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Expression of *BORIS* in melanoma: Lack of association with *MAGE-A1* activation

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Several genes with specific expression in germ cells show aberrant activation in different types of tumors. These genes, termed cancer-germline (CG) genes, encode tumor-specific antigens, which represent potential targets for therapeutic vaccination against cancer. The germline-specific gene *BORIS* (Brother Of the Regulator of Imprinted Sites), which encodes an 11-zinc-fingers transcriptional regulator, was recently qualified as a new CG gene, as it was found to be activated in a variety of tumor samples. Moreover, it was suggested that BORIS might be responsible for the activation of most other CG genes, including gene *MAGE-A1*, in tumors. In the present study, we evaluated the frequency of *BORIS* activation in melanoma by quantitative RT-PCR. *BORIS* activation was detected in 27% ($n = 63$) melanoma tissue samples. Surprisingly, many melanoma samples expressed *MAGE-A1* and other CG genes in the absence of *BORIS* activation, suggesting that BORIS is not an obligate factor for activation of these genes in melanoma. Consistently, forced expression of BORIS in melanoma cell lines did not induce expression of *MAGE-A1*. Our results indicate that BORIS may serve as a useful target for immunotherapy of melanoma. However, it appears that BORIS is neither necessary nor sufficient for the activation of other CG genes.

Key words: melanoma; cancer-germline genes; cancer-testis genes; BORIS/CTCFL; tumor antigens

Cancer-germline (CG) genes, also referred to as cancer-testis genes, are characterized by a unique expression pattern.[1,2] They are expressed in male germ cells and, in some cases, in female germ cells and in trophoblast, but not in adult somatic tissues. However, CG genes are activated in a number of human cancers of different histological types. Apart from this particular expression profile, CG genes share some other characteristics, including frequent existence as multigene families, frequent mapping to chromosome X and, for many of them, immunogenicity of their protein products. Indeed, the activation of several CG genes in tumors leads to the expression of antigens that can be recognized by autologous T-cells and by antibodies.[1] As germ line cells do not express the classical HLA class I molecules and therefore cannot present antigens to T cells, antigens encoded by CG genes are strictly tumor-specific. Consequently, these antigens represent promising targets for cancer immunotherapy and are currently used in therapeutic vaccination trials of cancer patients.[3]

The mechanisms underlying activation of CG genes in tumors are only partially understood. Growing evidence points to epigenetic modifications as the regulatory basis of selective expression of CG genes. DNA methylation, in particular, appears to have an essential role in the repression of these genes in normal somatic tissues.[4–6] Consistently, expression of CG genes was shown to be induced by treatment with demethylating agents, such as 5-aza-2′-deoxycytidine (5-azadC), or by direct targeting of DNA methyltransferases using antisense oligonucleotides or siRNA.[7–9] Activation of CG genes in tumor cells is associated with demethylation of their promoters, which probably occurs in the course of the genome-wide demethylation process characteristic to tumors.[4,10] The processes leading to hypomethylation of DNA sequences in tumor cells remain obscure. We undertook to address this issue by using *MAGE-A1*, the founding member of the CG group of genes,[11] as a model. Our observations suggest that hypomethylation of CG gene promoters in tumors relies on a historical event of DNA demethylation, and on the presence of appropriate transcription factors to protect their promoter against remethylation.[8,12] The factors that are responsible for the initial DNA demethylation process and for maintaining CG gene promoters unmethylated remain to be identified.

CCCTC-binding factor (CTCF) is an 11-zing-fingers protein that binds multiple DNA sequences. Target sequences are generally unmethylated, and it has been proposed that CTCF binding might be essential to maintain this unmethylated status. As such, CTCF is involved in various aspects of gene regulation, including gene imprinting and X chromosome inactivation.[13,14] A testis-specific paralog of CTCF, known as BORIS (Brother of the Regulator of Imprinted Sites), has been isolated.[15,16] The amino acid sequence of the central 11-zinc-fingers region of BORIS exhibits high homology to that of CTCF, and both proteins were shown to bind overlapping DNA sequences.[16–19] However, BORIS and CTCF contain divergent amino- and carboxy-terminal domains, suggesting distinct functions. Although the exact role of BORIS remains unclear, it has been proposed that by replacing CTCF at specific genomic sites, BORIS may modify DNA methylation patterns in male germ line cells.[20]

Interestingly, the gene encoding BORIS qualifies as a member of the CG group of genes, as it was shown to be aberrantly activated in different malignancies.[17,19] The studies, however, analyzed only limited amounts of tumor samples. Nevertheless, *BORIS* mRNA was detected in a remarkably high proportion (up to 90%) of cancer cell lines (including melanoma cell lines) and primary tumors.[17,19] Like other CG genes, *BORIS* appears to be regulated by DNA methylation, as suggested by its induction following 5-azadC treatment.[19,21]

Given the presence of BORIS in tumor cells and its possible contribution to epigenetic processes, the role of BORIS in the regulation of other CG genes was recently investigated.[17,19] One study reported derepression of the *MAGE-A1* gene and partial demethylation of its promoter upon conditional ectopic expression of BORIS in cultured normal human dermal fibroblasts.[19] Consistently, *MAGE-A1* appeared to contain a CTCF/BORIS binding site near its transcription start region. Surprisingly, binding of CTCF to this site appeared to be insensitive to the methylation status. According to the study, the activation of *MAGE-A1* in BORIS-expressing cells was associated with a switching from CTCF to BORIS occupancy at this target sequence. In addition to *MAGE-A1*, several other CG genes, including *MAGE-A2, MAGE-A4, MAGE-B1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, were activated in response to the ectopic expression of BORIS. Moreover, induction of *BORIS* by 5-azadC appeared to precede that of *MAGE-A1*, and it was suggested that BORIS is the essential mediator of 5-azadC for *MAGE-A1* derepression.

