

MAGE-A, mMage-b, and MAGE-C Proteins Form Complexes with KAP1 and Suppress p53-Dependent Apoptosis in MAGE-Positive Cell Lines

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Abstract

The MAGE-A, MAGE-B, and MAGE-C protein families comprise the class-I MAGE/cancer testes antigens, a group of highly homologous proteins whose expression is suppressed in all normal tissues except developing sperm. Aberrant expression of class I MAGE proteins occurs in melanomas and many other malignancies, and MAGE proteins have long been recognized as tumor-specific targets; however, their functions have largely been unknown. Here, we show that suppression of class I MAGE proteins induces apoptosis in the Hs-294T, A375, and S91 MAGE-positive melanoma cell lines and that members of all three families of MAGE class I proteins form complexes with KAP1, a scaffolding protein that is known as a corepressor of p53 expression and function. In addition to inducing apoptosis, MAGE suppression decreases KAP1 complexing with p53, increases immunoreactive and acetylated p53, and activates a p53 responsive reporter gene. Suppression of class I MAGE proteins also induces apoptosis in MAGE-A-positive, p53^{wt/wt} parental HCT 116 colon cancer cells but not in a MAGE-A-positive HCT 116 p53^{-/-} variant, indicating that MAGE suppression of apoptosis requires p53. Finally, treatment with MAGE-specific small interfering RNA suppresses S91 melanoma growth *in vivo*, in syngenic DBA2 mice. Thus, class I MAGE protein expression may suppress apoptosis by suppressing p53 and may actively contribute to the development of malignancies and by promoting tumor survival. Because the expression of class I MAGE proteins is limited in normal tissues, inhibition of MAGE antigen expression or function represents a novel and specific treatment for melanoma and diverse malignancies. [Cancer Res 2007;67(20):9954–62]

Introduction

The cancer testes antigens are a group of proteins originally defined by their normal expression in testes and their aberrant expression in melanomas and other cancers. The first cancer testes

antigens discovered were members of the MAGE family of proteins, including the MAGE-A, MAGE-B, and MAGE-C subfamilies, which are encoded on the X-chromosome and which are now called class I MAGE antigens (1, 2). Because many class I MAGE genes are highly homologous and are coregulated in gametogenesis and in tumors, it has been suggested that many MAGE proteins have similar or complementary functions (3). Due to these factors and the difficulty in obtaining antibodies that differentiate between nearly identical subfamily members, most studies of MAGE gene expression rely on the use of antibodies recognizing common determinants or on the detection of mRNA, usually by reverse transcription followed by the PCR (RT-PCR; refs. 3, 4). MAGE gene expression can be caused by promoter region demethylation and is widespread in malignancies, being found in 50% or more of melanomas, synovial sarcomas, and primary carcinomas of the lung, head and neck, urinary bladder, and ovaries, as well as lesser percentages of primary breast carcinomas and myelomas (5–8). Despite their widespread expression, the functions of most class I MAGE molecules have not been determined, and it is not known whether their expression is a functionally irrelevant by-product of cellular transformation or could actually contribute to the development of malignancies (2).

KAP1, also known as TRIM28, Tif1 β , or Krip1, is an ~106 kDa protein with a RING-B-box coiled-coil (RBCC) domain near its amino-terminal end (9–11). Complete loss of KAP1 function in the homozygous KAP1 knockout mouse is lethal *in utero* in the presence of functional p53, and KAP1 is increasingly being recognized as a central molecule in gene regulation (12). Binding to the RBCC motif of KAP1 is required for function of all KRAB domain containing zinc finger transcription factors (13, 14). KAP1 seems to function as a molecular scaffold that coordinates at least four activities necessary for gene-specific silencing, including (a) targeting of specific promoters through the KRAB protein zinc finger motifs; (b) promotion of histone deacetylation via the NuRD/histone deacetylase complex; (c) histone 3-K9 methylation via SETDB1; and (d) recruitment of HP1 protein (14). Of particular interest in tumor biology is the fact that KAP1 acts as a corepressor of p53 by binding to MDM2, thereby suppressing p53 expression, p53 acetylation, and p53 function (15).

We recently reported that multiple MAGE proteins promote the viability of malignant mast cell lines, mostly by suppressing apoptosis, and other workers have shown that one MAGE molecule, MAGE-A2, binds to p53 (16, 17). We now extend our studies by showing that MAGE proteins suppress apoptosis in melanoma and colon cancer cell lines *in vitro* and suppress the

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-07-1478

growth of syngenic melanomas in DBA mice *in vivo*. Our studies support a novel mechanism in which MAGE proteins can act as corepressors of p53 by binding to KAP1 and enhancing its suppression of p53. These results suggest that MAGE proteins may contribute to the development of malignancies by providing a survival advantage and that interfering with MAGE expression or function may prove to be a novel avenue for therapeutic intervention in a wide variety of other malignancies.

Materials and Methods

Cell lines. The Hs-294T, A375, and S91 melanoma cell lines were purchased from American Type Culture Collection. p53RE-*bla* HCT-116 cell was purchased from Invitrogen, and HCT 116 p53^{wt/wt} or p53^{-/-} cell lines were kindly provided by Bert Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD). The HMC1 malignant mast cell line was kindly provided by Dr. J.H. Butterfield (The Mayo Clinic, Rochester, MN; ref. 18).

Small interfering RNA. All small interfering (siRNA) preparations were siStable or siSTABLE-Plus (conjugated to cholesterol at the 5' end of the sense strand) siRNAs purchased from Dharmacon, Inc. In some *in vivo* studies, we used polyethylenimine-complexed siRNA (JET-PEI, PolyPlus Transfection, Illkirch, France). The specific targets for each individual siRNAs were described by Yang et al. (17).

Antibodies. For human target validation studies and immunoprecipitation, we used pan-MAGE-A monoclonal (Zymed Laboratories, Inc.), anti-MAGE-A1 monoclonal (Santa Cruz Biotechnology), anti-MAGE-B2 polyclonal (Santa Cruz Biotechnology) or anti-human MAGE-C2 (monoclonal, Ludwig Institute for Cancer Research, New York, NY), anti-human KAP1 (polyclonal, Novus Biologicals, recognizing the NH₂-terminal region 1–50 amino acids of KAP1), anti-human KAP1 (polyclonal, supplied by Dr. Frank J. Rauscher III, recognizing the COOH-terminal PHD-bromo domains of KAP1), anti-human p53 (Biosource), and anti-human lamin B1 (Santa Cruz Biotechnology) antibodies. Anti-FLAG (monoclonal, Sigma) and anti-V5 (Invitrogen) antibodies were used to detect expression of recombinant proteins. Nonspecific mouse or rabbit IgG (monoclonal, Sigma) was used as a control for immunoprecipitation. Antibodies specific for mouse MAGE proteins expressed by the cells in these experiments were not available.

