, Rodabaugh K, Lele S, Shrikant P, Old LJ, Gnjatic S. Vaccination with an NY-ESO-1 peptide of HLA class I/II specificities induces integrated humoral and T cell responses in ovarian cancer. *Proc Natl Acad Sci U S A* 2007; **104:** 12837-12842. (PMID: 17652518)

4. Karbach J, Gnjatic S, Bender A, Neumann A, Weidmann E, Yuan J, Ferrara CA, Hoffmann E, Old LJ, Altorki NK, Jager E. Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. *Int J Cancer* 2010; **126:** 909-918. (PMID: 19728336)

5. Diefenbach CS, Gnjatic S, Sabbatini P, Aghajanian C, Hensley ML, Spriggs DR, Iasonos A, Lee H, Dupont B, Pezzulli S, Jungbluth AA, Old LJ, Dupont J. Safety and immunogenicity study of NY-ESO-1b peptide and montanide ISA-51 vaccination of patients with epithelial ovarian cancer in high-risk first remission. *Clin Cancer Res* 2008; **14:** 2740-2748. (PMID: 18451240)

6. De Smet C, Lurquin C, Lethé B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol* 1999; **19:** 7327-7335. (PMID: 10523621)

7. Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J, Rosenberg SA. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. *Cancer Res* 1994; **54:** 1766-1771. (PMID: 7511051)

8. Weiser TS, Guo ZS, Ohnmacht GA, Parkhurst ML, Tong-On P, Marincola FM, Fischette MR, Yu X, Chen GA, Hong JA, Stewart JH, Nguyen DM, Rosenberg SA, Schrump DS. Sequential 5-Aza-2 deoxycytidine-depsipeptide FR901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic T lymphocytes specific for NY-ESO-1. *J Immunother* 2001; **24:** 151-161. (PMID: 11265773)

9. Karpf AR. A potential role for epigenetic modulatory drugs in the enhancement of cancer/germ-line antigen vaccine efficacy. *Epigenetics* 2006; **1:** 116-120. (PMID: 17786175)

10. James SR, Link PA, Karpf AR. Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b. *Oncogene* 2006; **25:** 6975-6985. (PMID: 16715135)

11. Link PA, Gangisetty O, James SR, Woloszynska-Read A, Tachibana M, Shinkai Y, Karpf AR. Distinct roles for histone methyltransferases G9a and GLP in cancer germ-line antigen gene regulation in human cancer cells and murine embryonic stem cells. *Mol Cancer Res* 2009; **7:** 851-862. (PMID: 19531572)

12. Loriot A, De Plaen E, Boon T, De Smet C. Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells. *J Biol Chem* 2006; **281:** 10118-10126. (PMID: 16497664)

13. Wischnewski F, Friese O, Pantel K, Schwarzenbach H. Methyl-CpG binding domain proteins and their involvement in the regulation of the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 gene promoters. *Mol Cancer Res* 2007; **5:** 749-759. (PMID: 17634428)

14. Wischnewski F, Pantel K, Schwarzenbach H. Promoter demethylation and histone acetylation mediate gene expression of MAGE-A1, -A2, -A3, and -A12 in human cancer cells. *Mol Cancer Res* 2006; **4:** 339-349. (PMID: 16687489)

15. Akers SN, Odunsi K, Karpf AR. Regulation of cancer germline antigen gene expression: implications for cancer immunotherapy. *Future Oncology* 2010; **6:** 717-732. (PMID: 20465387)

16. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA. Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine. *Mol Pharmacol* 2004; **65:** 18-27. (PMID: 14722233)

17. Hoffmann MJ, Müller M, Engers R, Schulz WA. Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies. *Biochem Pharmacol* 2006; **72:** 1577-1588. (PMID: 16854382)

18. Rousseaux S, Khochbin S. New hypotheses for large-scale epigenome alterations in somatic cancer cells: a role for male germ-cell-specific regulators. *Epigenomics* 2009; **1:** 153-161.

19. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, Zhao M, Nguyen DM, Barrett JC, Lobanenkov VV, Schrump DS. Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. *Cancer Res* 2005; **65:** 7763-7774. (PMID: 16140944)

20. Klenova EM, Morse HC 3rd, Ohlsson R, Lobanenkov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. *Semin Cancer Biol* 2002; **12:** 399-414. (PMID: 12191639)

21. Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, Pugacheva E, Hong JA, Morse H 3rd, Schrump DS, Risinger JI, Barrett JC, Lobanenkov VV. Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. *Cancer Res* 2005; **65:** 7751-7762. (PMID: 16140943)

22. Woloszynska-Read A, James SR, Link PA, Yu J, Odunsi K, Karpf AR. DNA methylation-dependent regulation of BORIS/CTCFL expression in ovarian cancer. *Cancer Immun* 2007; **7:** 21. URL: http://www.cancerimmunity.org/v7p21/071122.htm

23. Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, Rasko JE, Docquier FM, Kistler M, Breen JJ, Zhuang Z, Quitschke WW, Renkawitz R, Klenova EM, Feinberg AP, Ohlsson R, Morse HC 3rd, Lobanenkov VV. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. *Proc Natl Acad Sci U S A* 2002; **99:** 6806-6811. (PMID: 12011441)

24. Suzuki T, Kosaka-Suzuki N, Pack S, Shin DM, Yoon J, Abdullaev Z, Pugacheva E, Morse HC 3rd, Loukinov D, Lobanenkov V. Expression of a testis-specific form of Gal3st1 (CST), a gene essential for spermatogenesis, is regulated by the CTCF paralogous gene BORIS. *Mol Cell Biol* 2010; **30:** 2473-2484. (PMID: 20231363)

25. Kholmanskikh O, Loriot A, Brasseur F, De Plaen E, De Smet C. Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. *Int J Cancer* 2008; **122:** 777-784. (PMID: 17957795)

26. Yawata T, Nakai E, Park KC, Chihara T, Kumazawa A, Toyonaga S, Masahira T, Nakabayashi H, Kaji T, Shimizu K. Enhanced expression of cancer testis antigen genes in glioma stem cells. *Mol Carcinog* 2010; **49:** 532-544. (PMID: 20082319)

27. Hines WC, Bazarov AV, Mukhopadhyay R, Yaswen P. BORIS (CTCFL) is not expressed in most human breast cell lines and high grade breast carcinomas. *PLoS One* 2010; **5:** e9738. (PMID: 20305816)

28. Smith IM, Glazer CA, Mithani SK, Ochs MF, Sun W, Bhan S, Vostrov A, Abdullaev Z, Lobanenkov V, Gray A, Liu C, Chang SS, Ostrow KL, Westra WH, Begum S, Dhara M, Califano J. Coordinated activation of candidate proto-oncogenes and cancer testes antigens via promoter demethylation in head and neck cancer and lung cancer. *PLoS One* 2009; **4:** e4961. (PMID: 19305507)

29. Suyama T, Shiraishi T, Zeng Y, Yu W, Parekh N, Vessella RL, Luo J, Getzenberg RH, Kulkarni P. Expression of cancer/testis antigens in prostate cancer is associated with disease progression. *Prostate* 2010; in press. (PMID: 20583133)

30. Woloszynska-Read A, Mhawech-Fauceglia P, Yu J, Odunsi K, Karpf AR. Intertumor and intratumor NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. *Clin Cancer Res* 2008; **14:** 3283-3290. (PMID: 18519754)

31. Zhou XC, Song CJ, Wang X, Tian Y, Jin BQ. [Preparation of the monoclonal antibodies against human BORIS and the expression pattern of BORIS in normal and diseased human mammary tissues]. [Article in Chinese]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2007; **23:** 353-355. (PMID: 17428394)

32. Song L, James SR, Kazim L, Karpf AR. Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Chem* 2005; **77:** 504-510. (PMID: 15649046)

33. De Smet C, Loriot A. DNA hypomethylation in cancer: Epigenetic scars of a neoplastic journey. *Epigenetics* 2010; **5:** in press. (PMID: 20305381)

34. Robertson KD. DNA methylation and human disease. *Nat Rev Genet* 2005; **6:** 597-610. (PMID: 16136652)

35. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, Baylin SB, Vogelstein B. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 2002; **416:** 552-556. (PMID: 11932749)

Materials and methods

Cell lines and drug treatments

Ovarian cancer cell lines (SKOV3, A2780, OVCAR3, OVCAR429) were propagated as described previously (22). IOSE121 cells (SV40 immortalized normal human ovarian surface epithelium cells) were obtained from the Canadian Ovarian Tissue Bank and Dr. Nelly Auersperg (University of British Columbia) and were grown as described previously (22). The human colorectal cancer cell lines HCT116 and *DNMT1-/-*, *DNMT3b-/-* HCT116 (DKO) cells were obtained from Dr. Bert Vogelstein (Johns Hopkins School of Medicine) and have been described previously (10, 35). Ovarian cell lines were treated with decitabine (DAC, 5-aza-2'-deoxycytidine, Sigma Chemical Company, St. Louis, MO) as described in the text.