Considering the reported high frequency of *BORIS* expression in melanoma cell lines,[19] we decided to assess the frequency of

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BORIS activation in a panel of primary and metastatic melanoma samples. The expression of *BORIS* was investigated by quantitative RT-PCR. We detected *BORIS* expression in a substantial fraction of the samples. The frequency of *BORIS* activation was, however, considerably lower than that of *MAGE-A1* and other CG genes. As a corollary, many melanoma samples expressed *MAGE-A1* and other CG genes in the absence of *BORIS*, suggesting that BORIS may not be involved in CG activation in melanoma. We therefore decided to assess the role of BORIS in the induction of *MAGE-A1* in melanoma cells, by testing the effect of forced expression of BORIS in 2 melanoma cell lines. Similar experiments were also performed in a sarcoma cell line, in immortalized human keratinocytes and in normal human fibroblasts.

Material and methods

Tissue samples and cell lines

Tissue samples were collected at resection. They were frozen immediately in liquid nitrogen and stored at −80°C until RNA isolation. EB16-MEL, SK-MEL-23 and LB23-SAR cell lines were grown in Iscove's medium supplemented with 10% fetal bovine serum (FBS; HyClone-Perbio, Brackley, UK), 0.55 mM L-arginine HCl, 0.24 mM L-asparagine, 1.5 mM L-glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of 8% CO_2. Immortalized human keratinocyte cell line HaCaT[22] (received from Dr. A. Moustakas, Ludwig Institute for Cancer Research, Uppsala, Sweden), normal human foreskin fibroblasts HFF2 (received from Dr. A. Decottignies, Institute of Cellular Pathology, Brussels, Belgium), and 293T cells were maintained in DMEM containing 10% FBS and antibiotics.

5-Aza-2′-deoxycytidine treatment

Cells grown to 60–70% confluence were exposed to a single dose of 2 μM 5-aza-2′-deoxycytidine (Sigma-Aldrich, St-Louis, MO), and maintained in culture for a period of time corresponding to at least 3 population doublings (4 days for SK-MEL-23, LB23-SAR and HaCaT cell lines, 5 days for HFF2 and 6 days for EB16-MEL cell line) before RNA or DNA extraction.

Construction of pBORIS plasmid

A plasmid carrying the *BORIS* cDNA sequence was isolated from a human testis cDNA library (Clontech, Palo Alto, CA) by PCR screening on successive subgroups. The isolated clone, however, contained a truncated *BORIS* cDNA sequence, which lacked about 1 Kb of the 5′ portion, including part of the open reading frame (ORF). The missing *BORIS* sequence was amplified from cDNA of an expressing nonsmall cell lung cancer cell line (LB61-NSCLC) using proofreading PrimeSTAR™ HS DNA polymerase (Takara Bio Europe, St-Germain-en-Laye, France) with a sense primer (GAGAATTCATTATGGCAGCCACTGA) overlapping the translation start site and containing a 5′ overhang with an EcoRI site, and an antisense primer (TACGAATGTGAGCGGTCATA) located in exon 9. We purified an EcoRI-Eco47III fragment (comprising exon 1–8) from this PCR product, and an Eco47III-XbaI fragment (exon 8–11) from the truncated BORIS cDNA clone. Both fragments were coligated within EcoRI-XbaI digested pcDNA3 vector (Invitrogen, Carlsbad, CA), which carries a geneticin resistance gene for selection of transfected cells. Plasmid DNA was purified with the Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany). The sequence of BORIS was verified using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). Compared with the human *BORIS* sequence published previously,[16] the sequence in pBORIS contains a G to C substitution at position 230 (relative to the transcription start site) leading to a Q50E amino acid substitution within the N-terminal part of the protein (outside of the 11-zinc-fingers domain). This nucleotide substitution corresponds to a frequent polymorphic variant (more than 30% of the population is homozygous for this allele, see NCBI SNP reference: rs 6070128). Homozygosity for this polymorphism was also detected in a *MAGE-A1*-expressing tumor, indicating that it is not incompatible with the activation of *MAGE-A1*.

Transfections

EB16-MEL and SK-MEL-23 cells (1×10^6) were seeded into 75 cm^2 flasks and transfected at 50–60% confluence with a total DNA amount of 20 μg (including carrier DNA), using ExGen 500 (Fermentas, Hanover, MD) according to the instructions of the manufacturer. LB23-SAR cells were plated at 1×10^6 cells in 75 cm^2 flasks 1 day before transfection by the calcium phosphate precipitation method.[23] HaCaT cells were transfected in 6-well plates with LipofectAMINE 2000 Reagent (Invitrogen) according to the instructions of the manufacturer. After 48 hr of incubation, geneticin was added in culture media and transfectants were selected for 3–4 weeks. For transient transfection experiments, cells were analyzed 72 hr after transfection.

