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Selective apoptosis of breast cancer cells by siRNA targeting of BORIS

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ABSTRACT

Brother of the regulator of imprinted sites (BORIS) is an epigenetically acting transcription factor which represses the tumor inhibitor functions of the tumor suppressor protein CTCF. *BORIS* expression has not been documented in adult females, making it an exciting molecular target for drug development in breast cancer. Previously, we demonstrated that vaccination of mice with zing-finger (ZF)-deleted non-functional BORIS results in regression of breast cancer and generation of potent anti-tumor immune responses. RNAi induction can be used as an alternative approach for selective tumor cell killing. Short interfering RNA (siRNA) molecules targeting *BORIS* were generated and their efficacy was tested in MDA-MB-231 breast cancer and non-malignant epithelial cell lines. Treatment with *BORIS*-specific siRNA, but not control siRNA led to a concentration-dependent reduction in *BORIS* expression and proportional apoptotic death of the cancer but not control cells. To our knowledge this is first report demonstrating a critical role of *BORIS* in maintaining tumor cell viability.

Selective targeting of tumors has previously been attempted with immunological, metabolic, and molecular biology approaches, although to date no widely applicable therapeutic solution exists. The identification of proteins selectively expressed by tumors, which are essential for the tumor phenotype, would theoretically offer novel directions in oncology drug development. Especially attractive are targets that function upstream of several oncogenes, so that inhibition of one protein would result in suppression of oncogenic cascades. It appears that brother of the regulator of imprinted sites (BORIS) may meet the above-mentioned criteria. BORIS is an 11-zing-finger (ZF) protein which is specifically expressed in neoplastically-transformed tissue (with exception of testis), both in cell lines and primary patient samples [1–4]. New data suggest a positive correlation between extent of *BORIS* expression and aggressiveness of the tumor phenotype in uterine cancers [5]. Additionally, it was shown that 70% of primary breast cancer cells and 100% of breast cancer cell lines express BORIS protein [14]. The physiological function of BORIS is believed to be associated with erasure of methylation patterns during the process of spermatogenesis and hence the only expression of this gene in normal tissues is in the testis [1]. In the context of neoplasia BORIS is believed to function as an epigenetic-acting oncogene via its ability to induce derepression of other oncogenes through inhibiting activity of the CCCTC-binding factor (CTCF).

CTCF was originally identified for its ability to suppress expression of the oncogene c-myc [6] in experiments which showed this protein bound to the c-myc transcription start site and deletion of this sequence was associated with upregulation of c-myc transcription. Other experiments have subsequently demonstrated that CTCF plays a role in transcriptional regulation of numerous oncogenes, tumor suppressor genes including p27, p21, p53, p19 (ARF), and telomerase [7,8]. The importance of CTCF as a tumor suppressor gene has been demonstrated by studies showing that its mutation/deletion occurs in tumors particularly the hot spot on 16q21 [9].

We have previously demonstrated the feasibility of treating cancer by inducing immune responses to BORIS in mouse model of breast cancer. In order to develop a therapeutic vaccination strategy, the 11 ZF domain of the wild-type murine BORIS protein was deleted by molecular biology techniques. The optimized construct containing modified (mBORIS) gene was used to immunize BALB/c mice utilizing biolistic delivery together with IL-12 and IL-18 expressing plasmids. Immunized mice generated CD4

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proliferative and Th1 responses against recombinant BORIS protein *in vitro*, as well as cytotoxic T cell responses which were capable of lysing BORIS expressing tumor cell lines of different histological origins in an MHC I-dependent manner [10]. It is important to note that the cytotoxic lymphocytes generated induced a high percentage of tumor cell apoptosis without *ex vivo* activation or expansion (direct CTL assay). Immunization of mice with the mBORIS antigen in the presence of IL-12 and IL-18 was capable of reducing tumor volume and significantly prolonging the survival of mice in the highly aggressive 4T1 mammary adenocarcinoma model using the Th2-prone BALB/c strain [10–12].

Here we demonstrated for the first time that the direct silencing of the BORIS gene by short interfering RNA (siRNA) induces apoptosis of MDA-MB-231 breast cancer cells, but not control epithelial cells, suggesting a critical role of BORIS in malignancy.

Materials and methods

Cell cultures and reagents. The MDA-MB-231 (human mammary gland ductal carcinoma) and ARPE-19 (human retinal pigment epithelium), cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cell cultures were maintained at 37 °C, with 5% CO_2 under fully humidified conditions in DMEM (Cellgro-Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin (Cellgro-Mediatech).

siRNA transfection. The cDNA sequence for human BORIS (GenBank Accession No. DJ033531, AF336042) was used to develop siRNAs targeting BORIS and not CTCF (GenBank Accession No. NM006565), confirmed by manual alignment (Fig. 1) and BLAST analysis. Double-stranded siRNAs targeting human BORIS were purchased through Ambion (Austin, TX). The OCM-8054 siRNA sequence is 5′-GGAAAUACCACGAUGCAAATT-3′. As controls, non-targeting scrambled siRNA (cat#4611G) and siRNA-targeting GAPDH (cat#AM4624) were purchased (Ambion). Transfections with siRNA concentrations ranging from 1.7 to 50 nM were performed using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions.