Expression vectors. V5-tagged mMage-b was expressed using pCDNA3.1/V5-TOPO vector from Invitrogen (19). V5-tagged MAGE-A3 was expressed using the T-Rex viral power lentivirus system (Invitrogen). FLAG-tagged MAGE-C2 was expressed using pFLAG-CMV-2 (Sigma). FLAG-tagged KAP1 cDNAs with various deletions were made in the Rauscher laboratory and expressed using pCDNA3.1.

siRNA transfection and cell viability. *In vitro* studies used Lipofectamine 2000 (Invitrogen) as a transfection reagent and all transfections were done under a RNase-free condition. The final siRNA concentrations were 50, 100, or 150 nmol/L. Cell viability was determined 72 h after transfection by counting cells that excluded trypan blue. In selected experiments, cell viability was also determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; Sigma], which showed similar results to the trypan blue assay (data not shown).

Target validation and specificity. Confirmation of cleavage of mRNA induced by siRNA was previously documented (17). Target protein suppression was validated by immunoblotting 48 h posttransfection (Fig. 1D).

Apoptosis assays. Apoptosis was determined by morphologic analysis after staining with acridine orange and ethidium bromide (17), or by APO-BrdU terminal deoxyribonucleotide transferase-mediated nick-end labeling assay (TUNEL; Molecular Probes). Both assays were done approximately one-half doubling time after transfection with a final siRNA concentration of 100 nmol/L.

Identification of MAGE binding partners. The human MAGE-C2 (NM_016249, previously called MAGE-E1) was used as a bait protein, and the Clontech YEASTMAKER kit was used to construct a cDNA library from mRNA of the HMC1.1 malignant mast cell line. Independent transformants

(1.2×10^6) were obtained, yielding 86 colonies, of which 51 showed β -galactosidase activity and were sequenced. KAP1 (Trim 28) was identified as a potential partner of MAGE-C2. The endogenous binding between KAP1 and MAGE proteins was confirmed in human melanoma cells by immunoprecipitation with anti-KAP1 or anti-MAGE antibody followed by immunoblotting with anti-MAGE or anti-KAP1 antibodies. Because there is neither mouse mMage-b nor individual human MAGE-A3 antibody available, we expressed V5-tagged mMage-b from an expression plasmid and induced V5-tagged MAGE-A3 expression by transducing lentivirus into mammalian cells. Cell lysates were then immunoprecipitated with anti-KAP1 antibody, and MAGE proteins were detected with anti-V5 antibody.

To identify the KAP1 binding domain for MAGE-C2 and mMage-b, COS-7 cells were cotransfected with MAGE-C2 or V5-mMage-b expression plasmids and with plasmids expressing full- or partial-length KAP1. Lysates were immunoprecipitated with anti-MAGE-C2 or V5 antibody and blotted with antibodies recognizing either the NH₂- or the COOH-terminal region of KAP1.

p53 and acetylated p53 cytotblots. To determine the amount of acetylated p53 and total p53 levels in cells treated with siRNA, we used the cytotblot technique (20). Cells are plated at 20,000 per well using a uFill reagent dispenser (Biotek Instruments, Inc.), allowed to attach overnight, and treated with siRNA. Following treatment, the cells are fixed by addition of 100 μ L of 3.7% formaldehyde using a Biomek FX liquid handler (Beckman Coulter, Inc.). The plates are incubated for 20 min at 4°C and cells are washed five times in 100 μ L of PBS (pH 7.4) containing 0.1% Triton X-100 using a Biomek FX liquid handler to permeabilize cell membranes. Following permeabilization, cells are incubated in 100 μ L/well of Odyssey Blocking Buffer (Licor, Inc.) for 1 h at room temperature with gentle rotation. Each well is incubated with 100 μ L per well anti-p53 antibodies (Cell Signaling Technologies) at a 1:1,000 dilution in Odyssey Blocking Buffer for 1 h, followed by incubation with anti-p53 or anti-acetylated p53 antibodies (Cell Signaling Technologies) at a 1:1,000 dilution in Odyssey Blocking Buffer for 1 h. Secondary antibody incubations are carried out simultaneously by addition of 100 μ L per well containing 1:5,000 dilution of 680 nmol/L dye conjugated anti-mouse secondary antibody (Licor) and 1:5,000 dilution of 800 nmol/L dye conjugated anti-rabbit secondary antibody (Licor) for 1 h. Following incubation, plates are washed six times using a Biotek Instruments microplate washer. All liquid is removed and plates are imaged on a Licor Odyssey microplate imager at the 700 and 800 nm channels. All raw data are quantified using the Licor In-Cell Western Analysis Software (Licor). To normalize the raw antibody values to cell number, the fixed cells are incubated with Sytox Green dye (Invitrogen) at a dilution of 1:10,000 in PBS for 15 min at 37°C. Sytox green is quantified by reading the plate on a Safire II plate reader (Tecan, Inc.) for green fluorescence (excitation 485 nm/emission 535 nm). All raw antibody data are normalized to the Sytox green signal. Fold differences are calculated by dividing control cells that were treated with solvent only by the normalized antibody data.

p53 activation assay. To determine if MAGE gene knockdown affects the activity of p53, we used GeneBLazer (Invitrogen) cell signaling pathway-specific CellSensor cell line, HCT-116 p53-*bla*, containing the GeneBLazer β -lactamase (*bla*) Reporter Technology. When the p53 pathway is activated or inhibited, β -lactamase reporter activity is modulated and can be measured quantitatively and selectively with the LiveBLazer-FRET B/G Loading Substrate (Invitrogen). Cells (12,000 per well) are plated in each well of the 384-well microplates using a uFill Reagent Dispenser (BioTek Instruments, Inc.). Cells are treated with siRNA and loaded with 8 μ L FRET B/G Loading Substrate (Invitrogen) engineered fluorescent substrate containing two fluorophores, coumarin and fluorescein. In the absence of *bla* expression, the substrate molecule remains intact. In this state, excitation of the coumarin results in fluorescence resonance energy transfer to the fluorescein moiety and emission of green light. However, when *bla* is expressed, the substrate is cleaved, separating the fluorophores and disrupting energy transfer. Excitation of the coumarin in the presence of enzyme *bla* activity results in a blue fluorescence signal. The resulting coumarin-to-fluorescein ratio provides a normalized reporter response which can minimize experimental noise that can mask the

underlying biological response of interest. β -Lactamase assays were read in a Safire II microplate reader (Tecan) at an excitation of 409 nm and emissions of 460 and 535 nm.

In vivo studies. S91 murine melanoma cells were injected s.c. into the flanks of syngenic DBA/2 mice. In one protocol, cells were transfected with 100 nmol/L siSTABLE-PLUS mMAGE-b siRNA or 100 nmol/L control siSTABLE-PLUS siRNA in Lipofectamine 2000 before inoculation. After 8 h, equal numbers of viable cells were injected s.c. into the flanks of DBA/2 mice and tumor growth was followed as described (17). In additional protocols, untreated S91 cells were injected s.c. on day 0 and then the mice were treated with multiple intratumoral or i.p. injections of stabilized siRNA complexed with polyethylenimine or conjugated to cholesterol.