Conventional and quantitative RT-PCR

Several RNA samples of normal tissues were purchased from Clontech or Ambion (Austin, TX). For the other samples, total RNA was extracted using TriPure Isolation Reagent (Roche Diagnostics GmbH, Manheim, Germany) or the guanidinium-isothiocyanate/cesium chloride procedure.[24] Total RNA extracted from transiently tranfected cell lines was treated with DNase (Turbo DNA-free, Ambion), to eliminate contaminating plasmid DNA. Reverse transcription was performed on 2 μg of total RNA using oligo-dT primer as described previously.[25] The different transcripts were amplified by PCR from 1/40th of the reverse transcription reaction. Conditions of conventional RT-PCR have been described elsewhere.[26] Primers used for amplification of *BORIS* and *ACTINB* were the same for both conventional and real-time quantitative PCR. Quantitative RT-PCR amplifications were performed, as previously described, using the reaction mix of Eurogentec (Seraing, Belgium).[27] The PCR primers and specific 5′-FAM/3′-TAMRA labeled probes synthesized commercially (Eurogentec) are described in Supplementary Table I. Samples for quantitative PCR were assayed in duplicate. Expression levels are represented as a ratio to 2,000 *ACTINB* mRNA copies (we estimated previously that 2,000 is the mean amount of *ACTINB* mRNA molecules in 1 cell). For the analysis of transient and stable transfectants, nonreverse transcribed RNA controls were included and the values obtained were subtracted from their reverse transcribed counterparts.

Western blot

Nuclear extracts were prepared using the Nuclear/Cytosol fractionation kit (BioVision Inc., Palo Alto, CA). Protein samples were separated on a NuPAGE® 4–12% Bis-Tris gel (Invitrogen) in MOPS Running Buffer, with NuPAGE® antioxidant added to the inner chamber, according to manufacturer's instructions (XCell *Sure Lock*™). Proteins from the gel were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Little Chaffold, UK) using XCell II™ Blot Module. Membranes were incubated in a phosphate-buffered saline blocking solution containing 2.5% dry milk and 0.1% Tween-20 for 1 hr and then probed with primary antibodies in a new blocking solution containing anti-BORIS rabbit polyclonal antibody, ab18337 (1:7,500 dilution; Abcam, Cambridge, UK) or anti-p80 Ku mouse monoclonal antibody, clone GE2.9.5 (1:1,000 dilution; Upstate Bio-Scientific, Lake Placid, NY). Membranes were washed and incubated with either donkey anti-rabbit IgG-HRP sc2313 (1:10,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-mouse IgG-HRP sc2005 (1:2,000 dilution; Santa Cruz Biotechnology) secondary antibodies and revealed using the SuperSignal West-Femto or West-Pico chemiluminescent substrates (Pierce, Rockford, IL). Reactivity was observed using CL-X posure™ autoradiographic film (Pierce).

