

FAST TRACKS

# Antitumor Efficacy of DNA Vaccination to the Epigenetically Acting Tumor Promoting Transcription Factor BORIS and CD80 Molecular Adjuvant

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**Abstract** Cancer testis (CT) antigens are promising candidates for tumor vaccines due to their immunogenicity and tissue-restricted expression. Recently, we identified a novel cancer testis gene, *BORIS*, whose expression is restricted to male testis after puberty and is strictly absent in non-malignant female tissue. *BORIS* encodes a DNA-binding protein that shares 11 zinc finger (ZF) with transcription factor *CTCF* and differs at the N- and C-termini. *CTCF* has been implicated in epigenetic regulation of imprinting, X chromosome inactivation, repression, and activation of cancer testis antigens. *BORIS* expression has been documented in cancers of diverse histological origin, including, but not limited to breast, prostate, ovary, gastric, liver, endometrial, glioma, colon, and esophagus. Interestingly, *BORIS* induces demethylation and subsequent expression of many cancer-testis genes, including *MAGE-A1* and *NY-ESO-1*, indicating that it is expressed very early in malignancy and might be an attractive candidate for immunotherapy. In this study we tested *BORIS* as a vaccine in a very aggressive, highly metastatic, and poorly immunogenic murine model of mammary carcinoma. Immunizations with a DNA encoding the mutant form of murine *BORIS* antigen (pm*BORIS* lacking DNA-binding function) significantly prolonged survival, and inhibited tumor growth in BALB/c mice inoculated with 4T1 cells. Priming with pm*BORIS* mixed with molecular adjuvant and boosting with adenoviral vector expressing m*BORIS* was generally more effective, suggesting that the vaccination protocol could be further optimized. This is the first report demonstrating the feasibility of vaccination with a cancer associated epigenetic regulator for the induction of tumor inhibition. *J. Cell. Biochem.* 98: 1037–1043, 2006. © 2006 Wiley-Liss, Inc.

**Key words:** *BORIS*; CT antigens; breast cancer; mammary tumor; vaccine

Abbreviations used: TAAs, tumor associated antigens; ZF, zinc finger; *BORIS*, Brother of the Regulator of Imprinted Sites; BrCA, breast cancer; CT, cancer testis; pm*BORIS*, plasmid encoding ZF-deleted (truncated) murine *BORIS*; Ad*BORIS*, recombinant adenoviral vector encoding truncated m*BORIS*.

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Current data indicate that successful immune protection against cancers requires recognition of epitopes on the tumor and the generation of a potent T-cell immune response targeted to those tumor associated antigens (TAAs). Several TAA-based vaccines for treating breast cancer (BrCA) and metastatic disease were developed. Such antigens may have been derived from the whole tumor cells (autologous or allogeneic tumors), or they may be overexpressed/alterd in the BrCA cells [Ko et al., 2003].

Since the 1980s, different TAAs have been identified and cloned, and today more than 60 human TAAs are known [Van den Eynde and Van der Bruggen, 2004]. The major target TAAs expressed in BrCA include: epidermal growth factor receptors (HER2), carcinoembryonic

antigen (CEA), mucin (MUC1), and tumor suppressor protein (p53). Functional immune responses against all of these BrCA TAAs may be limited through tolerance mechanisms because they are self-antigens. Thus, development of a BrCA vaccine based on such TAAs requires the breaking of immune tolerance to these immunogens without generation of autoimmunity capable of destroying normal tissues [Smyth et al., 2001].

Almost 15 years ago, the first cancer testis (CT) gene, *MAGE-A1* encoding the CT antigen was identified using CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) derived from a tumor-bearing patient [van der Bruggen et al., 1991; Van Pel et al., 1995]. Subsequently, more than 80 CT antigens have been identified and described [Simpson et al., 2005]. Expression of *MAGEA1* gene was not detected in normal tissues, but was detected in most melanomas, some breast carcinomas, and other types of cancer. In fact, expression patterns of many other CT antigens are also highly restricted in normal tissues, but are readily detected in different tumors [Simpson et al., 2005]. Since the testis is an immuno-privileged organ, these CT TAAs may be considered similar to non-self antigens that could induce strong anti-cancer immunity. Accordingly, several CT antigens have been widely used in different pre-clinical and clinical trials as targets for cancer vaccines [Scanlan et al., 2002].