Tumor growth was followed as previously described (17). Briefly, mice were palpated for tumors by two blinded independent investigators beginning on day 5. When tumors reached measurable size, a digital vernier caliper was used to take two measurements at 90° to each other and the square root of the product was calculated to give an estimate of the mean tumor diameter, and each mouse was followed either until tumor reached the target diameter or for 45 days, when animals were sacrificed as required by protocol. All procedures were approved by the institutional review board and done in accordance with the guidelines of the Animal Care Committee.

Statistical analysis. Student's *t* test was used for cell viability. The data show mean \pm SD from triplicates of each experiment, and each experiment was done at least thrice independently, except as indicated for some specificity studies that were done twice. For the *in vivo* experiments, the time for a tumor to reach the target mean tumor diameter of 13 mm was

defined as the elapsed time from the date of cell implantation to the date when a 13-mm target diameter was reached, or when the mouse was sacrificed, which is considered censored. Kaplan-Meier survival analysis with the corresponding log-rank analysis was done using S-plus Software (Insightful). Linear regression analysis was used to measure the rate of mean tumor diameter growth as a function of time using S-plus Software (Insightful).

Results

Suppression of MAGE genes inhibits tumor cell viability.

Working with human melanoma cell lines that express MAGE-A and MAGE-C2 proteins, and with a murine melanoma cell line that expresses mMAGE-b, we found that transfection with siRNAs targeting several MAGE genes decreased cell viability compared with the same cells transfected with control siRNA (Fig. 1). Because of the high degree of homology of many MAGE family members and the lack of antibodies recognizing specific MAGE family members, we used siRNAs that target whole subfamilies as well as siRNAs that target individual MAGE genes. Figure 1A shows significant growth inhibition of human melanoma cells by siRNAs targeting common regions of the human MAGE-A gene family or targeting the human MAGE-C2 gene, but no significant effect with siRNA targeting the MAGE-B family members, which are not significantly expressed by these cells (Fig. 1D). In contrast, mMAGE-b

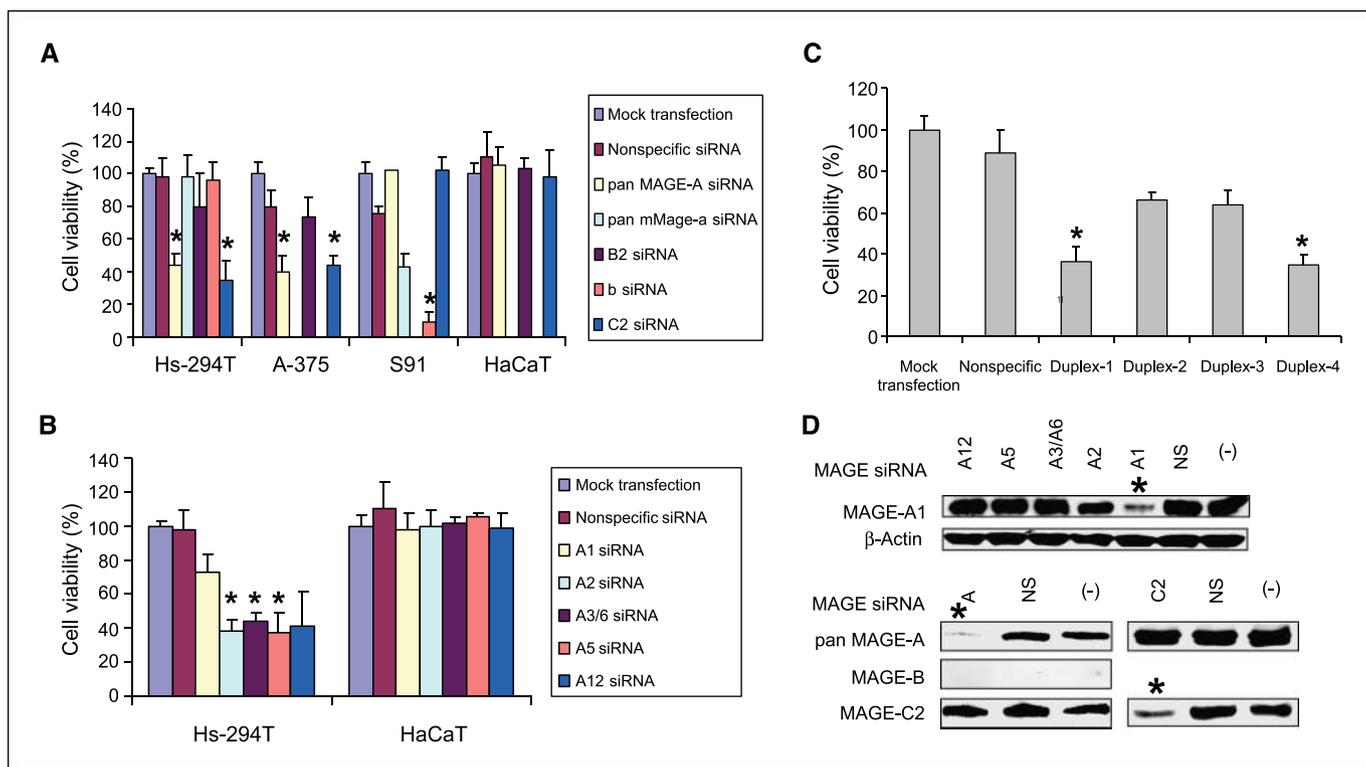


Figure 1. MAGE siRNAs inhibit melanoma growth *in vitro*. **A**, MAGE siRNA inhibits the growth of human (Hs-294T, A375) and murine (S91) melanoma cell lines but not the HaCaT human keratinocyte line. Trypan blue viability was determined 72 h posttransfection with 100 nmol/L siRNA. The growth of mock-transfected cells (no siRNA) and is considered as 100% viability. Pan-MAGE-A siRNA targets all human MAGE-A subfamily members except MAGE-A1, which has significant sequence variation, which makes the use of a small common set of siRNAs impossible. Pan-mMAGE-a and mMAGE-b siRNAs target all murine mMAGE-a and mMAGE-b family members, respectively. The human melanoma cell lines do not express MAGE-B and there is no murine mMAGE-c. The experiment to check the effect of murine mMAGE siRNA on A375 cell growth was not done. *, $P < 0.05$, a significant difference from nonspecific siRNA treated cells (*t* test). Bars, SD. Analyses were done from at least three individual experiments with triplicates for each group. **B**, the effect of siRNAs targeting individual MAGE-A family members. **C**, individual siRNA duplexes targeting different sequences in mMAGE-b have variable results, indicating sequence specificity. **D**, MAGE siRNAs decrease expression of target proteins. For protein target validation, cells were harvested 48 h posttransfection with 100 nmol/L of indicated siRNAs. Equal amount protein of whole-cell lysates was loaded. *, protein targeted by siRNA. NS, nonspecific control siRNA; (-), no siRNA.