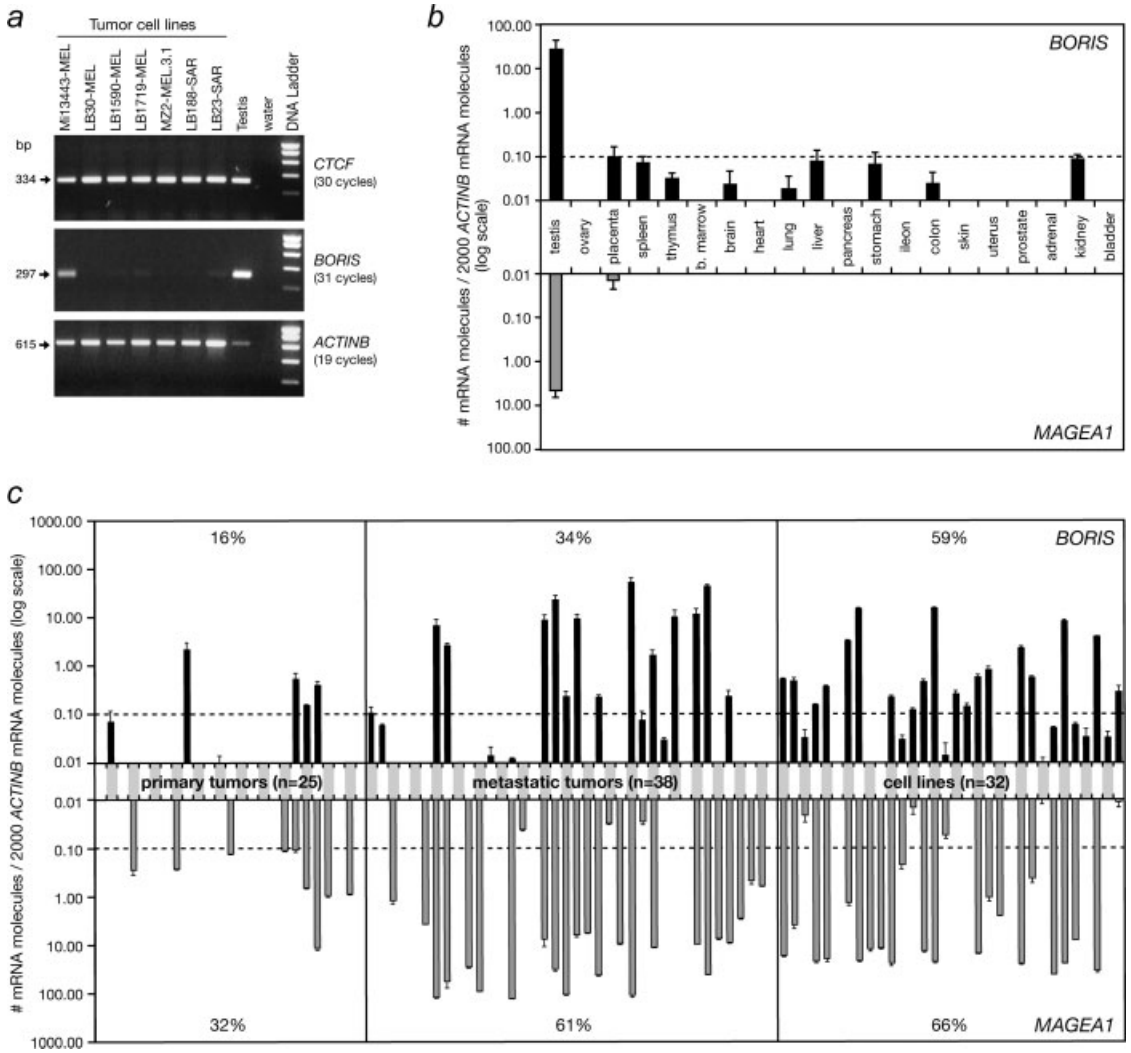

Figure 1 – Expression of *BORIS* and *MAGE-A1* in normal tissues, melanoma tissues and melanoma cell lines. (*a*) In an initial experiment, the expression of *BORIS, CTCF* and *ACTINB* (used as an internal control) was tested by conventional RT-PCR in the indicated samples, including 5 melanoma cell lines (-MEL) and 2 sarcoma cell lines (-SAR). The numbers of PCR cycles performed are indicated. (*b*) The level of *BORIS* and *MAGE-A1* expression was evaluated in a series of normal somatic tissues, by quantitative RT-PCR. Two different RNA samples were used for each tissue. A threshold level of activation was set for *BORIS* (dashed line). It corresponds to the highest level of BORIS mRNA detected among normal somatic tissues. (*c*) *BORIS* and *MAGE-A1* expression levels were also evaluated by quantitative RT-PCR in a series of primary melanoma tumors, melanoma metastases and melanoma cell lines. The percentages of samples showing activation of *BORIS* or *MAGE-A1* are indicated. Samples were scored positive for *BORIS* when the expression level of the gene was significantly higher than the threshold level defined in panel *b* (dashed line). The same threshold level was applied to the analysis of *MAGE-A1* activation. Each value represents mean ± SE. All quantitative RT-PCR data derive from 2 independent experiments.

Sodium bisulfite genomic sequencing

Sodium bisulfite genomic sequencing of the *MAGE-A1* 5′ region was performed as described previously.[12]

Retrovirus production and transduction of target cells

The retroviral vector pLN-BORIS was constructed by subcloning an Ecl136II-BamHI fragment from pBORIS, comprising the human BORIS coding sequence, into BglII and StuI sites of pLNCX2 vector containing a neomycin resistance gene (Clontech). Recombinant retroviruses were produced by transient cotransfection of 293T cells in 100-mm tissue culture dishes with 7.5 μg of each pVPack-GP, pVPack-VSV-G (both from Stratagene, La Jolla, CA) and pLNCX2 or pLN-BORIS retroviral expression vectors, using the calcium phosphate precipitation method. The medium was replaced 4 hr later, and virus-containing supernatants were harvested 48 and 72 hr posttransfection. Supernatants were centrifuged, filtered through a 0.45-μm filter (Millipore, Carrighwohill, Ireland) and directly used to transduce target cells. HFF2 fibroblasts (4×10^5 cells) were plated in 75 cm^2 flasks and 24 hr later, cell medium was removed, and 10 ml of specific and control viral supernatants were added, followed 3 hr later by the addition of 10 ml of cell-growing medium. The transduced cells were then incubated for an additional 48 hr at 37°C before selection with geneticin. Transduced cells were selected for 25 days. Western blot analyses on nuclear extracts from pLN-BORIS-transduced HFF2 cells indicated that the BORIS protein was efficiently produced and was translocated into the nucleus (supplementary Fig. S1).

Results

Analysis of BORIS expression in melanoma

A previous study using conventional RT-PCR reported aberrant derepression of the testis-specific gene *BORIS* in a vast majority

of tumor tissues and cell lines of different types.[19] The study included 10 melanoma cell lines, 9 of which scored positive for *BORIS* expression. Surprisingly, our preliminary RT-PCR experiments failed to detect a high frequency of *BORIS* expression in a series of tumor cell lines, including 5 melanoma and 2 sarcoma cell lines (Fig. 1*a*). In contrast, *CTCF* was strongly expressed in all tested samples. The discrepancy between our results and the previous study could be explained by the different number of amplification cycles performed (31 cycles instead of 40–45 cycles). High numbers of PCR cycles may, however, lead to the occurrence of false positives, resulting from the amplification of irrelevant background mRNA levels. To resolve this problem, we decided to use real-time quantitative RT-PCR to accurately determine the level of *BORIS* expression.

We first evaluated the level of expression of *BORIS* in normal tissues obtained from tumor-free individuals (Fig. 1*b*). As expected, only in testis did we detect a significant level of *BORIS* expression. Background levels of *BORIS* mRNA, not exceeding 0.3% of the level in testis, were detected in several somatic tissues. On the basis of these results, we defined a threshold level of *BORIS* activation, which corresponded to the highest level of *BORIS* mRNA in normal somatic tissues (equal to 0.1 copy of *BORIS* mRNA/2,000 copies of *ACTINB* mRNA). Tumor samples exhibiting *BORIS* mRNA levels above this threshold would be scored positive for *BORIS* activation. Using these criteria, we conducted the analysis of *BORIS* expression in tissue samples of primary and metastatic melanomas. *BORIS* was found to be activated in 16% ($n = 25$) of primary melanomas and in 34% ($n = 38$) of metastatic melanomas (Fig. 1*c*). The actual frequency of *BORIS* activation in melanoma cells may be underestimated when assessing melanoma samples, because of the heterogeneous nature of the tumor tissues. We therefore performed the analysis of *BORIS* expression in melanoma cell lines, which represent pure populations of tumor cells. Activation of *BORIS* was observed in 59% of the cell lines ($n = 32$; Fig. 1*c*). Altogether, these data indicate that, although *BORIS* is activated in a substantial fraction of melanoma samples, it does not appear to be so in the vast majority of the tumors.