Recently, we found the expression of another CT antigen, BORIS, in the testis of men after puberty, but not in any other normal tissues of men [Loukinov et al., 2002]. As we expected, this molecule was a TAA, since *BORIS* expression was activated abnormally in many different cancer cells, including but not limited to breast, prostate, ovary, gastric, liver, endometrial, glioma, colon, and esophagus [Loukinov et al., 2002; Hong et al., 2005; Vatolin et al., 2005]. More importantly, we demonstrated that BORIS is a transcriptional factor, and its expression results in demethylation and derepression of other CT genes including *MAGE-A1* and *NY-ESO-1* [Hong et al., 2005; Vatolin et al., 2005]. These data indicate that BORIS may be an ideal target for a cancer vaccine, specifically for BrCA vaccine, because it is a non-self antigen that is expressed in a wide range of different tumors very early in malignancy. Furthermore, the fact that BORIS is actually involved in maintaining the cancerous state indicates that immune

escape through BORIS mutation would compromise the ability of the tumor to maintain oncogenic potential. In this pilot study we evaluated a truncated form of murine BORIS antigen as a component of anti-cancer vaccine using the 4T1 mammary carcinoma-based mouse model of BrCA. Mice were vaccinated with mammalian and adenovirus expression vectors encoding the mutated murine BORIS molecule that lacks DNA-binding function, and protection against challenge with highly malignant and poorly immunogenic mouse mammary tumor cell line was assessed.

## MATERIALS AND METHODS

### Generation of Plasmid and Adenoviral Vector Encoding Truncated Murine *BORIS* Gene

To generate a safe BrCA vaccine based on murine BORIS (mBORIS), the DNA-binding motif of the BORIS molecule was disrupted in order to prevent possible adverse events due to BORIS oncogenic activity [Hong et al., 2005; Vatolin et al., 2005] and possible overlapping with CTCF gene [Loukinov et al., 2002]. Thus, we constructed a plasmid encoding a ZF-deleted murine BORIS (pmBORIS). The PCR-amplified N- and C-terminal regions of the *mBORIS* gene were connected through a specifically designed short spacer and cloned into the pORF plasmid at the *NcoI* and *EcoRI* sites under the control of the hEF1-HTLV promoter (Invivogen, CA). The resulting pmBORIS was isolated using the EndoFree Plasmid maxi kit (Qiagen, CA). Purity of the plasmid DNA was confirmed using UV spectrophotometry and gel electrophoresis.

Recombinant adenoviral vector encoding the same truncated mBORIS (AdBORIS) was prepared using the AdEasy XL Adenoviral Vector System (Stratagene, CA). The shuttle vector was constructed by subcloning the mBORIS fragment into the plasmid pShuttle-CMV by a PCR technique using the appropriate primers. To generate AdBORIS for immunization, the shuttle vector containing *mBORIS* was linearized with *PmeI*, purified using an agarose gel and utilized for transformation of BJ-5183-Ad-1 cells. Selected positive recombinant Ad plasmid DNA was amplified in XL-10 cells, linearized with *PacI* restriction enzyme and propagated in AD-293 cells. The viral stock was titrated and

purified on a CsCl gradient. The purified virus was dialyzed against PBS-5% sucrose and used for immunization of mice.

#### Detection of Expression of Truncated mBORIS in CHO Cells Transiently Transfected by pmBORIS or AdBORIS

CHO cells ( $1 \times 10^6$ ) were transiently transfected with 2  $\mu\text{g}$  of pmBORIS or pORF vector using Lipofectamine Plus Reagent (Invitrogen, CA). The expression of the pmBORIS construct was analyzed by Western blot (WB) in the lysate of transiently transfected CHO cells as previously described [Vasilevko et al., 2003]. Expression of mBORIS was visualized by staining with rabbit anti-BORIS IgG antibodies (1  $\mu\text{g}/\text{ml}$ ) prepared in our laboratory using different peptides spanning this antigen. CHO cells transfected with pORF vector were used as a control.

The expression of mBORIS was also analyzed and confirmed by WB using the same antibodies in CHO cells transfected with pShuttle-mBORIS.