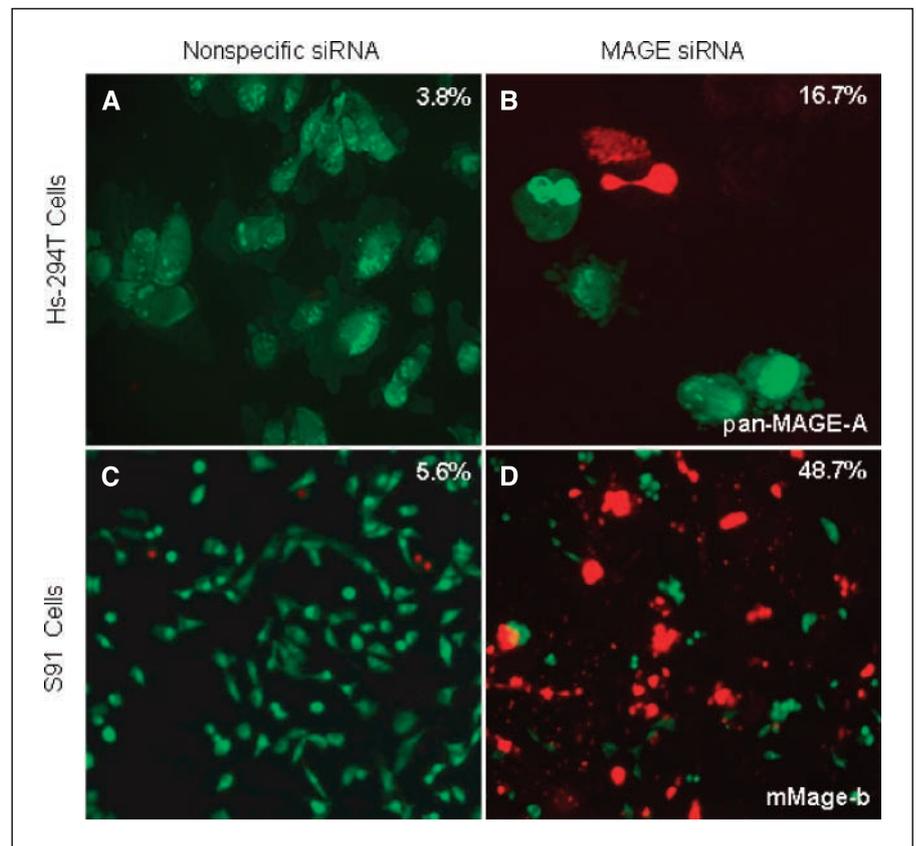


Figure 2. Class I *MAGE* gene knockdown induces apoptosis in melanoma cells. Fluorescence microscopy of acridine orange/ethidium bromide-stained cells shows green fluorescence of live cells and red fluorescence of dead cells. Apoptotic cells show condensed or fragmented nuclei. *A* and *B*, Hs-294T cells; *C* and *D*, S91 cells. Original magnification, $\times 200$ (*A* and *B*) or $\times 100$ (*C* and *D*). Percentage of apoptotic cells is given in upper right hand corner of each panel. Differences between numbers of apoptotic cells in MAGE siRNA-treated cells versus nonspecific siRNA-treated cells were significant ($P < 0.05$, *t* test for both Hs-294T and S91; $n = 3$).

siRNA inhibits the viability of the murine S91 cell line, which expresses significantly more mMAGE-b than mMAGE-a (Supplementary Fig. S1). Figure 1*B* shows siRNAs targeting unique sequences in several individual *MAGE-A* family members also effectively inhibit cell viability, except for *MAGE-A1*, which differs significantly from the other *MAGE-A* family members. Figure 1*C* shows individual siRNA duplexes targeting different sequences in mMAGE-b have variable effects, indicating sequence specificity. Note that these siRNA reagents are species specific and have been previously shown to specifically destroy their target mRNAs (17). Figure 1*D* shows that the siRNAs specifically suppress the targeted MAGE proteins.

Suppression of *MAGE* genes induces apoptosis. We have previously shown that suppression of *MAGE* genes has only modest effects on cell cycle progression in malignant mast cells (17), and preliminary studies showed similar results with melanoma cell lines (data not shown). Therefore, we focused on suppression of apoptosis as the most likely mechanism by which MAGE expression promotes survival in melanomas. We first used acridine orange/ethidium bromide staining with morphologic analysis and found that MAGE siRNA induced significant apoptosis in both human and murine melanoma cell lines (Fig. 2). TUNEL analysis with flow cytometry confirmed that MAGE siRNAs caused apoptosis (Supplementary Fig. S2). Interestingly, the apoptosis was not decreased by caspase inhibitors.

KAP1 is a binding partner of multiple MAGE proteins. To identify the mechanism by which *MAGE* gene expression could suppress apoptosis, we looked for potential MAGE binding partners using a yeast two-hybrid assay with MAGE-C2 as bait. KAP1 bound to MAGE-C2 in the yeast assay (Supplementary

Fig. S3*A*) and was confirmed as an endogenous MAGE-C2 binding partner by coimmunoprecipitation from human melanoma cells (Fig. 3*A*), and from human malignant mast cells (data not shown). We next sought to determine whether other MAGE molecules could bind to KAP1 by immunoprecipitation of endogenous and expression-tagged MAGE proteins. We found that we could bring down KAP1 with endogenous MAGE-A using a pan-anti-MAGE-A antibody and that we could also bring down V5-tagged human MAGE-A3 and V5-tagged murine mMAGE-b with KAP1 (also Fig. 3*A*).

To determine which portions of KAP1 are necessary for MAGE binding, we cotransfected COS-7 cells with plasmids encoding MAGE-C2 and plasmids encoding various truncated KAP1 proteins. Immunoprecipitation of MAGE-C2 followed by immunoblotting with KAP1 antibodies recognizing either the NH₂ or COOH-terminal regions of KAP1 showed that the KAP1 BB-coiled-coil region was necessary and sufficient for MAGE binding (Fig. 3*B* and *C*). Similar studies showed mMAGE-b binds to the BB-coiled coil region of KAP1 (Supplementary Fig. S3*B*), indicating that multiple MAGE proteins are capable of binding to the same region of KAP1. Overall, our data allow us to conclude that multiple different MAGE proteins, including members each of the three class I MAGE families, can bind to KAP1, and that the ability of MAGE proteins to suppress apoptosis may involve interactions with KAP1. Our data also suggest that binding to KAP1 may be a common function of class I MAGE molecules.