MAGE-A1 and BORIS are not always coexpressed in melanoma samples

Given the proposed role of BORIS in the activation of other CG genes,[19] we decided to investigate whether CG genes were activated exclusively in melanomas expressing *BORIS*. We first assessed the expression of the *MAGE-A1* gene, because the involvement of BORIS in the regulation of this CG gene has been the most extensively studied. In normal tissues, *MAGE-A1* transcripts were found to be present in testis, and at a lower level in placenta, but were undetectable in normal somatic tissues (Fig. 1*b*). In melanoma samples, *MAGE-A1* was activated at a higher frequency than *BORIS* (p-value = 0.0192, double-sided t-test; Fig. 1*c*). As a consequence, *MAGE-A1* activation was detected in 17 melanoma tissues and 6 cell lines that scored negative for *BORIS* activation (Fig. 1*c*; Table I). We also analyzed the expression of other CG genes, namely *MAGE-A2, MAGE-A4, MAGE-B1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, in a fraction of the metastatic melanoma tissue samples. Activation of these CG genes, which have been proposed to be regulated by BORIS as well, was found in a substantial proportion of samples, including samples that were negative for *BORIS* activation (Table I). Taken together, these results indicate that sustained transcription of *MAGE-A1* and of other CG genes in melanoma does not rely on the presence of BORIS. There was, however, some level of correlation between the expression of BORIS and other CG genes in the melanoma samples (Table I). This is expected for a group of genes that share a common mechanism of activation.

TABLE I – LACK OF A STRICT ASSOCIATION BETWEEN THE EXPRESSION OF *BORIS* AND OTHER CG GENES IN MELANOMA TISSUE SAMPLES

Expression of other CG genes[1]		*BORIS* expression[1]		Fisher's exact test (p-value)
		+	−	
MAGE-A1 ($n = 63$)	+	14 (22)	**17 (27)**	0.001
	−	3 (5)	29 (46)	
MAGE-A2 ($n = 32$)	+	8 (25)	**11 (34)**	0.035
	−	1 (3)	12 (38)	
MAGE-A4 ($n = 22$)	+	6 (27)	**6 (27)**	0.012
	−	0	10 (46)	
MAGE-B1 ($n = 32$)	+	4 (13)	**1 (3)**	0.008
	−	4 (13)	23 (72)	
LAGE-2$^{NY\text{-}ESO\text{-}1}$ ($n = 32$)	+	5 (16)	**4 (13)**	0.040
	−	4 (13)	19 (59)	

Values in parantheses are in percentages.
[1]The expression status was determined by quantitative RT-PCR, as described in Figure 1. Associations between the expression of a particular CG gene and *BORIS* were assessed with Fisher's exact test (p-values). Although significant associations were found, tumor samples expressing *MAGE* or *LAGE* genes but not *BORIS* were commonly detected (bold).

Exogenous expression of BORIS in melanoma cells does not lead to CG gene activation

One could still argue that BORIS is required only for the initial event of activation of CG genes and that other transcription factors take over to maintain the genes active. According to this view, a transient phase of BORIS expression would be sufficient to induce permanent activation of CG genes. This may explain the lack of coexpression of *BORIS* and other CG genes in certain melanomas in which *BORIS* expression would have been lost at some stage of the tumor development. We decided therefore to evaluate the potential of BORIS in the activation phase of *MAGE-A1* in melanoma cells, by testing the effect of forced expression of BORIS. For the experiments, we chose EB16-MEL and SK-MEL-23 melanoma cell lines, because they were initially negative for both *BORIS* and *MAGE-A1* activation. Both genes however, were induced following 5-azadC treatment, indicating that DNA methylation is involved in their repression in these cells (Fig. 2*a*). Moreover, activation of *MAGE-A1* by this treatment indicated that both cell lines possess all required factors to support expression of this gene upon demethylation. EB16-MEL and SK-MEL-23 cells were transfected with increasing amounts of a BORIS-encoding plasmid (pBORIS) or with the empty vector and were analyzed 72 hr after transfection. Quantitative RT-PCR analyses revealed high levels of BORIS expression upon transfection with pBORIS (Fig. 2*a*). Furthermore, Western blot analyses on nuclear protein extracts of BORIS-transfected cells indicated that the protein was efficiently produced and was translocated into the nucleus (Fig. 2*b*). Exogenous expression of BORIS, however, did not induce significant increases in the expression of *MAGE-A1*, as measured by quantitative RT-PCR (Fig. 2*a*).

One possible explanation for the lack of activation of *MAGE-A1* in transfected cells is that the time of exposure of the gene to BORIS was insufficient to support its activation. Derepression of epigenetically silenced genes may indeed require longer time and more cell generations. We therefore decided to derive stable transfectants to test the effect of long-term expression of BORIS on the activation of *MAGE-A1*. EB16-MEL and SK-MEL-23 cells that had been transfected with pBORIS were selected according to their resistance to geneticin, which was conferred by the transgene vector. Pools of transfectants, representing several hundreds of clones, were used for subsequent analyses without further subcloning to avoid the possibility of selecting clones not representative of the entire transfected population. Quantitative RT-PCR analyses on EB16-MEL and SK-MEL-23 transfected populations indicated that the levels of *BORIS* expression in these cell cultures were comparable to those observed in cell lines in which the gene is constitutively expressed (compare Fig. 2*c* and Fig. 1*c*). As

