Melanoma Antigen-11 Inhibits the Hypoxia-Inducible Factor Prolyl Hydroxylase 2 and Activates Hypoxic Response

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Abstract

Activation of hypoxia-inducible factors (HIF), responsible for tumor angiogenesis and glycolytic switch, is regulated by reduced oxygen availability. Normally, HIF-α proteins are maintained at low levels, controlled by site-specific hydroxylation carried out by HIF prolyl hydroxylases (PHD) and subsequent proteasomal degradation via the von Hippel-Lindau ubiquitin ligase. Using a yeast two-hybrid screen, we identified an interaction between melanoma antigen-11 (MAGE-11) cancer-testis antigen and the major HIF-α hydroxylating enzyme PHD2. The interaction was confirmed by a pull-down assay, coimmunoprecipitation, and colocalization in both normoxic and hypoxic conditions. Furthermore, MAGE-9, the closest homologue of MAGE-11, was also found to interact with PHD2. MAGE-11 inhibited PHD activity without affecting protein levels. This inhibition was accompanied by stabilization of ectopic or endogenous HIF-1α protein. Knockdown of MAGE-11 by small interfering RNA results in decreased hypoxic induction of HIF-1α and its target genes. Inhibition of PHD by MAGE-11, and following activation of HIFs, is a novel tumor-associated HIF regulatory mechanism. This finding provides new insights into the significance of MAGE expression in tumors and may provide valuable tools for therapeutic intervention because of the restricted expression of the MAGE gene family in cancers, but not in normal tissues. [Cancer Res 2009;69(2):616–24]

Introduction

During tumor growth, delayed blood vessel expansion is responsible for the limited amount of oxygen and glucose required for tumor growth. Tumors adapt to this condition by rapidly stabilizing hypoxia-inducible factors (HIF1–HIF3), which activate transcription of angiogenic factors and a plethora of enzymes supporting anaerobic glycolysis and high-glucose consumption. HIFs are heterodimeric complexes composed of regulated HIF-α subunits and constitutively expressed HIF-β subunits or ARNT (1–3). HIF-α subunits are constantly transcribed and translated, but under normal oxygen conditions, they are hydroxylated at two prolyl residues located in the oxygen-dependent degradation domain (ODDD). This hydroxylation allows for an interaction of HIF-α with the E3-ubiquitin ligase pVHL, followed by rapid degradation (4–6). When oxygen is limited, the HIF-α subunits are stabilized, translocate into the cell nuclei, heterodimerize with HIF-β, and activate transcription of their target genes.

Increase of HIF-α subunits under low oxygen is critically dependent on the three HIF prolyl 4-hydroxylases termed PHD1, PHD2, and PHD3 (also called EGLN2, EGLN1, and EGLN3 or HPH3, HPH2, and HPH1, respectively), simultaneously discovered by several groups (4, 5, 7, 8). They belong to the conserved family of dioxygenases that use oxygen and α-ketoglutarate to hydroxylate prolines Pro402 and Pro564 in the ODDD of HIF-1α and the corresponding proline residues of HIF-2α (4, 5, 9). These proteins use nonheme iron for their catalytic activity, and iron-chelating compounds are inhibiting PHD activity, leading to increased HIF-α protein stability (10–12). The low affinity of PHDs for oxygen ensures a highly sensitive oxygen-regulating mechanism, in which small changes in oxygen concentration result in a pronounced decrease in reaction rate and rapid HIF-α up-regulation (9). Another dioxygenase family member, factor-inhibiting HIF, controls HIF transcriptional activity by hydroxylating asparagine 803, thereby preventing HIF interaction with transcriptional coactivators p300/CBP (13–16). This dual control mechanism provides a tight regulation of HIF activity in response to oxygen availability.

The three mammalian HIF PHD isoforms differ in their expression, cellular localization, and substrate specificity (9, 17–21). Experimental evidence indicates that PHD2 is the major PHD isoform controlling HIF-α protein stability (22).

In addition to regulating substrate availability, several proteins modifying PHD activity have also been identified. Among them, two cellular oncogenes, v-Src and activated Ras, are capable of inducing HIF by blocking PHD (23). In contrast, other proteins are important for the proper functioning of PHDs. The ubiquitously expressed OS-9 protein was found to facilitate interaction between HIF-1α and PHD, thereby promoting HIF hydroxylation and degradation (24). PHD1 and PHD3 protein turnover is regulated by the E3 ubiquitin ligases SIAH1/SIAH2, which are induced by hypoxia, and lowers availability of PHD1/PHD3 to ensure full HIF-α induction (25). The PHD2 protein stability, however, seems to be regulated by an interaction with FK506-binding protein 38 (FKBP38), leading to destabilization of PHD2 (26). Interestingly, PHD2 protein has a hydroxyprolyl-independent function in hypoxic cell nuclei, where it binds through HIF to hypoxia-responsive promoters and down-regulates their transcription (27). Although the precise mechanism has not yet been identified, there is a possibility that interaction of PHD2 with tumor suppressor ING4, a component of a chromatin-remodeling complex, impairs the ability of HIF to activate transcription (28).

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

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To better understand the regulation of PHDs in hypoxic tumor cells, we performed a yeast two-hybrid screen, using PHD2 as a bait and a library prepared from PC3 prostate cancer cells subjected to 24 hours of hypoxia (1% oxygen). This screen identified some of the known PHD2 interacting proteins. Importantly, we found a novel interaction of PHD2 with melanoma antigen-11 (MAGE-11 or MAGE-A11). MAGE-11 is a member of a family of 12 MAGE-A proteins, representing a group of cancer-testis antigens expressed in some embryonic tissues but not in normal adult organs, with the exception of testis and placenta, and aberrantly reexpressed in different types of tumors. We focused on discovering what effects MAGE-11 protein may have on PHD2 because the MAGE-A family represents an ideal target for therapeutic intervention due to its restricted expression pattern. We found that MAGE-11 interacts with PHD2 in both normoxic and hypoxic conditions. MAGE-11 protein itself is not regulated by hypoxia and does not change PHD2 protein levels but rather suppresses PHD2 activity. Down-regulation of MAGE-11 by RNA interference results in diminished HIF-1α induction and transcriptional activity in hypoxia. Given the common re-expression of MAGE and other cancer-testis antigens in cancers, this finding provides a novel mechanism of action of these proteins.