#### Expression of BORIS in Non-Transfected 4T1 Mammary Carcinoma Cells

Aberrant reactivation of murine BORIS in 4T1 mammary carcinoma cells have been detected by RT-PCR, as we previously described [Vatolin et al., 2005] using the following primers: 5'-ATGGCTGCCGCTGAGGTCCCTGTCCCTTCTGGGTAC (forward), 5'-CACTGAAAGCTCTGAGGCTTCCCTTGGTCTGCC-TC (reverse).

#### Animals, Immunizations, and Tumor Challenge

Eight- to ten-week-old female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in a temperature and light cycle-controlled facility and their care was under the guidelines of the National Institutes of Health and the approved IACUC protocol at the University of California, Irvine. Gold beads were coated with the purified pmBORIS or mixture of pmBORIS with plasmid encoding CD80 molecular adjuvant (pCD80) and used for gene gun immunization, as previously described [Vasilevko et al., 2003]. Two groups of mice were injected four times at biweekly intervals with pmBORIS or pmBORIS mixed with pCD80 received the booster injection with  $1.5 \times 10^8$  PFU AdBORIS intravenously (i.v.). Another experimental group of mice was immunized four times biweekly with

pmBORIS, whereas control animals received pORF (vector) followed by the booster injection with AdBORIS. Additional control groups of mice were either non-immunized or injected once with Ad expressing the irrelevant  $\beta$ -GAL gene.

Ten days after the last immunization, experimental and control mice were challenged with  $1.5 \times 10^4$  4T1 cells (100  $\mu\text{l}$  in PBS) injected into the mammary fat pad. Tumor volumes were determined by bidimensional measurement and calculated three times weekly, as previously described [Vasilevko et al., 2003]. Animals were sacrificed when the mouse appeared moribund, or the tumors reached approximately 2  $\text{cm}^3$ , the maximum size recommended by the Institutional Animal Care and Use Committee, University of California, Irvine guidelines for tumor research using a mouse model.

#### Statistical Analysis

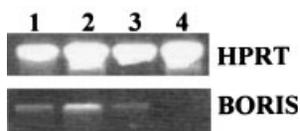
All statistical parameters (average tumor volume, standard deviations (SD), significant differences between groups) were calculated using GraphPad Prism 3.0 Software. Statistical significance between groups was determined by one-way ANOVA with Tukey's multiple comparison post-test ( $P$  values less than 0.05 were considered significant.). Kaplan-Meier curves were generated to indicate survival data.

## RESULTS AND DISCUSSION

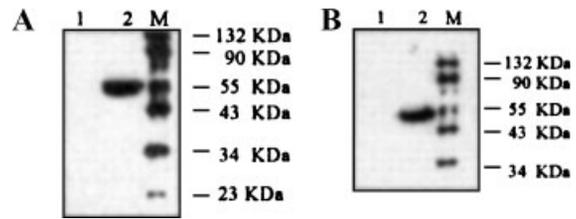
Numerous tumor antigens have been used for immunotherapeutic purposes with mediocre clinical results [Rosenberg et al., 2004]. A potential explanation is the relative immunogenicity of numerous animal tumor models utilized for pre-clinical development in contrast to the poorly immunogenic spontaneous human tumors [Le Poole et al., 2003]. Additionally, the ability of tumor cells to rapidly lose antigenic markers leads to the possibility of tumor mutation in response to immunological pressure, resulting in vaccine inefficiency [Browning and Dunnion, 1997]. Therefore, in this study we chose to use BALB/c mice and the 4T1 murine model of spontaneous metastatic mammary carcinoma as a stringent system [Miller et al., 1983; Pulaski and Ostrand-Rosenberg, 1998]. It has been described in the literature that the 4T1 cell line elaborates a variety of immune suppressive molecules similar to the