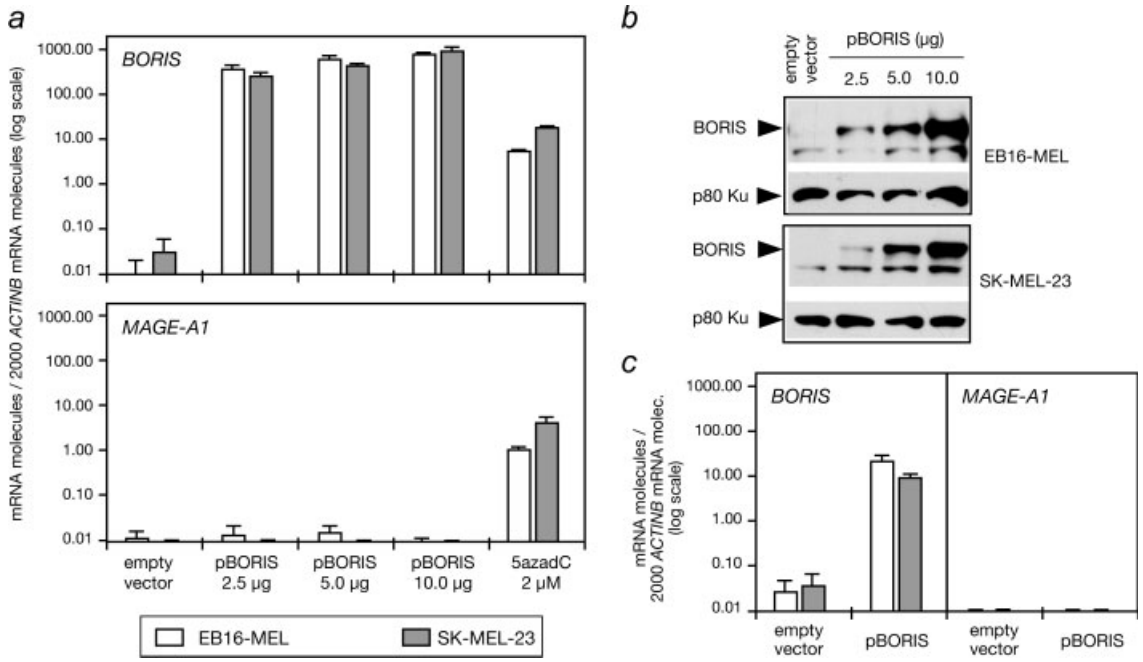

FIGURE 2 – Forced expression of BORIS does not lead to *MAGE-A1* activation in melanoma cell lines. (*a*) EB16-MEL and SK-MEL-23 melanoma cell lines were transfected with increasing amounts of the vector pBORIS, which contains the entire open reading frame of *BORIS*, or with the empty vector. *BORIS* and *MAGE-A1* expression levels were assessed by quantitative RT-PCR 72 hr after transfection. The expression level of these genes was also measured after treatment of the cells with the methylation inhibitor 5-azadC. Each value represents mean ± SE. (*b*) Western blot analyses with antibodies directed against BORIS were performed on nuclear extracts from EB16-MEL and SK-MEL-23 transfectants. Detection of p80Ku was performed to verify equal loading in the different lanes. (*c*) EB16-MEL and SK-MEL-23 cells were transfected with pBORIS or the empty vector, and selected according to their resistance to geneticin, which was conferred by the vector. Four weeks after transfection, the stable transfectants were assessed for *BORIS* and *MAGE-A1* expression levels by quantitative RT-PCR. Each value represents mean ± SE. All quantitative RT-PCR data derive from 2 independent experiments.

shown in Figure 2*c*, even 1 month after transfection, forced expression of *BORIS* in both melanoma cell lines did not result in the activation of *MAGE-A1*. We also tested the expression of other CG genes, including *MAGE-A2, MAGE-A4, MAGE-B1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, in the transfected populations, but none of them were induced in the *BORIS*-expressing cells (supplementary Fig. S2). Taken together, these data indicate that overexpression of BORIS is not sufficient to induce activation of CG genes in melanoma cells.

MAGE-A1 methylation patterns following ectopic expression of BORIS in melanoma cells

We examined whether the ectopic expression of *BORIS* in both melanoma cell lines induced methylation changes in the 5′ region of *MAGE-A1*. To this end, DNA was extracted from EB16-MEL and SK-MEL-23 cells that had been stably transfected with either pBORIS or the empty vector. DNA was also obtained from both cell lines exposed to 5-azadC. We applied the sodium bisulfite genomic sequencing method to analyze the methylation status of 17 CpG sites located between positions −81 and +169 relative to the transcription start site of *MAGE-A1*. Although this region of *MAGE-A1* showed marked demethylation following 5-azadC treatment, its overall methylation level was not significantly affected by the ectopic expression of *BORIS* (Fig. 3). In SK-MEL-23 cells, however, a cluster of 5 CpGs located within the first intron of *MAGE-A1,* between positions +129 and +169, showed a reduced level of methylation in the pBORIS-transfected group (6.7% methylated CpGs) when compared with the empty vector control group (21.1%, p-value = 0.005; χ^2 test). This observation is in accordance with the study by Vatolin *et al*., which showed partial demethylation of the same 5 CpGs in human fibroblasts that were induced to express BORIS.[19] However, demethylation of these intronic CpG sites does not appear to influence the transcriptional status of *MAGE-A1*. We showed previously that the activation of *MAGE-A1* is strongly associated with the demethylation of 6 CpGs located closer to the transcription start site, between postions −30 and +30.[4,12] This cluster of CpGs was mostly methylated (>80%) in both *MAGE-A1*-negative melanoma cell lines, and remained so even after long-term expression of *BORIS* (Fig. 3). In contrast, these CpGs were significantly demethylated in the 5-azadC-treated cells (<45% methylated CpGs), where *MAGE-A1* is activated. Together, these data indicate that the CpGs that impact on the transcription of *MAGE-A1* remain methylated in melanoma cells that were induced to express *BORIS*.