Materials and Methods

Yeast two-hybrid screen. Twelve different PHD2 fragments, cloned into pGBT.superB vector (Myriad Genetics), were used as bait constructs in the yeast two-hybrid analysis. All baits were first tested in a yeast one-hybrid assay to eliminate self-activation of transcription. The activation domain prey library was made in pGAD.PN2 vector (Myriad Genetics) using the PC3 prostate cancer cell line exposed to 1% oxygen for 24 h. Yeast strain PNY200 was used to maintain the bait plasmids, and yeast strain BK100 was used to maintain the prey constructs. After mating, colonies were selected using two auxotrophic marker genes, HIS3 and ADE2. To confirm the interactions, the bait and prey plasmids were cotransfected into yeast and interaction was verified using the third reporter gene, LacZ.

Cell lines and reagents. HeLa, U2OS, A549, 786-O, MDA-MB435, and 293T cells were maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and penicillin/streptomycin (Invitrogen) in humidified air containing 5% CO_2 at 37°C. Hypoxia treatment was carried out in a modular incubator chamber (Billaps-Rothenberg) with the mixture containing different percentage of oxygen and 5% CO_2 and balanced with nitrogen. Deferoxamine mesylate (DFO), *O*-phenanthroline, proteasomal inhibitor MG132, and dimethyloxallyl glycine were purchased from Sigma-Aldrich, Inc.

Plasmids. MAGE-11 amino acids 2-429 were PCR-amplified from a human testis cDNA library (Clontech) and subcloned into the 3× Flag-CMV10 expression vector (Sigma) or pCR2.1-TOPO (Invitrogen) for *in vitro* translation or into pGEX5x-1 (Amersham) for expression in *Escherichia coli*. Full-length MAGE-4 and MAGE-9 clones were part of the Open Biosystems Human ORF collection in the pDONR223 gateway entry vector. These clones were transferred into the destination vector pDEST-510 with a COOH terminal Flag tag (National Cancer Institute), using homologous recombination with a Gateway LR Clonase II enzyme mix (Invitrogen). All plasmids were sequenced to verify the authenticity.

The pSG5-MAGE-11 plasmid was kindly provided by Dr. Elizabeth Wilson (University of North Carolina at Chapel Hill). The GHO-plasmid was from Dr. M.C. Simon (University of Pennsylvania). V5-PHD2 was from Dr. R. Bruick (University of Texas Southwestern Medical Center), and Flag-tagged PHD1-PHD3 were from Dr. F.S. Lee (University of Pennsylvania).

Cell transfection, immunoprecipitation, and immunoblotting. Cells were transfected with an Effectene reagent (Qiagen). Briefly, cells were trypsinized and counted, and 2×10^6 for 293T or 1.5×10^6 cells for other cell lines were mixed with the DNA-transfection reagent complex made with 1 to 2 μg DNA, according to the manufacturer's instructions, and plated on a 60-mm plate. For immunoprecipitation experiments, the transfection reactions were scaled up to 100-mm plates. For coimmunoprecipitation, cells were lysed in NP40 buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, and protease (Sigma) and phosphatase (Pierce) inhibitors. Total protein (300 μg) was used for immunoprecipitation. Cell lysates were precleared with normal mouse IgG and protein A/G Sepharose (Santa Cruz). The agarose conjugated with anti-V5 antibody (Invitrogen) was washed several times with NP40 buffer and blocked with 5% bovine serum albumin (BSA) for 1 h, and 30 μL were used to collect the protein complexes for 4 to 16 h at 4°C. The agarose was washed with NP40 buffer thrice, and bound proteins were resolved in 10% SDS-PAGE. Immunoprecipitation of endogenous PHD2 and MAGE-11 from HeLa cells was performed with ~1 mg of total protein. The lysates were precleared with A/G Sepharose (Santa Cruz), and complexes of PHD2 were immunoprecipitated with 5 μg of anti-PHD2 antibody (Novus Biologicals) The complexes were collected with 40 μL of protein A/G Sepharose for 1 h at 4°C and washed four times with lysis buffer supplemented with 1 mg/mL BSA and twice with the same buffer without BSA.

The nuclear extracts were prepared by scraping cells into cold PBS followed by a low-speed centrifugation. Cell pellets were resuspended in 400 μL of cold hypotonic buffer [10 mmol/L Tris-HCl (pH 7.5), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF)] and allowed to swell on ice for 10 min. After centrifugation, the supernatant fraction was discarded and the pellets were resuspended in 50 to 70 μL of high-salt buffer [20 mmol/L of Tris-HCl (pH 7.5), 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L PMSF] and incubated on ice for 20 min followed by high-speed centrifugation.

The antibodies used for Western blotting were anti–Flag-horseradish peroxidase (HRP; Sigma), anti–V5-HRP (Invitrogen), anti-PHD2 rabbit polyclonal (Novus Biologicals), anti–HIF-1α (BD Biosciences), anti-HA (Cell Signaling), anti–MAGE-11 (MabAb94, kindly provided by Dr. Wilson, University of North Carolina at Chapel Hill), anti–von Hippel-Lindau (VHL; BD Biosciences), anti–α-tubulin (Sigma), and anti-actin (Sigma).

Small interfering RNA transfections. Small interfering RNAs (siRNA) for MAGE-11 knockdown were purchased from Dharmacon (D-017280-03), as well as nonsilencing control siRNA 2. The duplexes were transfected into HeLa or U2OS cells, as described before (29), except that HeLa cells were transfected with 100 nmol/L of siRNA duplex. Stable transfection of MDA-MB435 cell line was performed with retroviral vector pSM2, expressing shMAGE11 in the context of mir-30 (Open Biosystems, clone V2HS_37013) or with the nonsilencing short hairpin RNA mir control (Open Biosystems, RHS1703) followed by selection with 2 μg/mL of puromycin.

VHL-binding assay. After transfection, cells were scraped in ice-cold reaction buffer containing 20 mmol/L Tris-HCl (pH 7.5), 5 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and protease inhibitor cocktail and disrupted by sonication. Synthetic peptide containing 19 HIF-1α amino acids, DLDLEMLAPYIPMDDDFQL or DLDLEMLAP(OH)YIPMDDDFQL, biotinylated on NH_2 terminus (2 μg/reaction) were first captured with streptavidin-conjugated magnetic beads in 200 μL of NP40 buffer for 2 h. The beads were washed twice in NP40 buffer and twice in the reaction buffer and incubated with 50 to 100 μg of total protein in the presence of 0.5 mmol/L α-ketoglutarate, 0.1 mmol/L $FeCl_2$, and 0.5 mmol/L ascorbate for 30 min at 30°C. The beads were washed thrice with NP40 buffer and incubated with 10 μL of *in vitro* translated HA-VHL for 1 h at 4°C. The beads were washed thrice with NP40 buffer, and bound HA-VHL was released with SDS loading buffer, resolved in 10% SDS-PAGE, and identified with anti-HA antibody.