MAGE proteins facilitate KAP1/p53 complex formation and p53 suppression. KAP1 is known to corepress p53 by several mechanisms, including decreasing p53 expression by facilitating its degradation and by blocking p53 acetylation and DNA binding

(transcriptional activating) function (15). Therefore, we next looked for interactions between p53, MAGE, and KAP1. Unlike Monte et al., we did not detect direct binding of MAGE proteins to p53 (data not shown). However, we did find that KAP1 formed

complexes with p53 and that MAGE knockdown decreased KAP1/p53 binding (Fig. 4A-C). MAGE knockdown also resulted in increased immunoreactive p53 and acetylated p53 (Fig. 4D; Supplementary Fig. S4), suggesting that MAGE binding facilitates

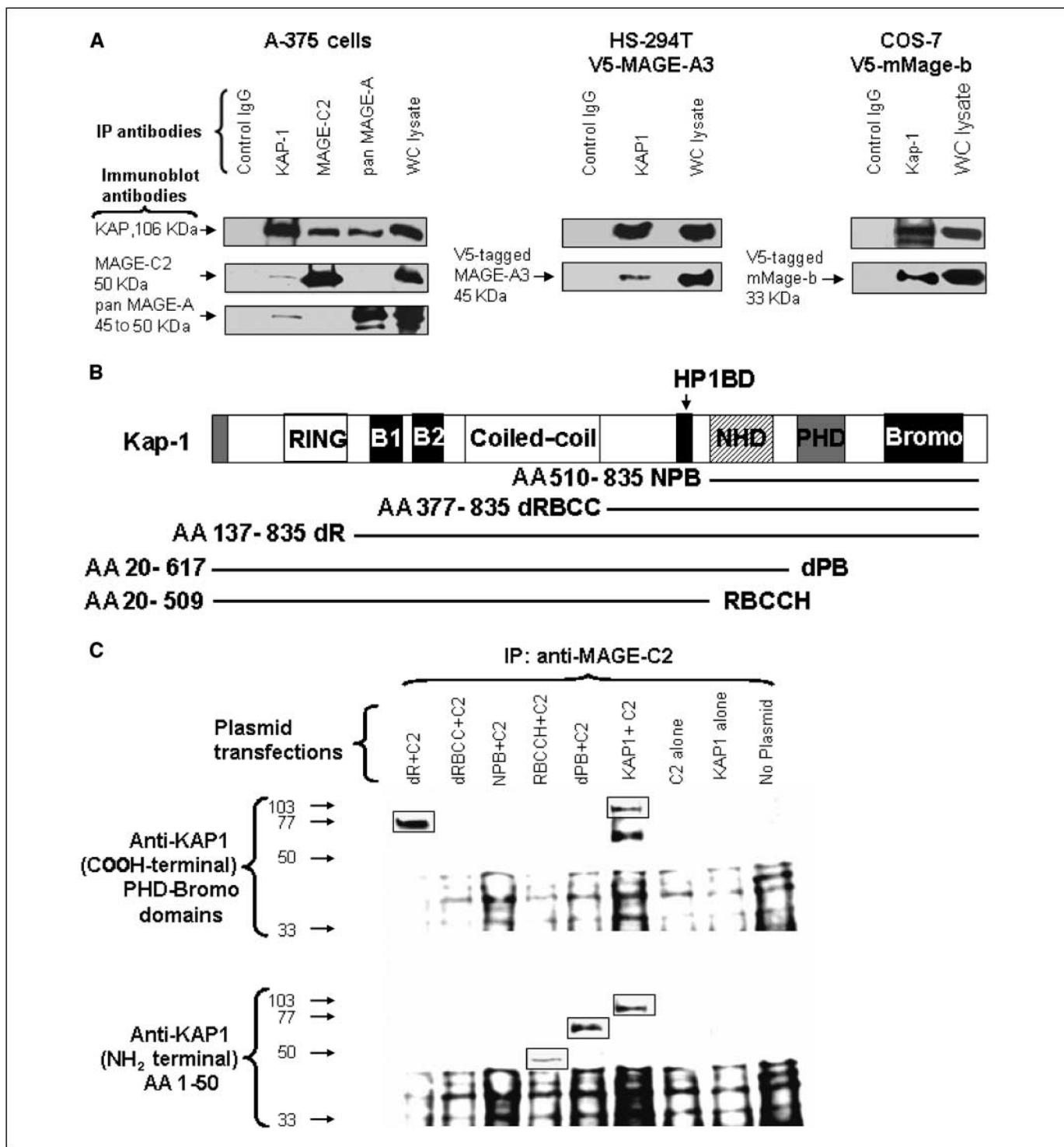


Figure 3. MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1. *A*, immunoprecipitations (IP) show that KAP1 coprecipitates with endogenous MAGE-A and MAGE-C2 proteins, and with expressed MAGE-A3 and mMage-b proteins. *B*, map of KAP1 deletion mutants and functional domains. *C*, MAGE-C2 and KAP1 deletion mutant expression plasmids were cotransfected into COS7 cells, and lysates were immunoprecipitated with anti-MAGE-C2 antibody. *Top blot*, immunoblotting with antibody against the COOH-terminal region of KAP1 shows that immunoprecipitation of MAGE-C2 only pulls down proteins containing the BB and coiled-coil regions (boxed bands, full-length KAP1 and the dR construct). *Bottom blot*, immunoblotting with antibody recognizing the NH₂-terminal region of KAP1 shows that MAGE-C2 pulls down proteins lacking any part of the COOH-terminal: NHD, PHD, and bromo regions (boxed bands, full-length, dPB, and RBCCH constructs).