clinical situation, including PGE-2 [Mitsuhashi et al., 2004], TGF- $\beta$  [Muraoka et al., 2002; Kobie et al., 2003], and other factors [Danna et al., 2004]. Additionally, it is known that the BALB/c strain possesses a Th2 propensity as noted by susceptibility to *Listeria* [Miura et al., 2000] and prolonged allograft survival [Wang et al., 2003] in contrast to other strains, due in part to upregulated production of the Th1 inhibiting enzyme arginase [Mills et al., 2000]. Due to the central role of BORIS in maintaining the oncogenic phenotype [Loukinov et al., 2002; Hong et al., 2005; Vatolin et al., 2005], we sought to determine the feasibility of inducing a relevant anti-4T1 response to this transcription factor by immunization. In previous studies, we demonstrated aberrant reactivation of BORIS in many different human and murine cancer cell lines and primary tumors [Loukinov et al., 2002; Vatolin et al., 2005]. However, we did not previously determine expression of full-length BORIS in 4T1 mammary carcinoma cells. Therefore, to be able to use this well-characterized mouse model of BrCA in this study, we first analyzed the expression of BORIS in the 4T1 cell line. As expected, 4T1 mammary carcinoma cells demonstrated expression of *BORIS* mRNA by RT-PCR (Fig. 1).

For DNA vaccination the 11 ZF deleted form of murine BORIS (mBORIS) was utilized in order to eliminate its functional activity and potential undesirable effects. Thus we first constructed a plasmid encoding truncated form of BORIS (pmBORIS). This plasmid was used for transfection of CHO cells, and expression of mBORIS was confirmed (Fig. 2A). Next it was decided to use a prime-boost vaccination strategy, where mice primed with DNA immunogen were boosted with recombinant adenovirus vector. Previously, such immunization strategies have proven to be more effective than DNA immunization alone. Accordingly, we also prepared AdBORIS using the AdEasy XL Adenoviral Vector System (Stratagene). The mBORIS



**Fig. 1.** Detection of full-length BORIS mRNA in 4T1 mouse breast cancer cells by RT-PCR (Lane 2). Expression of BORIS mRNA in mouse GL261 glioma (Lane 1) and P815 mastocytoma (Lane 3) cell lines. Mouse splenocytes (Lane 4) were used as non-malignant negative control.



**Fig. 2.** Expression of mBORIS in both mammalian and adenoviral vectors. Expression of mBORIS is detected in the CHO cells transfected with pmBORIS (A) or pShuttle-mBORIS. Lysates of transiently transfected CHO cells were analyzed on 10% Tris SDS-PAGE followed by Western blot with rabbit-anti BORIS antibodies. A: Lysate of CHO cells transfected with pORF vector (Lane 1) or pmBORIS (Lane 2); (B) lysate of CHO cells transfected with Shuttle vector (Lane 1) or pShuttle-mBORIS (Lane 2). Protein markers (Lane M).

gene was cloned into the pShuttle-vector and the expression of mBORIS was analyzed in CHO cells transfected with pShuttle-mBORIS. CHO cells expressed high level of mBORIS molecules (Fig. 2B). Next we transferred mBORIS gene into the adenoviral vector by homologous recombination and selected recombinant adenovirus by restriction analysis with *PacI* restriction endonuclease.