Quantitative real-time PCR. Total RNA was purified with Trizol reagent (Invitrogen), and 4 μg were converted into cDNA using M-MLV reverse transcriptase and oligo dT primer (Invitrogen). cDNA (1 μL) was used for PCR with PCR master mix (Applied Biosystems), and 1.25 μL of 20× primer-probe mix were used for individual TaqMan assays (Applied Biosystems). Every measurement was done in triplicate. The amount of β-actin transcripts was determined in separate reactions, and the final data were normalized to the amount of β-actin and presented as a fold change compared with control cells. MAGE-11 primers used for PCR in quantitative reverse transcription–PCR (RT-PCR) reaction were spanning the boundaries

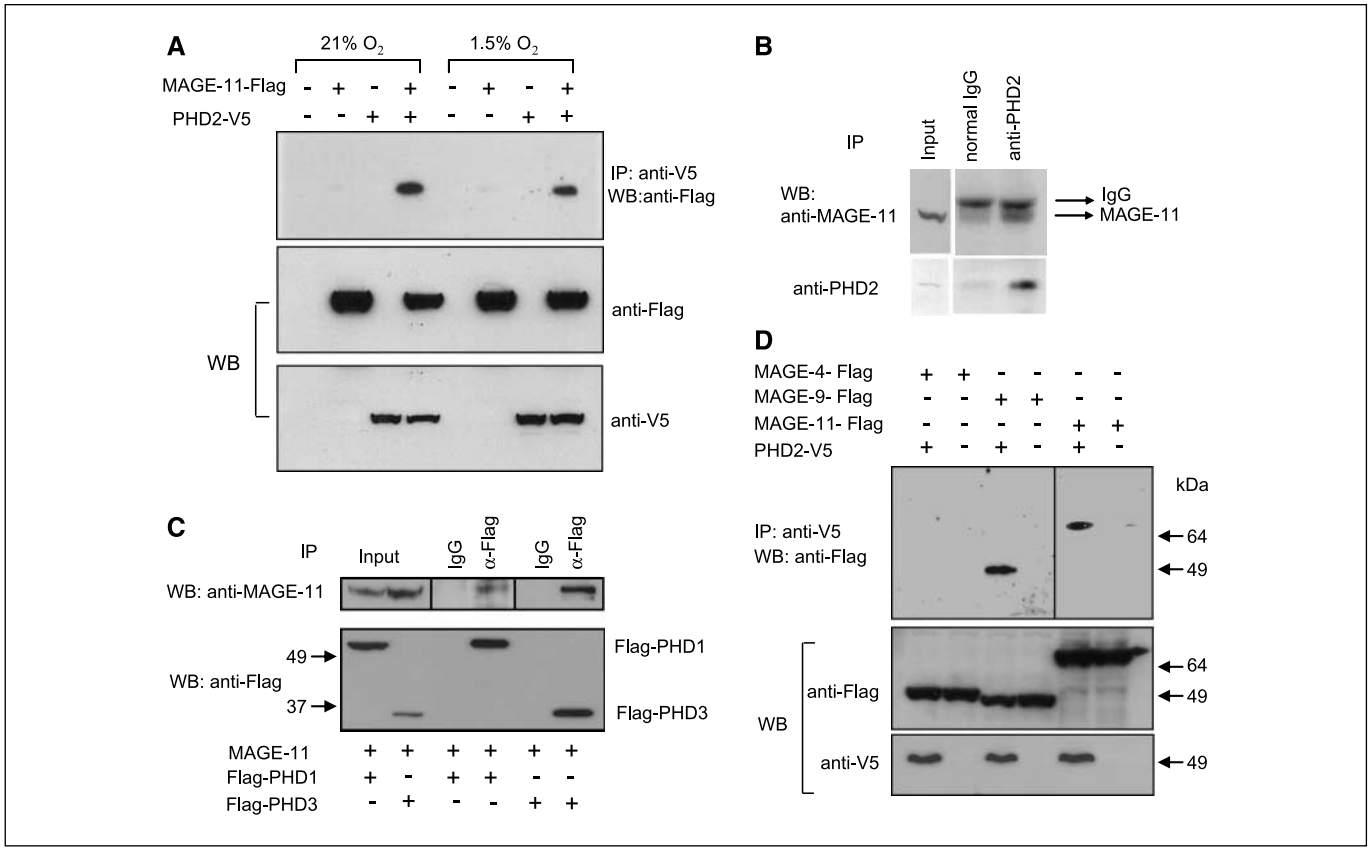

Figure 1. MAGE-11 interaction with PHD2. *A,* Flag-MAGE-11 and V5-PHD2 were cotransfected into 293T cells and, 2 d later, treated with 1.5% oxygen or left untreated for 16 h. Immunoprecipitation was performed with anti-V5 agarose, followed by Western blotting with anti-Flag antibodies. *B,* coimmunoprecipitation of endogenous MAGE-11 and PHD2 from HeLa cell extracts. Protein complexes were immunoprecipitated with anti-PHD2 antibody or normal rabbit IgG, as a negative control, followed by Western blotting with anti-MAGE-11 (*top*) or anti-PHD2 (*bottom*) antibody. *C,* MAGE-11 was overexpressed in 293T cells with Flag-PHD1 or Flag-PHD3 and immunoprecipitated with anti-Flag or normal mouse IgG, followed by Western blotting with anti–MAGE-11 antibody. *D,* three different Flag-tagged MAGE-A genes were overexpressed in 293T cells alone or in combination with V5-PHD2 and immunoprecipitated with anti-V5 agarose followed by Western blotting with anti-Flag antibody.

of exons 2 and 3 (Applied Biosystems, Hs00377815_m1), bHLHB2 (exons 2 and 3; Applied Biosystems, Hs00186419_m1), and NDRG1 (exons 13 and 14; Applied Biosystems, Hs00608389_m1).

Luciferase reporter assay. Cells were split at 0.5×10^5 into 12-well plates and transfected on the next day with 200 ng of luciferase-reporter plasmid, 5 ng of RL-CMV, 200 ng of HIF-1α, 10 ng of V5-PHD2, and 100 ng of Flag-MAGE-11 using Fugene 6 Reagent (Roche). Whenever one of the DNA components was eliminated, the total amounts of DNA were balanced with pcDNA3 empty vector. All transfections were performed in triplicates. Two days after transfection, cells were lysed in passive lysis buffer (Promega) and 20 μL were used to measure the activity of firefly and *Renilla* luciferase with dual luciferase reporter assay system (Promega).