Lack of BORIS-induced activation of MAGE-A1 in other cell types

To extend the transfection experiments to other cell types, we decided to assess the effect of BORIS overexpression in a human sarcoma cell line (LB23-SAR), in immortalized human keratinocytes (HaCaT) and in normal human fibroblasts (HFF2). None of these cells expressed *BORIS* nor *MAGE-A1* above background levels before treatment (Fig. 4). *MAGE-A1* was induced by 5-azadC in all 3 cell cultures. In contrast, induction of *BORIS* was very weak in 5-azadC-treated HaCaT and HFF2 cells (Fig. 4). This observation is inconsistent with the model proposed by Vatolin *et al*., in which CG gene induction by 5-azadC relies on prior activation of *BORIS*.[19] LB23-SAR and HaCaT cells were transfected with pBORIS, whereas HFF2 fibroblasts were transduced with a BORIS-expressing retroviral vector (pLN-BORIS). Cells were thereafter selected for their resistance to geneticin, which was conferred by the vectors. Quantitative RT-PCRs, which were performed 3–4 weeks after transfection, indicated that all 3 selected populations expressed *BORIS*. However, forced *BORIS* expression in these cells did not result in the activation of *MAGE-A1* (Fig. 4). These data indicate that the lack of induction of

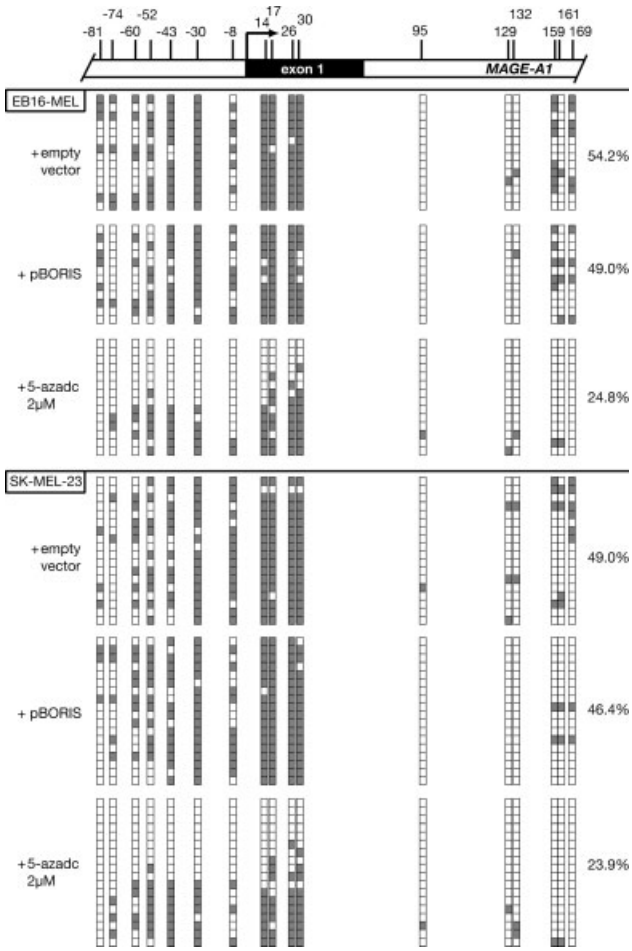

FIGURE 3 – Methylation status of the 5′ region of *MAGE-A1* in melanoma cells transfected with pBORIS. DNA was extracted from EB16-MEL and SK-MEL-23 cells either 4 weeks after transfection with pBORIS or the empty vector, or 72 hr after exposure to 5-azadC. DNA samples were treated with sodium bisulfite, and the 5′ region of *MAGE-A1* was amplified by PCR. The amplified region of *MAGE-A1* is represented at the top with vertical bars at the location of CpG dinucleotides, a broken arrow at the transcription start site and a black box corresponding to the first exon. PCR products were cloned, and several clones were sequenced. The deduced CpG methylation patterns are represented by the squares: filled squares, methylated cytosines; empty squares, unmethylated cytosines. Each line derives from the sequence of one clone and gives the methylation patterns of a single DNA molecule. The overall level of methylation within the region is given (percent methylated CpGs).

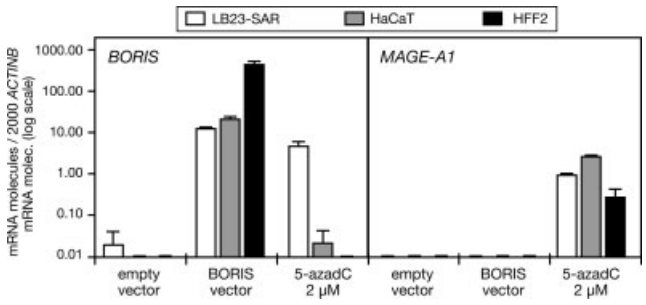

FIGURE 4 – Lack of induction of *MAGE-A1* by BORIS in nonmelanoma cell types. LB23-SAR sarcoma cells, HaCaT immortalized human keratinocytes and HFF2 normal human fibroblasts were transfected or transduced with a BORIS-expressing vector or with the corresponding empty vector, and selected according to their resistance to geneticin, which was conferred by the vectors. Three to four weeks after transfection or transduction, the selected cell populations were assessed for *BORIS* and *MAGE-A1* expression levels by quantitative RT-PCR. The level of expression of these genes was also assessed in LB23-SAR, HaCaT or HFF2 cells that had been exposed to 5-azadC. Each value represents mean ± SE. All quantitative RT-PCR data derive from 2 independent experiments.