Ectopic BORIS expression

A BORIS adenoviral expression vector was generated using ViraPower™ Adenoviral Gateway® Expression Kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, we cloned the human *BORIS* open reading frame (ORF) into the entry vector pENTR/D-TOPO (Invitrogen) using the Gateway system. Subsequently, the *BORIS* ORF was sub-cloned into the pAd/CMV/V5-DEST expression construct. This vector was transfected into 293A cells using Lipofectamine2000 (Invitrogen) to produce recombinant adenovirus. An adenovirus expression plasmid containing the *lacZ* gene was used as a negative control. Adenovirus titers were determined by plaque formation assay, and recombinant viral supernatants were stored at 80°C. Ovarian cells were infected with recombinant virus at a multiplicity of infection (MOI) of 50, and BORIS and LacZ (β-galactosidase) expression were detected by Western blot or IF.

Western blotting

Western blot analyses were performed as described previously (10), using 20 μg of protein per sample. Anti-BORIS antibody has been described previously (31) and was used at a 1:20000 dilution. The anti-V5 mouse monoclonal antibody 1:5000 (Invitrogen) was used to detect β-galactosidase, and anti-α-tubulin 1:2500 (Accurate Chemical and Scientific Corporation, Westbury, NY) was used as a loading control. HRP-conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, Piscataway, NJ) were used as secondary antibodies.

Immunofluorescence staining of BORIS in OVCAR429 cells

Cells were grown on glass coverslips and infected with BORIS adenovirus at a MOI of 50. 48 hours post-infection, coverslips were washed in PBS, fixed in 2% formaldehyde for 15 min, and washed twice with 1x PBS. The cells were then incubated with anti-BORIS mouse monoclonal antibody (31) (1:500) in 1x PBS containing 0.2% Triton X-100 (PBS/Triton) for 1 h at room temperature. Following washes in PBS/Triton, cells were incubated with TRITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Westgrove, PA) (1:200) for 30 min. Coverslips were dipped for 20 seconds in 100 ng/ml DAPI solution, washed in PBS, and mounted with Aquamount (Polysciences, Inc., Warrington, PA). Samples were viewed using a Zeiss Axioskop (Carl Zeiss, Thornwood, NY) equipped with a Hamamatsu digital CCD camera (Hamamatsu Corporation, Bridgewater, NJ).

Reverse-transcriptase PCR (RT-PCR) and quantitative real-time reverse transcriptase PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). RT-PCR and qRT-PCR for *BORIS* (NCBI Entrez GeneID 140690), *MAGE-A1* (GeneID 4100), *NY-ESO-1* (GeneIDs 1485 and 246100), and *XAGE-1* (GeneIDs 653219, 653220, 653048, 9503, and 653067) was accomplished as described previously (10, 22). High homology did not allow specific quantification of *NY-ESO-1* and *XAGE-1* isoforms. qRT-PCR was done on

triplicate samples, and expression analysis of each gene was performed a minimum of two times.

Quantitative sodium bisulfite DNA pyrosequencing

Genomic DNA was isolated using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN), and methylation of the *BORIS*, *MAGE-A1*, *NY-ESO-1*, and *XAGE-1* promoters was analyzed by pyrosequencing as described previously (11, 22, 30). High homology did not allow specific quantification of *NY-ESO-1* and *XAGE-1* isoforms. To verify efficient bisulfite conversion, non-CpG cytosines served as internal controls, and unmethylated and methylated DNAs were run as additional controls. Pyrosequencing was done on duplicate samples, and pyrosequencing assays were performed a minimum of two times.

Global DNA methylation analyses

Genomic DNA was isolated as described above, and 5mdC levels were determined as described previously (32). Pyrosequencing of *LINE-1* was done as described previously (30).

BORIS siRNA knockdown

Knockdown of BORIS in OVCAR3 cells was achieved using siRNA oligos (Ambion, Austin, TX). Two different BORIS siRNAs were utilized: 5'-GGAUCAACCUACAGCUGGUtt-3' (siBORIS1), and 5'-GGCCUGAAGGAGGAGGAAAtt-3' (siBORIS2). Cells were transfected with 50 nM BORIS siRNAs or a control non-targeting siRNA (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen).

Contact

Address correspondence to:

Dr. Adam R. Karpf
Department of Pharmacology and Therapeutics
Roswell Park Cancer Institute
Elm & Carlton Streets
Buffalo, NY, 14263
USA
Tel.: + 1 716 845-8305
Fax: + 1 716 845-8857
E-mail: adam.karpf@roswellpark.org