Confocal microscopy. HeLa cells were grown on coverslips and, on the next day, were placed in 0.5% oxygen for 16 h or left untreated. Cells were fixed with 4% paraformaldehyde for 10 min and then treated with 0.1 mol/L glycin for 1 h. After three washings with PBS, cells were permeabilized with 0.2% Triton X-100 for 4 min, washed again, and blocked with 10% normal goat serum in PBS. Primary antibody anti–MAGE-11 (affinity purified rabbit polyclonal) and anti-PHD2 (mouse polyclonal, Novus Biologicals) at 1:100 dilution in the blocking solution were applied overnight at 4°C. Secondary antibody anti-mouse labeled with Alexa 486 or anti-rabbit labeled with Alexa 568 (both from Invitrogen) was diluted at 1:500 with the blocking solution and incubated with cells for 1 h at room temperature. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole using Prolong Gold Antifade Reagent (Invitrogen). Stained cells were observed on a Zeiss Axiovert 100M microscope equipped with a 100×/1.3oil Plan NeoFluar objective. Confocal images were obtained using an LSM510 scanning laser microscope (Zeiss). Controls containing no primary antibody were performed in parallel samples and showed no staining.

Results

Identification of PHD2-MAGE-11 interaction. Most solid tumors exhibit some degree of oxygen deprivation. Therefore, we sought to identify proteins that regulate PHD activity under conditions of limited oxygen supply. We performed a yeast two-hybrid analysis using PHD2 as bait, and a prey library made from hypoxic PC3 prostate cancer cells exposed to 1% oxygen for 24 h. A total of 12 fragments of PHD2 was used in an effort to maximize likely binding partners. Of these, we found multiple interactions of PHD2 bait constructs (spanning the region of amino acids 70-420) with the HIF-2α protein. Interestingly, we did not find an interaction with HIF-1α in this screen, implying either that the interaction of PHD2 with HIF-2α protein is more stable than with HIF-1α or that HIF-1α may require some additional modifications compromised in the yeast strains used in the study. Furthermore, in agreement with the recent publication of Barth and colleagues (26), we found three different PHD2 baits, all located between amino acids 1 and 180, interacting with multiple FKBP38 clones. In addition, we found a novel interaction of PHD2 (amino acids 202-301) with the melanoma antigen MAGE-11. The normal or tumor-specific functions of MAGE-A proteins are poorly understood. MAGE-11 was recently identified as a scaffolding

protein involved in the androgen receptor (AR) signaling pathway (30), but neither a link to hypoxia nor a regulation of HIFs has been identified and tested.

We confirmed the interaction between PHD2 and MAGE-11 by a pull-down assay using purified GST-MAGE-11 fusion protein and ^{35}S-labeled *in vitro* translated PHD2 (Supplementary Fig. S1*A*) or lysates of 293T cells overexpressing PHD2 (Supplementary Fig. S1*B*). We further confirmed the association between MAGE-11 and PHD2, using 293T cells cotransfected with expression vectors encoding Flag-tagged MAGE-11 and V5 epitope-tagged PHD2. Furthermore, we examined the effect of oxygen on the interaction. Immunoprecipitation with anti-V5 antibodies showed specific binding of PHD2 and MAGE-11, whereas no MAGE-11 was found in the immunoprecipitates of 293T cells transfected with an empty vector instead of PHD2 (Fig. 1*A*). Interestingly, the oxygen level had no effect on the interaction, as MAGE-11 coprecipitated with PHD2 equally well in hypoxic and in normoxic conditions. To coprecipitate endogenous MAGE-11 and PHD2, we first tested expression of MAGE-11 protein in a wide panel of cell lines. We found that HeLa cells expressed the highest levels of MAGE-11 (data not shown). For this study, we used a rabbit polyclonal antibody raised against a unique polypeptide in the NH_2 terminus of MAGE-11 (31). Immunoprecipitation with anti-PHD2 antibody followed by Western blotting with anti–MAGE-11 antibody confirmed that these two proteins bind to each other *in vivo* (Fig. 1*B*).

The bait PHD2 fragment used in the yeast two-hybrid screen (amino acids 202-301) is located outside of the catalytic domain in the region of moderate conservation between PHD paralogues involved in substrate recognition (32). Therefore, we tested two other PHD family members and found that both PHD1 and PHD3 also interact with MAGE-11 (Fig. 1*C*).

The fragment of MAGE-11 identified in the yeast two-hybrid screen spanned the region of amino acids 212-421. This fragment represents a MAGE homology domain conserved among all MAGE family members. To find out if other members of the MAGE-A family would also interact with PHD2, we cloned two other genes, MAGE-4 and MAGE-9. The phylogenetic analysis of the MAGE domains showed that MAGE-9 is the closest homologue of the MAGE-11 protein (33). We found that MAGE-9 was readily detected in a complex with PHD2 by coimmunoprecipitation, whereas MAGE-4 was not (Fig. 1*D*). This study showed that there is certain selectivity in the interaction of different MAGE-A proteins with PHD2 and that other MAGE family members may act in the same manner as MAGE-11.

MAGE-11 colocalizes with PHD2. To further characterize the interaction between MAGE-11 and PHD2, we examined their cellular localizations. Previous studies have shown that MAGE-11 is localized in both nuclear and cytoplasmic fractions with higher amounts found in cell nuclei (30, 34). To investigate MAGE-11 localization under low oxygen, HeLa cells were grown under normoxic or hypoxic conditions and immunostained with affinity purified rabbit polyclonal antibody raised against MAGE-A11 (31) and a commercially available polyclonal anti-PHD2 antibody produced in mouse. Confocal microscopy showed that, in agreement with previous publications, MAGE-11 is located in both nuclei and cytoplasm of HeLa cells with preferential nuclear staining that does not change in the conditions of low oxygen. Normoxic HeLa cells show robust colocalization of MAGE-11 with PHD2 proteins in cytoplasm and less, but still obvious, colocalization was observed under hypoxic conditions (Fig. 2).