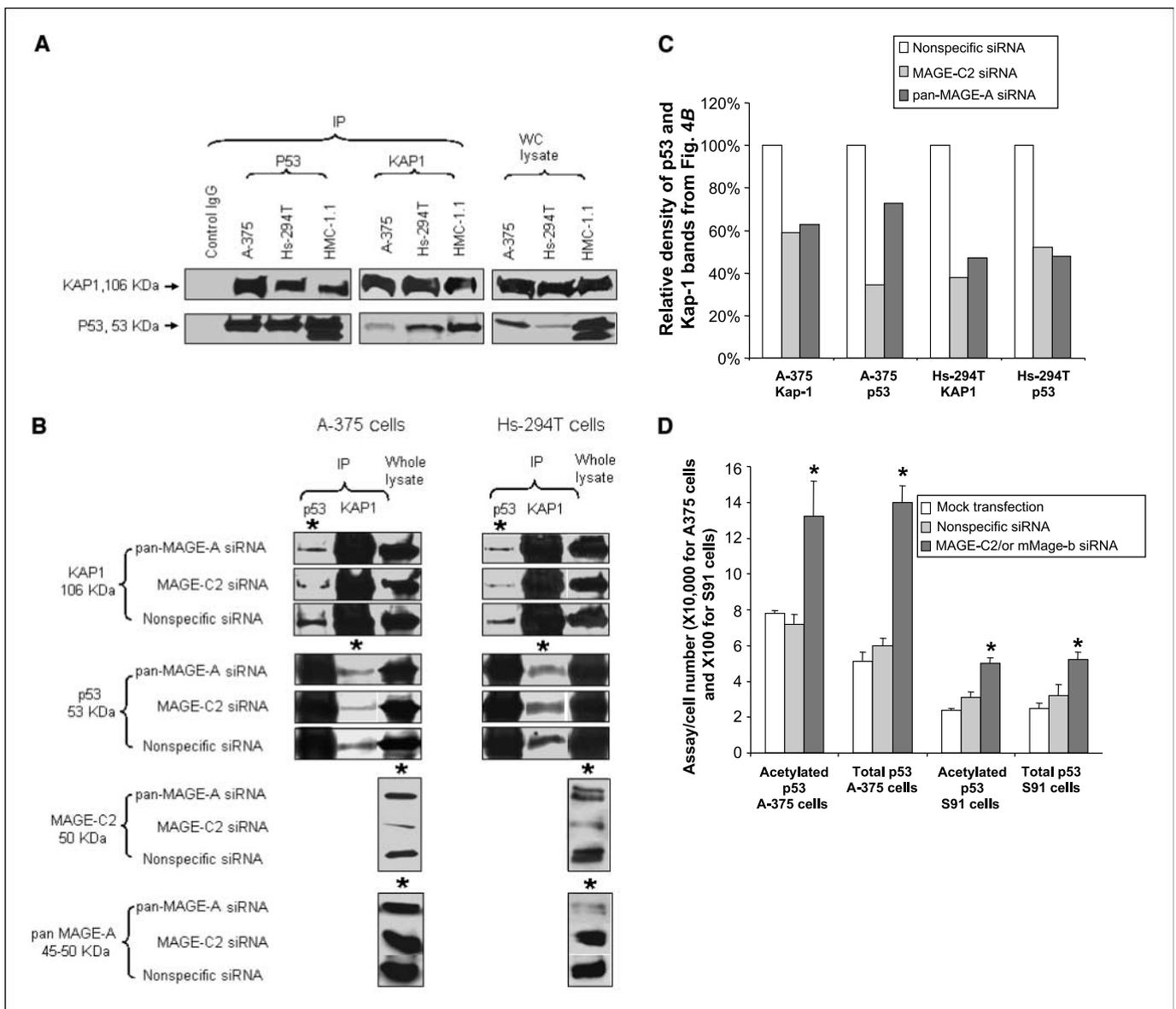


Figure 4. MAGE protein expression augments KAP1/p53 complex formation and suppression of p53. *A*, coimmunoprecipitation of endogenous KAP1 and p53. *B*, the columns indicated with * show decreases in coimmunoprecipitated KAP1 and p53, and decreased MAGE protein in whole-cell lysates after treatment with MAGE siRNA. *C*, relative densities of the bands of coimmunoprecipitated p53 and Kap-1 shown in *B*. *D*, MAGE knockdown increases levels of total p53 and acetylated p53 determined by the cytotol technique. *, $P < 0.05$, significantly different from MAGE-siRNA-transfected and control siRNA-transfected cells (t test, $n = 6$).

KAP1 repression of p53 expression and function. To test the hypothesis that MAGE proteins suppress apoptosis by suppression of p53, we used variants of the HCT116 colon cancer cell line, which express moderate levels of total MAGE-A protein (Supplementary Fig. S5). As we have shown for malignant melanoma and mast cell lines, MAGE siRNA suppressed the viability and induced apoptosis of the parental HCT116 cell line (viability data Fig. 5A; apoptosis data not shown). However, MAGE knockdown could not induce apoptosis in the absence of p53 because it has no significant effect on viability or apoptosis of HCT116 cells that are p53^{-/-}. Furthermore, MAGE knockdown activated an integrated β -lactamase reporter gene controlled by a consensus p53 responsive element in the p53RE-*bla* variant of the HCT116 cell line (Fig. 5B). Finally, if MAGE suppression of apoptosis is dependent on the function of KAP1, knockdown of KAP1 should also decrease cell viability. KAP1

knockdown did indeed suppress cell viability (Fig. 5C). Thus, our data allow us to conclude that expression of select class I MAGE proteins promotes cell viability by preventing apoptosis and that a likely mechanism of action is that MAGE proteins function as cofactors supporting KAP1-dependent suppression of p53.

MAGE suppression inhibits tumor growth *in vivo*. To establish whether suppressing MAGE genes can interfere with melanoma growth *in vivo*, we inoculated DBA mice with syngenic S91 melanoma cells that had been transfected with mMAGE-b siRNA. Figure 6A shows by Kaplan-Meier plot that pretreatment with mMAGE-b siRNA significantly suppresses tumor growth and improves survival. (See Supplementary Fig. S6 for additional analysis.) Furthermore, although all control siRNA-treated mice developed tumors, three of the mice receiving mMAGE-b siRNA-treated cells never developed tumors.

Early human melanomas and other solid tissue malignancies can be cured by complete excision, and it is usually only when the tumor has already metastasized that systemic treatment is required. To simulate this clinical situation, we administered siRNA parenterally when the tumors were not yet palpable, 4 days after s.c. inoculation. In one experiment, we gave a series of five i.p. injections of 12 nmol mMAGE-b siRNA in polyethylenimine, and found that it prolonged the time to reach the target tumor size by an average of 5 days compared with mice treated with similar doses of nonspecific siRNA ($P < 0.01$ by log-rank, $P = 7.587 \times 10^{-6}$ by regression analysis, $n = 11$ for treatment group and $n = 8$ for control group). In a second experiment, i.p. injections of mMAGE-b siRNA directly conjugated to cholesterol prolonged the time for tumors to grow to the target diameter by 8 days compared with injections of similar preparations of nonspecific siRNA (Fig. 6B; Supplementary Fig. S7; $P < 0.01$ by regression analysis and $P < 0.002$ by log-rank analysis, $n = 5$ for both mMAGE-b and control siRNA groups). In separate experiments, RT-PCR target analysis showed loss of amplifiable mMAGE-b mRNA in established tumors removed from mice 48 h after treatment with i.p. mMAGE-b siRNA, but not in tumors removed from mice treated with nonspecific siRNA (Fig. 6C).

Discussion

Combined with our previous work (17), our knockdown studies fulfill criteria for demonstrating a classic RNA interference response including showing specificity of reagents and reduction of gene expression at the mRNA and protein levels (21). Our studies of individual siRNAs targeted to different mMAGE-b mRNA sequences serve as multiplicity controls by demonstrating similar biological effects with two or more siRNAs, and together with the irrelevant control siRNAs and cross species studies indicate the sequence specificity of induction of apoptosis. The biological result is significant, with suppression of melanoma cell growth *in vitro* and *in vivo*, and we have identified the basic mechanism as the induction of apoptosis by suppression of *MAGE* gene expression. We have further shown that this phenomenon is likely mediated by MAGE binding to KAP1 and suppression of p53. Our data clearly show that members of all three class I MAGE protein families can promote cell viability and therefore can provide a growth advantage to melanomas and other malignancies.