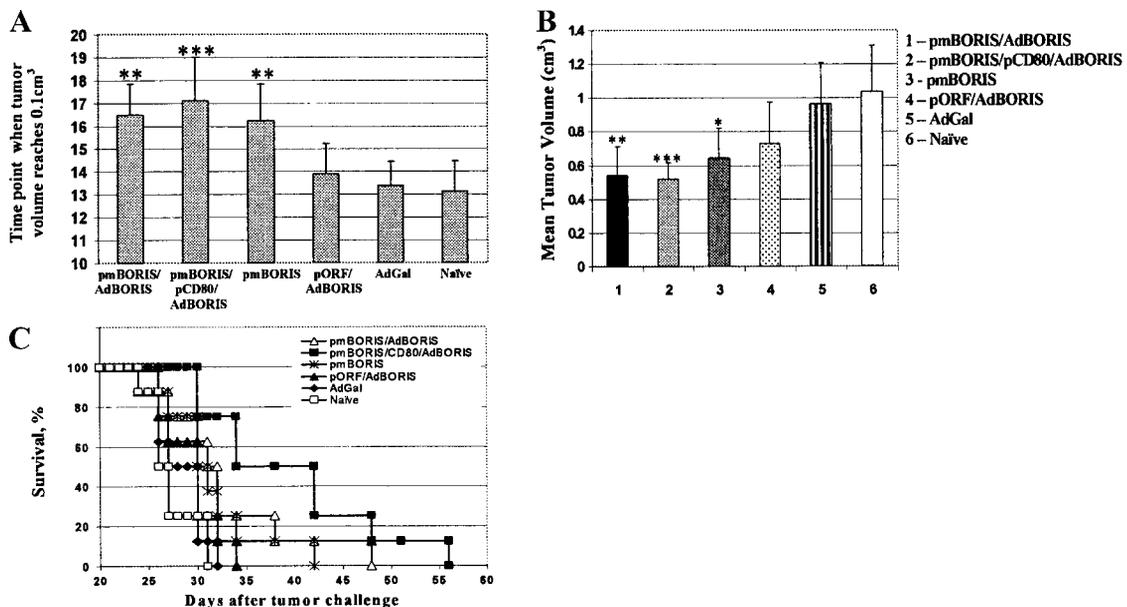
The results presented above provide all the necessary components for testing the efficacy of DNA and adenoviral-based mBORIS vaccines in the 4T1 BrCA model. Accordingly, pilot protective studies with pmBORIS and AdBORIS were initiated. Two groups of mice ( $n = 8$  per group) were injected with pmBORIS whereas the other group of experimental animals ( $n = 8$ ) was immunized with a mixture pmBORIS and pCD80. After four biweekly immunizations, one group of mice primed with pmBORIS and animals from the group injected with pmBORIS plus pCD80, received also the booster injection with AdBORIS. Control groups of mice were administered with pORF and boosted with AdGal, an adenovirus vector encoding irrelevant antigen, or remained non-immunized. Ten days after the last immunization, mice were challenged with  $1.5 \times 10^4$  4T1 cells (100  $\mu$ l in PBS) implanted into the mammary fat pad. Tumor growth and survival of mice were analyzed as we described above and previously [Vasilevko et al., 2003].

The time points at which tumor volume reached 0.1  $\text{cm}^3$  were compared in control and experimental groups. The tumor volume of 0.1  $\text{cm}^3$  was chosen because of it correctly measurable size. We found that this period in mice immunized with pmBORIS, as well as in

mice immunized and boosted with pmBORIS/AdBORIS or pmBORIS/pCD80/AdBORIS, was significantly extended relative to the controls ( $P < 0.01$  for groups pmBORIS/AdBORIS and pmBORIS and  $P < 0.001$  for group pmBORIS/pCD80/AdBORIS) (Fig. 3A). The longest delay in tumor growth was in the group of mice injected with pmBORIS/pCD80/AdBORIS. Mice immunized with pmBORIS or primed and boosted with pmBORIS/AdBORIS also showed a delay in the development of measurable tumors relative to animals that received the vector control (Fig. 3A). Thus, all experimental groups demonstrated delay in tumor growth relative to the controls.

Vaccinated mice also demonstrated significant inhibition of tumor growth relative to the controls (Fig. 3B). On day 24 when all mice were still alive, the average tumor sizes in the animals from the pmBORIS, pmBORIS/AdBORIS, and pmBORIS/pCD80/AdBORIS groups were significantly smaller ( $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively) than the average tumor sizes in animals from control groups (Fig. 3B). Importantly, the average long-term survival of

vaccinated mice was significantly prolonged relative to that of the control groups (Fig. 3C). Mice injected with pmBORIS/pCD80/AdBORIS had the longest delay in mortality or in growth of tumors to a volume of  $2.0 \text{ cm}^3$  ( $P < 0.01$ ). Other vaccinated mice also survived longer than naïve mice or animals injected with vectors only. On day 34 when all mice in the control groups have succumbed to tumor burden, 25% of mice immunized with pmBORIS, or mice primed with pmBORIS and boosted with AdBORIS, and 50% of mice from the pmBORIS/pCD80/AdBORIS group were still alive (Fig. 3C). Of note, in these experiments relatively stringent conditions were used with mice being challenged with  $1.5 \times 10^4$  unmodified 4T1 cells. Importantly  $(5-7) \times 10^3$  of the same cells resulted in local growth of mammary tumors in all experimental animals (data not shown and [Pulaski and Ostrand-Rosenberg, 1998]). In fact, naïve BALB/c mice challenged with  $1.5 \times 10^4$  or  $1 \times 10^5$  4T1 cells developed tumors almost simultaneously, whereas appearance of tumors in mice injected with  $7 \times 10^3$  4T1 cells was significantly delayed (data not shown). However, even using