MAGE-11 is degraded by the ubiquitin-proteasome system but not through PHD-VHL pathway. Having confirmed that PHD2 and MAGE-11 physically interact in the cell, we sought to study what function this interaction may have. First, we examined whether MAGE-11 was a hydroxylation target of PHD2. Although it is well established that HIF-α family members are substrates of PHD hydroxylation required for VHL E3 ubiquitin ligase recognition, there may be other proteins that are degraded through the same mechanism. Indeed, the large subunit of RNA polymerase II requires hydroxylation of a specific proline residue and VHL binding to undergo polyubiquitination and degradation (35). All known PHD substrate proteins consist of a LXXLAP consensus motif in which the last proline is the hydroxyl group acceptor. The primary sequence of MAGE-11 has six amino acids starting at position 160, LXXLPA, similar to the HIF consensus but differing in the position of the acceptor. Due to the fact that several studies have shown a certain tolerance divergence in this consensus (36, 37), we tested if MAGE-11 may be a substrate of PHD2 and may degrade through VHL-dependent ubiquitination. We examined MAGE-11 levels in 293T cells after treatment with the proteasomal inhibitor MG132 and found that the abundance of MAGE-11 protein increased, and it was also associated with a ladder of high molecular weight bands typical for polyubiquitinated proteins (Supplementary Fig. S2*A*). This phenomenon was independent of

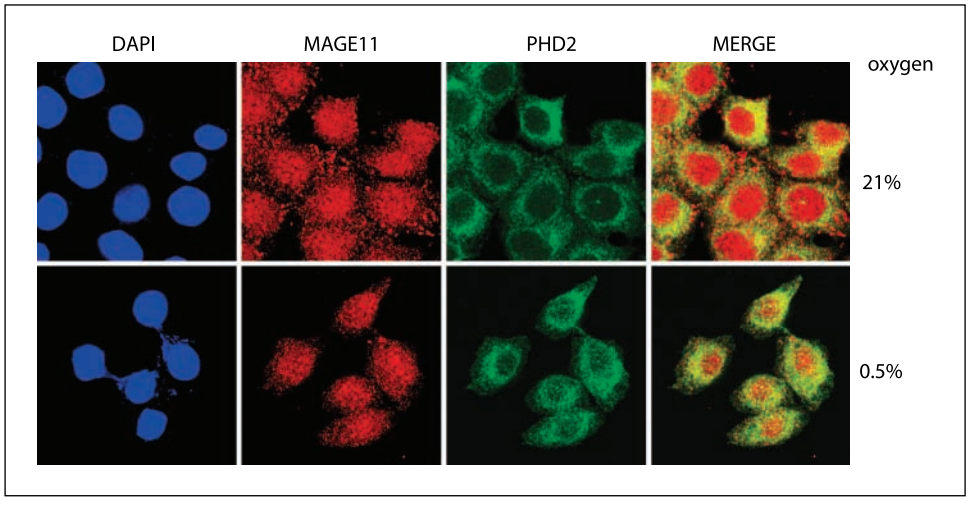

Figure 2. PHD2 colocalizes with MAGE-11 in HeLa cells. Confocal microscopy of HeLa cells exposed to 0.5% or 21% oxygen and stained with affinity purified anti–MAGE-11 rabbit antibody and anti-PHD2 mouse antibody followed by secondary anti-rabbit Alexa 568 (*green fluorescence*) and anti-mouse-Alexa 486 (*red fluorescence*) antibody.

whether or not PHD2 was overexpressed together with MAGE-11 (Supplementary Fig. S2*A*). The inhibition of endogenous PHD activity by hypoxia or by the iron-chelating agents DFO and *O*-phenanthroline did not affect MAGE-11 accumulation either (Supplementary Fig. S2*B*). When we expressed MAGE-11 in the VHL-negative 786-O cell line, we also observed induction of MAGE-11 protein by proteasome inhibition, implying that VHL is not required for proteasomal degradation of MAGE-11 (Supplementary Fig. S2*C*). We further expanded this observation to endogenous MAGE-11 and noted that, in HeLa cells, MAGE-11 levels were also induced by MG132 (Supplementary Fig. S2*D*) but were unresponsive to hypoxia or PHD inhibition (Supplementary Fig. S2*E*).

MAGE-11 inhibits PHD activity. Several proteins that modulate PHD activity either by bridging PHDs with HIF-α (OS-9; ref. 24), destabilizing the PHD2 protein (FKBP38; ref. 26), or causing suppression of HIF transcriptional activity under hypoxia (ING4; ref. 28) have been identified. To understand the function of MAGE-11 in regulation of PHD2, we first studied the effect of MAGE-11 on the protein levels of PHD2. There was no effect on PHD2 protein levels when it was ectopically coexpressed with the increasing amounts of MAGE-11 in HeLa or Hep3B cells (Fig. 3*A* and *B, bottom*). Similarly, there was no change in endogenous PHD2 protein levels in normoxic or hypoxic MDA-MB435 (Fig. 3*C*) or 293 cells overexpressing MAGE-11 (data not shown). We then tested the effect of MAGE-11 on PHD activity by using a VHL-binding assay. This *in vitro* reaction uses a 19–amino acid peptide derived from the ODDD of HIF-1α containing Pro564, which was hydroxylated by cell extracts expressing increasing amounts of MAGE11, and the extent of hydroxylation was measured by interaction with the *in vitro* translated HA-VHL protein. Figure 3*A* shows that an increase in MAGE-11 in cell extracts results in decreased binding of VHL (*top*). This effect was specific because MAGE-4 did not change PHD activity (Fig. 3*B*). Because pVHL binds only to the hydroxylated peptide, these results indicate that PHD activity is suppressed by MAGE-11 protein.

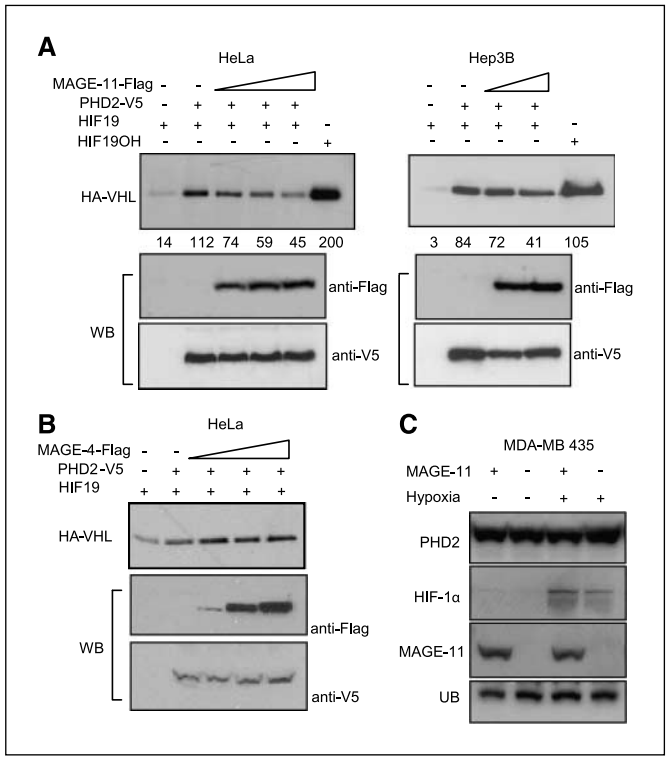

Figure 3. MAGE-11 suppresses enzymatic activity of PHD2. *A,* V5-PHD2 and increasing amounts of Flag-MAGE-11 were coexpressed in HeLa and Hep3B cells, and 48 h later, 100 μg of protein was used to hydroxylate HIF-1α–derived 19–amino acid peptide (*HIF19*), followed by binding with *in vitro* translated HA-VHL (*top*). The last lanes in both panels show VHL binding to the same peptide that was chemically hydroxylated on Pro564 and served as a positive control. The numbers under the top panel show the intensity of each HA-VHL band obtained by gel densitometry. Bottom panels show that increase in MAGE-11 protein does not affect PHD2 protein levels. *B,* MAGE-4 does not change PHD activity when used instead of MAGE-11. *C,* overexpression of MAGE-11 in the MDA-MB435 cells does not change the levels of endogenous PHD2. *UB,* unspecific band that serves as a loading control.