The anti-MAGE-A antibody we used for immunoprecipitation recognizes a common antigen present on all MAGE-A proteins so we cannot yet determine whether all of the individual MAGE-A proteins interact with KAP1 in the cell lines that we used. However, our data on cell viability using siRNAs specific for individual MAGE-A family members suggest that suppression of apoptosis is a common function of multiple MAGE molecules, and we speculate that this may be a function of binding between the MAGE common homology domain and the KAP1 BB-CC region. Our data also support a previous proposal that the existence of multiple nearly identical MAGE family members enables a single critical function to be expressed under different transcriptional controls (1). It might be expected that the highly related MAGE family members that are not down-regulated by specific siRNA targets would still bind to KAP1 and still be able to inhibit p53 activity. However, close examination of the reciprocal immunoprecipitations in Figs. 3A and 4B show that only a percentage of KAP1 is bound to particular MAGE molecules at any one time and that only a percentage of individual MAGE molecules are bound to KAP1. The fact that

apoptosis occurs in this context suggests that the total amount of MAGE protein available affects the level of suppression and that MAGE/KAP1 binding may be in a dynamic equilibrium. Precedent for such a system exists in the regulation of p53 by KAP1 and MDM2 in which p53 expression is tightly controlled with a feedback loop in which p53 stimulates transcription of MDM2 and MDM2 binds directly to p53, blocking its transcription-related functions and increasing degradation of p53 by polyubiquitination (15). Alternatively, the formation of complexes between different

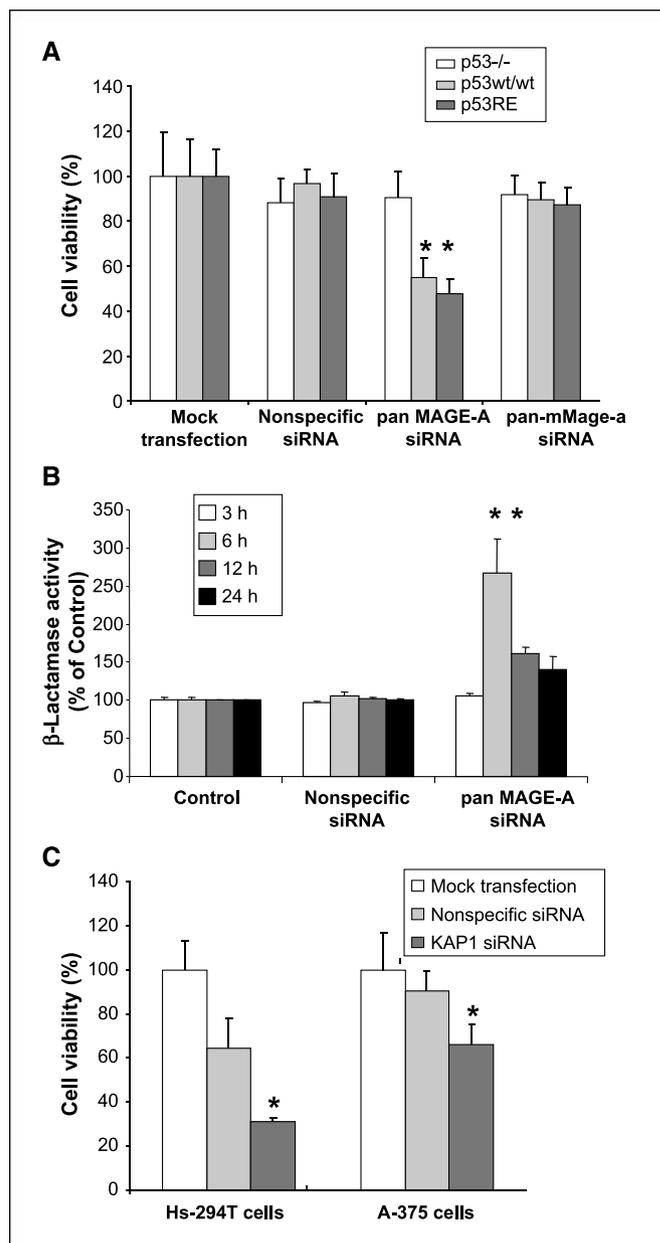


Figure 5. A, MAGE siRNA suppresses the viability of the parental and p53RE-bla HCT116 cell lines, but not in the p53^{-/-} variant of HCT116. B, MAGE-A knockdown activates a β -lactamase reporter gene driven by a p53 consensus sequence promoter in the p53RE-bla HCT116 cell line. Activation of the reporter gene indicates transcriptional activity of endogenous p53. The assays were done at different time points posttransfection as shown above, and p53 activity reached to peak 6 h after MAGE gene was knocked down. C, KAP1 siRNA inhibits growth of human melanoma cells. *, $P < 0.05$, *t* test, compared with nonspecific siRNA treatment; this analysis was obtained from two individual experiments with triplicates for each group.