MAGE-A1 following forced expression of BORIS is not restricted to melanoma cells.

Discussion

Tumor-specific antigens encoded by CG genes represent attractive targets for cancer immunotherapy. The germline-specific gene *BORIS* was recently defined as a new CG gene, as it was found to be activated in different malignancies. Because our group is currently focusing on antitumor vaccinations in melanoma patients, we decided to assess the frequency of activation of *BORIS* in melanoma. We found activation of *BORIS* in a substantial fraction of melanoma tissue samples (27%). The level of *BORIS* mRNA in several melanoma samples was similar to that in testis, where the gene is naturally expressed, and where the protein could be readily detected.[16,21] This level of expression should be sufficient to allow efficient recognition of BORIS-derived antigenic peptides by T lymphocytes. BORIS peptide sequences that can be processed properly to be presented at the cell surface by HLA class I molecules, remain to be identified.

The frequency of *BORIS* activation appeared to be higher in metastatic melanoma samples when compared with primary lesions. This tendency has already been observed for other CG genes.[1] This may reflect a positive role of *BORIS* and other CG genes in the metastatic process, and therefore selection for expression of these genes during progression of the disease. However, to date there is no clear evidence that CG genes play such a role. Another explanation for the higher frequency of CG gene expression in metastases may relate to the process of activation of these genes. Thus, the DNA demethylation process, which underlies the activation of CG genes and probably of *BORIS* as well, may coincide with one of the numerous steps associated with tumor progression. In this case, the chance that this demethylation process occurred would be higher in tumors that are at later stages of progression. Of note, the increased frequency of CG gene expression in advanced tumors supports the notion that CG gene expression in tumors is due to an activation process associated with malignant development, rather than the expansion of precursor cancer stem cells, in which these genes were constitutively unmethylated and active.

It was previously reported that the frequency of *BORIS* activation in tumors is generally higher than that of other CG genes.[19] While this appears to be true in certain types of tumors, such as uterine cancers,[28] our study indicates that the frequency of activation of *BORIS* in melanoma is significantly lower than that of *MAGE-A1*. Another recent study also reported limited activation of *BORIS* in prostate and bladder carcinoma.[21] Contradictory results about the frequency of *BORIS* activation in tumors may be due to leaky transcription of the gene in several tissues, which may lead to the occurrence of false positives when analyzing tumor samples with a sensitive but not quantitative RT-PCR assay.

While numerous studies confirmed that DNA demethylation is an essential step toward CG gene activation in tumors, the mechanisms underlying this demethylation process remain unknown. The recent proposal that BORIS could be involved in this epigenetic process, relies on the observation that CG genes became demethylated and activated in normal human fibroblasts that were forced to express BORIS.[17,19] This effect was attributed to the ability of BORIS to bind nearby the promoter of CG genes, and to

antagonize the transcriptional silencing imposed by CTCF, when bound to the same DNA sites. Demethylation and activation of CG genes in tumors was therefore proposed to result from the aberrant activation of BORIS, and the consequent CTCF to BORIS switch in occupancy of regulatory regions. As such, induction of *BORIS* expression in tumors appeared as a novel interesting strategy to increase antitumor immunity *via* activation of numerous antigen-encoding CG genes.[29,30]

Several results in our study indicate that BORIS is not the primary regulator of CG gene expression. First, our detailed expression analysis in a large panel of melanoma tissues revealed that a substantial proportion of tumors express *MAGE-A1* and other CG genes in the absence of *BORIS* activation. Therefore, BORIS does not appear to be necessary to maintain expression of CG genes in melanoma. Second, forced expression of BORIS in 2 melanoma cell lines by either transient or stable transfection of a BORIS-encoding vector did not result in the activation of *MAGE-A1* or other CG genes. Both cell lines evidently possessed all required factors to support expression of CG genes upon demethylation, since these genes were efficiently induced by 5-azadC treatment. Therefore, the lack of CG gene induction by BORIS appears to be due to its inability to direct DNA demethylation within CG gene promoters. We conclude that BORIS is not sufficient to induce CG gene expression in melanoma cells and that other, yet unidentified, factors are involved in this process. These conclusions do not apply only to melanoma cells, since forced expression of BORIS failed to induce *MAGE-A1* and other CG genes (data not shown) in other cell types as well. Our data, which show lack of induction of CG genes in *BORIS*-expressing human fibroblasts, differ from those previously reported.[19] The reason for this discrepancy is unclear. Altogether, our results indicate that overexpression of BORIS may not serve as a general means to induce CG genes in tumors.

Besides its putative role in the activation of CG genes in tumors, BORIS was also proposed to induce expression of CG genes in normal male germ cells. However, the timing of expression of BORIS and of the other CG genes during germ cell development does not fit with this suggestion. Thus, whereas BORIS was detected exclusively in the nucleus of spermatocytes in adult human testis,[16,21] most CG genes, including *MAGE-A1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, are already expressed in fetal gonocytes and become silenced at the spermatogonia to spermatocyte transition stage.[31–33] Therefore, the appearance of BORIS in human testicular germ cells coincides with repression rather than activation of CG genes. This implies that factors other than BORIS are involved in the activation of CG genes during early male germ cell development. These factors, as well as those underlying CG gene derepression in tumors, still need to be identified.

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