When PHD2 activity is blocked by RNA interference or small molecules, the stability of HIF-α proteins is expected to increase. We, therefore, tested the effect of MAGE-11 on the stability of the recombinant protein (GHO), which consists a GAL4 DNA-binding domain and a HA-tagged HIF-1α ODDD fragment. This recombinant protein is hydroxylated by all three PHDs, undergoes a proteasomal degradation after ubiquitination by pVHL, and previously served as a useful model to study HIF-α proline hydroxylation (38). Figure 4*A* shows that when overexpressed in 293T cells, GHO protein abundance was greatly increased by coexpression with MAGE-11. This effect was further magnified by treatment of cells with the proteasomal inhibitor MG132. The recombinant GHO protein migrates as two separate bands: the slower migrating band represents the unhydroxylated form and the faster migrating band represents the hydroxylated form (38). Densitometry of the two forms of GHO revealed that MAGE-11 increased the amount of unhydroxylated band from 41% to 60.5% in MG132-treated cells (Fig. 4*B*). The increase in GHO stability was specific because coexpression of PHDs, together with GHO and MAGE-11, diminished the ability of MAGE-11 to stabilize GHO (Fig. 4*C*). The degree to which each PHD abrogates the effect of MAGE-11 seems to be different, with PHD2 protein being the most potent (Fig. 4*C*). We then tested an effect of MAGE-11 on full-length HIF-1α. The protein levels of HIF-1α was also increased when it was overexpressed with MAGE-11, but not MAGE-4; however, the stable form of HIF-1α, mutated at both Pro402 and Pro564, was unaffected by MAGE-11 (Fig. 4*D*).

We then tested the effect of MAGE-11 on endogenous HIF-1α protein. We found that in the PC3 prostate cancer cell line, where the detectable amounts of HIF-1α are present even in normoxia (due to HIF-1α gene amplification; ref. 39), MAGE-11 overexpression resulted in increasing the amounts of HIF-1α protein (Fig. 5*A*). In A549 lung cancer cell line, MAGE-11 overexpression was not enough to achieve HIF-1α induction in normoxia, but when PHD activity was partially compromised by DFO, the HIF-1α levels were higher in the presence of MAGE-11 (Fig. 5*B*). Transfection with MAGE-4, which did not interact with PHD2 (Fig. 1*D*), did not affect the levels of HIF-1α induction by DFO in A549 cells (Fig. 5*B*). To estimate if ectopic levels of MAGE-11 were in the average range of endogenous levels observed in tumor cells, we performed immunoblotting with equal amounts of protein from PC3 cells transfected with MAGE-11 and from HeLa cells that express high amounts of endogenous MAGE-11. We found that PC3 cells produce about half of the amount of MAGE-11 protein compared with untransfected HeLa cells (data not shown).

We also tested the effect of MAGE-11 on HIF-1α transcriptional activity by using a 3× HRE-luciferase construct in the reporter assay. The reporter luciferase activity was increased four-fold by

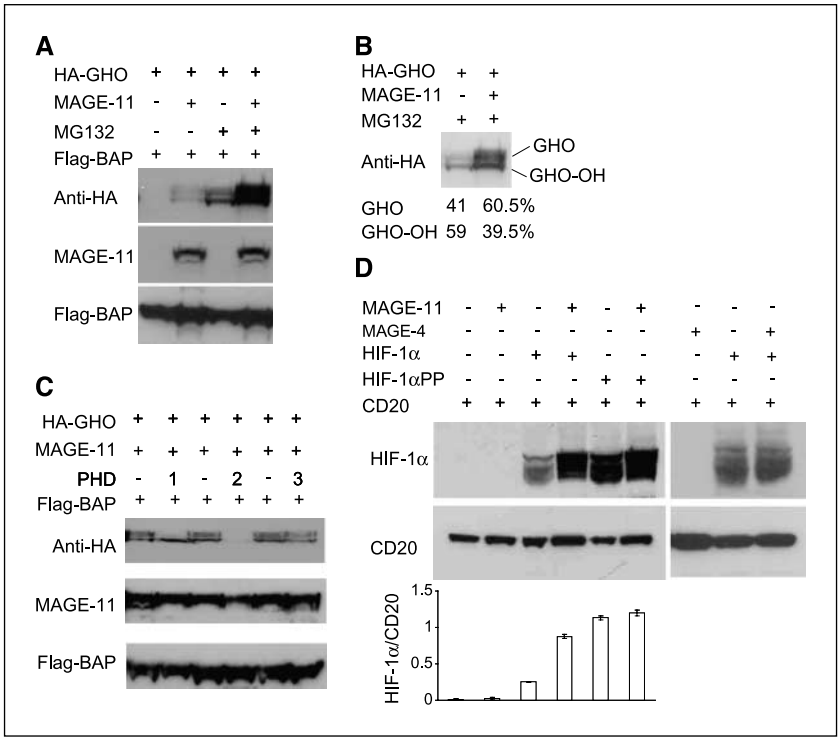

Figure 4. MAGE-11 increases the stability and transcriptional activity of HIF-1α. *A,* a plasmid containing Gal4 DNA-binding domain and HA-tagged HIF-1α ODDD (amino acids 496-626; HA-GHO) was cotransfected with pSG5-MAGE-11, and 2 d later, cells were treated with 10 μmol/L MG132 for 4 h. Each transfection reaction contained equal amounts of Flag-BAP (bacterial alkaline phosphatase) that served as a transfection efficiency control. *B,* shorter exposure of the fragment shown in the top panel of *A* allowed the separation and quantitation of the two bands. Numbers under the picture show the percentage of unhydroxylated band (GHO) and hydroxylated band (GHO-OH) in cells with and without MAGE-11. *C,* coexpression with PHDs completely (PHD2) or partially (PHD1 and PHD3) eliminates the ability of MAGE-11 to stabilize GHO protein. MAGE-11 and HA-GHO were cotransfected into 293T cells with or without Flag-tagged PHD1, PHD2, or PHD3. *D,* MAGE-11, but not MAGE-4, stabilizes full-length HIF-1α protein but has no effect on PHD-insensitive HIF-1α mutant. pSG5-MAGE-11 was cotransfected with the full-length wild-type HIF-1α or HIF-1α with two proline residues (Pro402 and Pro564) mutated to alanine (HIF-1αPP), and the stability of HIF-1α was analyzed with Western blotting. Equal amounts of pCMV-CD20 plasmid were cotransfected in each condition and served as a transfection efficiency control. *Bottom,* amounts of HIF-1α protein normalized to CD20.