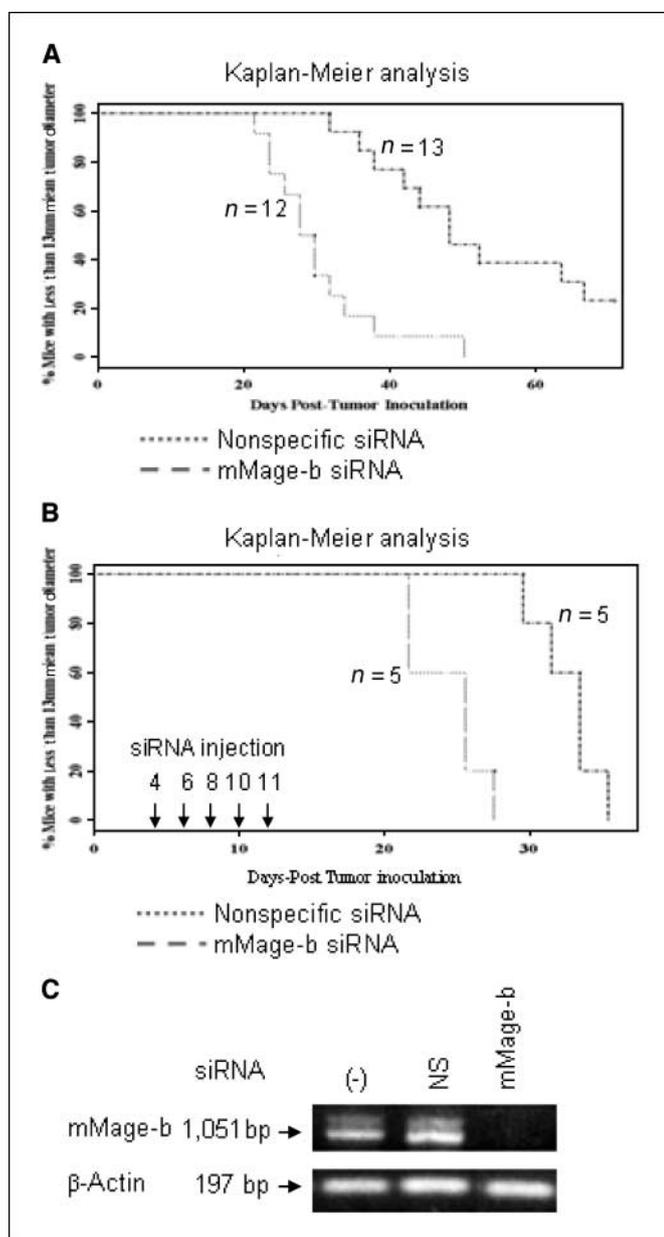


Figure 6. MAGE siRNAs suppress the growth of melanoma *in vivo*. **A**, Kaplan-Meier plot for mice injected with S91 cells transfected preinoculation with 100 nmol/L control or mMAGE-b siSTABLE-PLUS siRNA. **B**, Kaplan-Meier plot for mice inoculated with S91 cells then given i.p. injections of mMAGE-b siSTABLE-PLUS siRNA, directly conjugated to cholesterol, on days 4, 6, 8, 10, and 11 after tumor inoculation. **C**, validation study shows loss of mMAGE-b target mRNA in tumor cells 48 h after mMAGE-b siRNA treatment.

classes of MAGE proteins might explain this phenomenon; however, there is no experimental support for such a model because neither our own yeast two-hybrid screen nor those reported by others have implicated MAGE-MAGE binding or the formation of MAGE homodimers or heterodimers (22–24).

MAGE-A1 seems to be an exception in our studies because suppression of MAGE-A1 does not significantly reduce cell viability. MAGE-A1 has the least common homology among the MAGE-A proteins and unlike other class I MAGE proteins has been shown to bind to and inhibit the activity of the intracellular portion of Notch1, a SKIP-interacting transactivator (23). Thus, our data are in

agreement with previous studies showing MAGE-A1 has a different binding partner and a different specific function than those we find for other class I MAGE molecules.

Our results differ somewhat from those of Monte et al. (16), who recently reported that MAGE-A2 binds to p53 and suppresses p53 function. Using immunoprecipitation of endogenous MAGE and immunoblotting, we were unable to confirm their finding of direct binding of MAGE-A molecules to p53, but our reagents and cell lines differ significantly from theirs and our overall findings agree with theirs in that we also find (a) MAGE proteins bind to complexes that include p53 and (b) MAGE proteins promote cell viability via suppression of p53. Our studies, however, show that class I MAGE proteins of all three subfamilies may function to suppress apoptosis in tumor cells. Furthermore, we find anti-apoptotic function in specific MAGE proteins, including MAGE-A2, MAGE-A3, MAGE-A5, and MAGE-A6, mMAGE-b, and MAGE-C2. Although Monte et al. did not see increased survival with transfection-mediated expression of MAGE proteins in select cell lines, our results are not incompatible with theirs because there is no a priori reason to believe that those cell lines need p53 suppression by MAGE proteins for survival. Our findings also fit nicely with those of White et al. (25), who found that KAP1 rapidly localizes to sites of DNA strand breakage, establishing a link between KAP1, chromatin-mediated transcriptional repression, and recognition/repair of DNA damage. Combined with our discoveries of MAGE suppression of apoptosis and MAGE binding to KAP1, these data fit well with the hypothesis that select MAGE proteins can interfere with p53-dependent DNA damage responses and thus offer a survival advantage to tumor cells.

A minor point of interest is that the apoptosis suppressed by MAGE proteins is not affected by caspase inhibitors (Supplementary Fig. S2). This result is unusual but not unheard of because p53-dependent, caspase-independent apoptosis has been reported in several systems including neurons (26, 27) and other cell types (28–30). Perhaps of greater interest are the implications these studies have for the role of MAGE proteins in normal biology. Normal spermatogenesis requires p53-dependent cellular proof-reading because p53^{-/-} male mice have decreased germ cell apoptosis, an increased percentage of morphologically abnormal sperm, and reduced fertility (31). It is known that both the KIT tyrosine kinase and multiple MAGE proteins are expressed in developing sperm during meiosis and we recently reported that activation of the KIT receptor tyrosine kinase promotes expression of MAGE genes by maintaining their promoter regions in a hypomethylated state, the first report of epigenetic regulation of specific genes by a tyrosine kinase (7, 17, 32, 33). A clue to the normal function of MAGE proteins comes from the observation that the males but not the females of kit^{W-v/W-v} partial loss of function mutant mice are infertile and exhibit increased germ cell apoptosis and decreased germ cell viability (33–35). Surprisingly, this defect is rescued in double-mutant p53^{-/-} kit^{W-v/W-v} mice, indicating that KIT normally regulates a p53-dependent apoptotic pathway in developing male germ cells (35). We speculate that KIT suppression of p53 in the testes is mediated through regulation of MAGE expression and MAGE binding to KAP1. We also note that KIT seems to be relevant to human fertility because decreased KIT expression is seen in testes of subfertile adult humans and is associated with increased apoptosis in spermatocytes (36).

MAGE proteins were the first cancer testes antigens discovered, and their limited tissue distribution has long been recognized as a potential key to tumor-specific treatment of many different

malignancies (2). The fact that *MAGE* genes are normally expressed in cells of the spermatogenic series during meiosis suggests that they may be members of the family of germ line antiapoptotic genes that are involved in the maintenance of genomic stability and fertility in mammalian germ cells, and may protect cells from triggering an apoptotic response during meiosis (37, 38). The hypothesis that developing neoplastic cells can co-opt these functions and use them to gain a growth advantage and resistance to apoptosis would help explain the increasing amount of correlative data which suggest that expression of class I MAGE proteins and other cancer testes antigens may actually contribute to the development of malignancies (4, 6). However, work published thus far has not shown whether *MAGE* gene expression is a functionally irrelevant by-product of cellular transformation or actually contributes to the development of melanoma and other malignancies. In answer to this question, our studies show unequivocally that inhibition of selected MAGE

proteins can decrease the viability of melanoma cells *in vitro* and *in vivo*. Our findings support a new paradigm in which interference with the expression or function of select class I MAGE antigens may by itself be useful for the suppression of growth of melanomas and other malignancies.

Acknowledgments

Received 4/25/2007; revised 7/5/2007; accepted 8/13/2007.

Grant support: NIH grant AR043356 and the Paul P. Carbone Comprehensive Cancer Center, University of Wisconsin Medical School (B.J. Longley); NIH/NIA grant 1R01 AG023096-01 and American Federation for Aging research grant A000106 (C. Gravekamp); University of Wisconsin Comprehensive Cancer Center (B.J. Longley, Principal Investigator); subsidized use of The W.M. Keck Small Molecule Screening Facility; and USPHS grant RO1AR043356 (B.J. Longley).

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MAGE-A, mMage-b, and MAGE-C Proteins Form Complexes with KAP1 and Suppress p53-Dependent Apoptosis in MAGE-Positive Cell Lines

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Cancer Res 2007;67:9954-9962.

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