**Fig. 3.** Survival of mice and delays in growth of unmodified mouse mammary 4T1 tumors in mice vaccinated with mBORIS. Mice were immunized with pmBORIS, pmBORIS plus pCD80 or injected with pORF (control vector) following by booster injection with AdBORIS (eight mice per group). Other groups were not vaccinated (naïve), vaccinated with pmBORIS or injected with AdGal (Ad expressing irrelevant antigen) ( $n = 8$ ). All mice were challenged with  $1.5 \times 10^4$  4T1 tumor cells. **A:** The time point at which tumor volume reached  $0.1 \text{ cm}^3$ . Differences between experimental and control groups were significant

( $**P < 0.01$  for groups pmBORIS/AdBORIS and pmBORIS versus control groups and  $***P < 0.001$  for group pmBORIS/pCD80/AdBORIS versus control mouse groups); **(B)** average tumor volumes of groups on day 24 when all mice within groups were alive. The differences between experimental (pmBORIS/AdBORIS, pmBORIS/pCD80/AdBORIS and pmBORIS) and control groups (AdGal and Naïve) were significant ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ); **(C)** Kaplan-Meier survival curves for different mouse groups.

this number of very aggressive, highly metastatic, and poorly immunogenic 4T1 cells [Wu et al., 2001], vaccination with mBORIS inhibited growth of tumors, and prolonged the survival of mice (Fig. 3). Also of note was the fact that vaccination of mice with pmBORIS/pCD80 was more effective than vaccination with pmBORIS alone. In fact, animals from this group generated the best protection against challenge with 4T1 cells after boosting with AdBORIS. These results were not surprising because tumors avoid destruction by the immune system in various ways, including failure to express costimulatory molecules such as CD80/CD86, which are necessary to elicit a primary T-cell response. Previously, we and others observed that expression of B7 molecular adjuvant provides important activation signals to murine T cells following DNA vaccination [Iwasaki et al., 1997; Kim et al., 1997; Tsuji et al., 1997]. Also, incorporation of the CD80 molecular adjuvant in an epithelial mucin (MUC1) vaccine formulation significantly improved protection against the challenge with 4T1 cells expressing human MUC1 molecules [Vasilevko et al., 2003].

In conclusion, this study demonstrates the feasibility of using BORIS, an epigenetically acting, cancer promoting transcription factor as a tumor antigen for vaccination. Recent data with other CT antigens suggests that they could be used as ideal targets for cancer vaccines because their expression is restricted or they are not expressed in normal tissues, while being expressed in wide range of human cancers [Simpson et al., 2005]. Importantly, it is shown that intracellular CT antigens could be processed for class I presentation in a broad range of human tumors [Vonderheide et al., 1999], and therefore could directly activate CD8<sup>+</sup> CTLs that play role in anti-tumor immune responses. In fact, our recent unpublished data supported the use of BORIS as a cancer vaccine candidate that is capable of inducing strong cellular immunity. More specifically, we demonstrated that BALB/c mice vaccinated with pmBORIS induced: (a) recall proliferation of antigen-specific CD4<sup>+</sup> cells; (b) predominant Th1 cytokine production by antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> cells; and (c) antigen-specific and MHC I-restricted direct cytotoxicity. Importantly, freshly isolated anti-BORIS CTLs from immunized mice lysed a wide variety of tumor cell lines, regardless of histological origin. Vaccina-

tion also significantly prolonged the survival and inhibited tumor growth in BALB/c mice challenged with 4T1 cells [Ghochikyan et al., 2006]. Further pre-clinical trials in different mouse cancer models may help to develop novel BORIS-adjuvant configurations with the potential to generate a potent protective and/or therapeutic anti-cancer vaccine. Today several CT antigens or peptides spanning these molecules are in clinical trials [Scanlan et al., 2002; Ikeda, 2003; Chen et al., 2004], and it is likely that a combination of such CT antigens, for example, MAGE-A1 and NY-ESO-1 will be required for development of more potent cancer immunotherapy that have thus far not proven very successful in clinical trials [Rosenberg et al., 2004]. We believe that the novel BORIS antigen that regulates expression of different CT genes, including *MAGEA1* and *NY-ESO-1* [Hong et al., 2005; Vatolin et al., 2005], could be an important and even a key component of such immunotherapeutic strategies.

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