HIF-1α and decreased almost back to the control levels by PHD2 (Fig. 5*C*). MAGE-11 partially reversed this down-regulation by inactivating ectopically expressed PHD2. Similar results were obtained with the HIF-responsive CITED2 promoter-luciferase construct (data not shown). However, when we replaced wild-type HIF-1α with the double mutant HIF-1αPP (Pro402A and Pro564A), which cannot be hydroxylated by PHDs, the luciferase reporter activation by HIF-1αPP was not affected by PHD2 or MAGE-11 (Fig. 5*D*).

Knockdown of MAGE-11 reduces HIF-1α induction by hypoxia and decreases activation of HIF target genes. The MAGE gene family was first discovered in melanoma patients but has subsequently been found in a broad range of tumors. We tested several cell lines routinely used in our study and found that HeLa, U2OS, and MDA-MB435 cells express significant levels of MAGE-11 by quantitative RT-PCR (data not shown). These cell lines were further used to confirm the inhibition of PHD2 activity by siRNA down-regulating MAGE-11. We first identified an siRNA

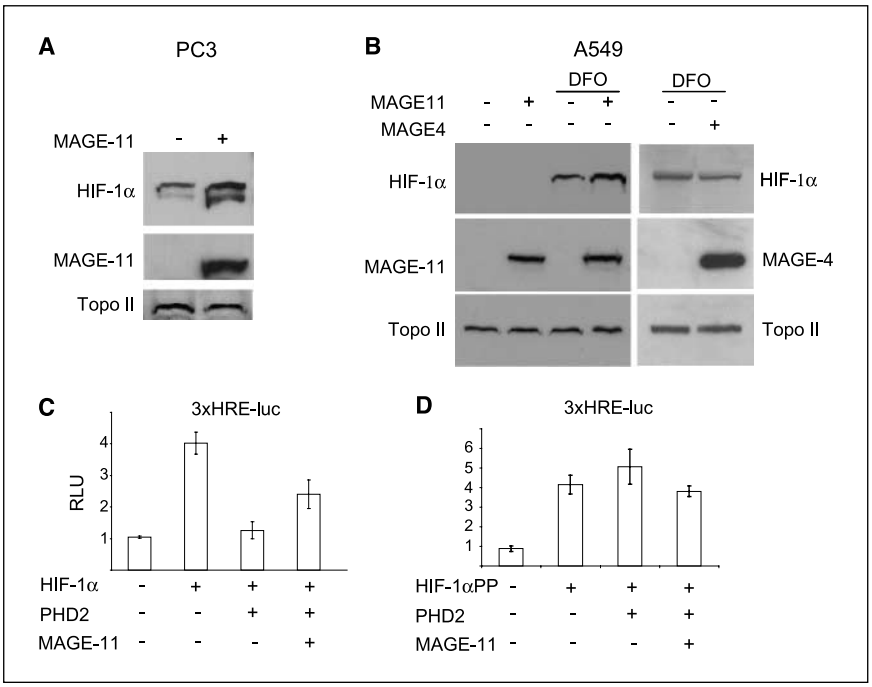

Figure 5. MAGE-11 increases the protein abundance and transcriptional activity of endogenous HIF-1α. Endogenous HIF-1α protein was analyzed after overexpression of MAGE-11 in PC3 cells (*A*) or A549 cells treated with DFO at 100 μmol/L for 16 h (*B*). HIF-1α (*C*) or HIF-1αPP (*D*) were cotransfected into 293T cells with or without PHD2 and MAGE-11 and equal amounts of 3× HRE-luciferase and pCMV-RL as an internal control. The experiments were performed in triplicate and repeated twice. *Columns,* mean; *bars,* SE.