Western blot. Cultures of human mammary gland ductal carcinoma cells (MDA-MB-231), or human retinal pigment epithelial (ARPE-19) cells were directly lysed in SDS sample buffer and subjected to electrophoresis on 10% SDS gels. Separated proteins were subsequently transferred onto BioTrace NT nitrocellulose blotting membranes (PALL, Pensacola, FL), and target proteins were detected by incubating the membrane with the following antibodies: Anti-BORIS (600-401-907; Rockland Immunochemicals, Gilbertsville, PA) and Peroxidase Conjugated secondary antibody (611-703-127; Rockland Immunochemicals). An antibody to β-actin (sc-47778; Santa Cruz Technology, Santa Cruz, CA) was also used to verify that equal amounts of protein were loaded into each lane. A Pierce enhanced chemiluminescence detection kit was purchased from Thermo Scientific, Rockford, IL and the membrane was exposed to Kodak X-Omat LS film.

Cell viability assay. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) was used to determine cytotoxicity effects of siRNA transfection per the manufacturer's protocol. Absorbance at 490 nm was determined on a Molecular Devices (Sunnyvale, CA) SpectraMax M5^e. All transfections and conditions were conducted in triplicate and repeated at least twice. All values were normalized to the viability of scramble siRNA transfected MDA-MB-231 cancer cells which were similar to untransfected controls.

Caspase 3/7 assay. Caspase 3/7 activation in transfected cells was measured using the Caspase-Glo 3/7 Assay (Promega) as recommended by manufacturer at the either 24- or 48-h post-transfection. As a positive control, 20 μM Hydrogen Peroxide (Sigma–Aldrich) was added for 24 h. Luminometric data, in a 96-well format, were collected using a Molecular Devices SpectraMax M5^e (Sunnyvale, CA). All transfections and conditions were conducted in triplicate and repeated at least twice. All values were normalized to the viability of scramble siRNA transfected MDA-MB-231 cancer cells which were similar to untransfected controls.

Statistical analysis. The error bars in histograms represent means ± standard deviation (SD) and significance was calculated using ANOVA software.

Results and discussion

BORIS expression in MDA-MB-231 breast cancer cells

BORIS expression has previously been identified in numerous cell lines and primary cancers of diverse histological origin [13,14]. Here we first decided to analyze expression of BORIS in the MDA-MB-231 breast cancer cell line, an established *in vitro* system for assessment of novel cancer therapeutic approaches, especially in the area of RNAi [15,16]. Using the Western blot technique we observed the full length 75 kDa BORIS protein in the lysates of MDA-MB-231, but not control human retinal pigment epithelium (ARPE-19) cells or human mammary epithelial (HMEC) cells (Fig. 2A and data not shown). Expression appeared consistent and was present at various time points during passage of the cells. Furthermore, different batches of MDA-MB-231 also demonstrated a consistent level of expression (data not shown).

OCM-8054 siRNA reduces BORIS expression

To determine whether RNAi induction may specifically be useful for inhibition of BORIS protein, we have generated numerous oligonucleotides targeting various regions of the BORIS molecule and delivered these molecules to the MDA-MB-231 cells as described in [15,16]. Of 21 siRNAs screened, one (OCM-8054) was the most effective in inhibiting expression of BORIS protein as assessed by Western blot (Fig. 2B) and densitometry analysis (Fig. 1C). Importantly, this inhibition was specific since it was not observed with scrambled siRNA sequences or with control GAPDH sequences.

OCM-8054 siRNA selectively reduces breast cancer cell viability

Next we analyzed the dependency of viability of MDA-MB-231 breast cancer cell viability on *BORIS* expression by silencing *BORIS* with OCM-8054 siRNA. At the 24-h time point there was no significant reduction of viability in MDA-MB-231 or control cell lines. Only the positive control, hydrogen peroxide, induced a 66% decrease in cell viability at this time point (data not shown). However, at 48 h, we detected significant concentration-dependent decrease in viability of cancer, but not control ARPE-19 (Fig. 3) or HMEC (data not shown) cell lines. Breast cancer cells transfected with 50 nM, 25 nM, 5 nM, and 1.7 nM OCM-8054 siRNA showed 86%, 61%, 43%, and 15% reduction in viability, respectively. Neither control scrambled siRNA transfected nor untransfected cells, demonstrated any change in viability over the two day time course. Of note, the viability of both non-malignant control cell lines (ARPE-19 and HMEC) that are not expressing BORIS were not affected by transfection with any concentration of OCM-8054 siRNA (Fig. 3 and data not shown), excluding a non-specific cellular killing by interfering with ubiquitous cellular processes. These data demonstrated that expression of BORIS is required for viability of the breast cancer, but not non-malignant cells assessed.

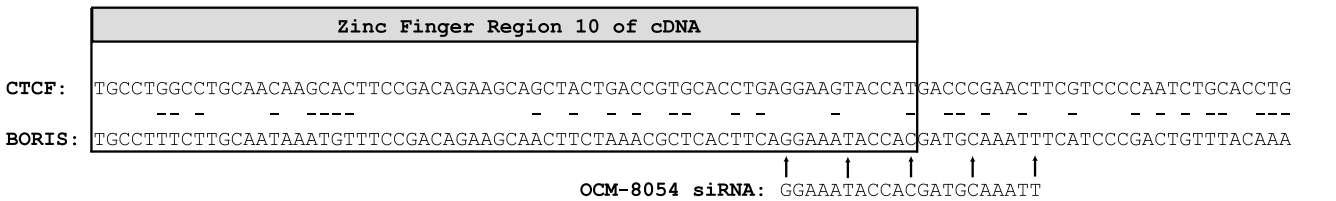

Fig. 1. Diagram of OCM-8054 siRNA targeting to BORIS zinc-finger region 10. OCM-8054 siRNA shares complete homology to the BORIS cDNA sequence but not to CTCF.