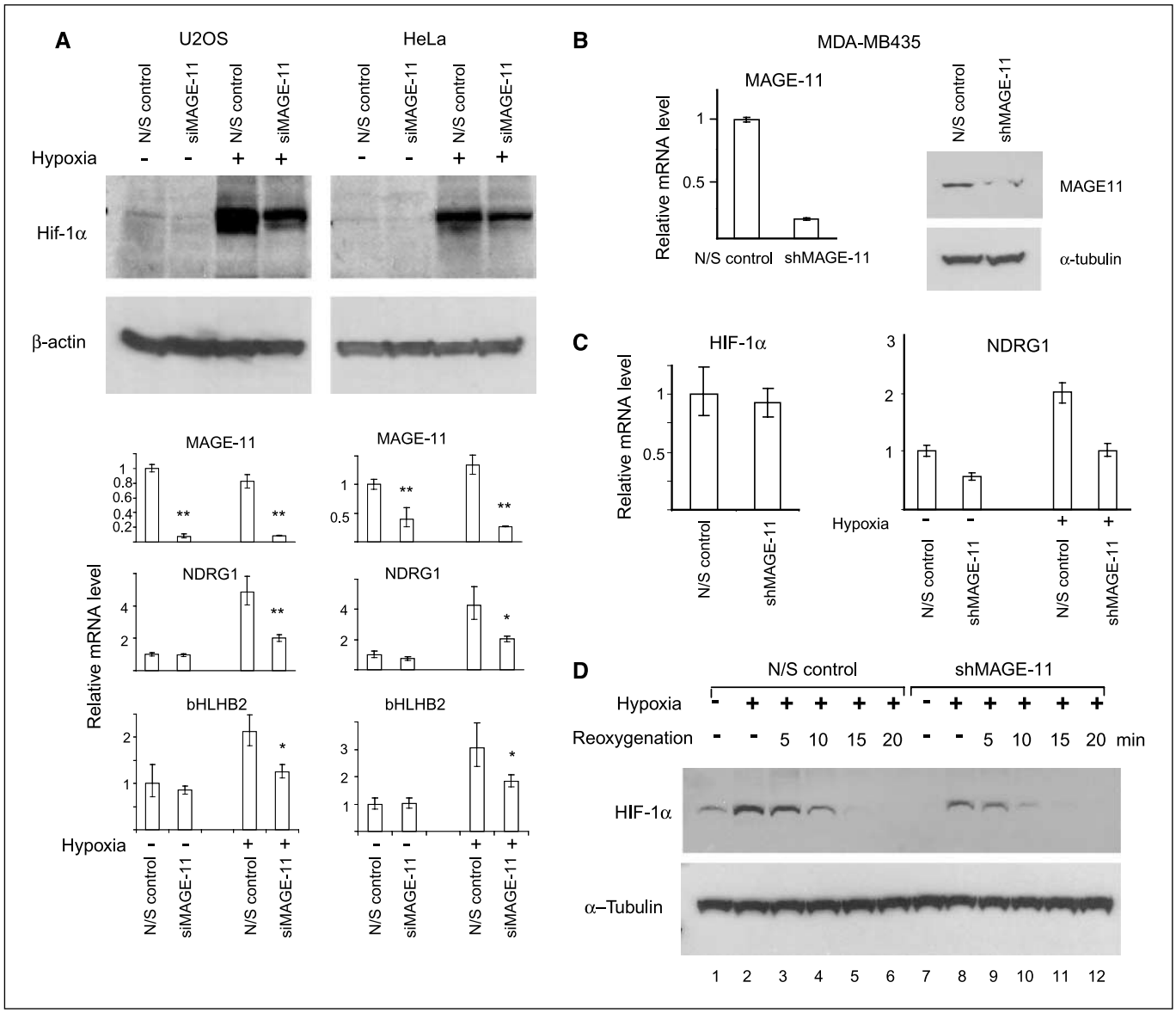

Figure 6. Down-regulation of MAGE-11 by siRNA results in impaired HIF-1α induction by hypoxia and inhibition of hypoxia-induced transcription of HIF target genes. *A,* the siRNA targeting MAGE-11 was transfected into U2OS or HeLa cells for two consecutive days, and in 24 h samples were collected for Western blotting (*top*) or quantitative RT-PCR analysis (*bottom*). *Columns,* mean of relative mRNA induction normalized to β-actin of at least three experiments; *bars,* SE. *P* values were obtained by paired *t* tests. **, $P < 0.005$; *, $P < 0.01$. *B,* stable expression of shMAGE-11 in MDA-MB435 cells results in down-regulation of MAGE-11 mRNA (*left*) and protein (*right*). *C,* expression of shMAGE-11 did not affect mRNA for HIF-1α but impaired the hypoxic induction of HIF target gene NDRG1. *D,* HIF-1α immunoblot analysis of 25 μg of nuclear extract after hypoxia-reoxygenation of shMAGE-11 MDA-MB435 cells. The experiment was repeated thrice. The representative experiment is shown.

oligonucleotide that suppresses MAGE-11 mRNA by 90% in U2OS cells and by 60% in HeLa cells (Fig. 6*A*). Western blot analysis confirmed that this siRNA down-regulated the MAGE-11 protein as well (Supplementary Fig. S3). When cells expressing siMAGE-11 were treated with hypoxia, we observed a lower HIF-1α protein stimulation (Fig. 6*A, top*) and a decrease in hypoxic induction of two HIF-regulated genes, NDRG1 and bHLHB2 (Fig. 6*A, bottom*) or HRE-driven luciferase reporter (Supplementary Fig. S3*B*).

Considering the predominant localization of MAGE-11 in cell nuclei identified here (Fig. 2) and by others (30, 34), it was conceivable that MAGE-11 may regulate the degradation of HIF-α subunits after reoxygenation of hypoxic cells. To test this hypothesis, we created the MDA-MB435 cell line, stably expressing shMAGE-11 hairpin under U6 promoter. Figure 6*B* confirms significant decrease in the levels of MAGE-11 mRNA and protein. Expression of shMAGE-11 did not affect the HIF-1α transcript levels but decreased the transcriptional activity of HIF, as supported by the low induction of the HIF target gene NDRG1 in hypoxic cells (Fig. 6*C*). To assess the effect of MAGE-11 on HIF-1α degradation rate, shMAGE-11 expressing and control cells subjected to hypoxia (1% oxygen) were collected after reoxygenation every 5 minutes during a 20-minute time period (Fig. 6*D*). The results show that, first, shMAGE expression decreased the protein levels of HIF-1α in the nuclei of normoxic cells (Fig. 6*D*, compare *lanes 1 and 7*), second, the magnitude of HIF-1α induction by hypoxia was significantly decreased (Fig. 6*D*, compare *lanes 2 and 8*),

and third, the half-life of HIF-1α was significantly shortened (Supplementary Fig. S4).

Taken together, these data indicate that MAGE-11 can modulate cellular responses to hypoxia. When overexpressed in cancer, it may augment HIF function by inhibiting prolyl hydroxylation and stabilizing HIF-α proteins even in the conditions of relatively normal oxygen concentrations.

Discussion

Several pathways that control stability and transcriptional activity of HIF in normal and pathologic conditions have been identified. Here, we found that the major HIF-1α regulator, PHD2, interacts with the melanoma antigen MAGE-11; this interaction results in the inhibition of PHD activity and an increase in the stability of HIF-1α. MAGE-11 belongs to the MAGE-A group of cancer-testis antigens that are normally expressed in male germ cells (some in the placenta of adult tissues) and aberrantly expressed in cancers (33, 40). Only recently, MAGE-A gene functions started to unravel. Recent publications showed that some members of the MAGE-A family are able to repress p53 transactivation (41). Specifically, MAGE-2 protein was able to physically interact with p53 and recruit transcriptional repressor histone deacetylase 3 to the MAGE/p53 complexes, resulting in a strong inhibition of p53 transcriptional activity and p53-dependent resistance of cells to etoposide (41). In agreement with these data, another group showed that several members of the MAGE-A family (A2, A3, A6, A11, and A12) were overexpressed in the ovarian cancer cell line SKOV3 as an early event in developing paclitaxel/doxorubicin resistance (42).

Yeast two-hybrid analysis identified MAGE-11 as a partner of AR (30). Further study has shown that MAGE-11 interacts with an NH_2 terminal FXXLF motif in AR that increases the ability of AR to interact with transcriptional coactivators and stimulate AR transcriptional activity. When a prostate-specific antigen (PSA) promoter was used as part of a reporter construct, MAGE-11 significantly stimulated reporter activation by AR. Interestingly, when the FXXLF consensus sequence of AR was mutated, MAGE-11 was still capable of partially activating the PSA promoter, independent of its interaction with AR (30). It is known that PSA is induced by hypoxia through a hypoxia-response element in the promoter of PSA by HIF-1, in cooperation with AR (43). Therefore, in addition to AR stimulation, activation of the hypoxia pathway by MAGE-11 could account for the induction of the PSA reporter.

We also found that MAGE-11 protein is regulated by proteasomal degradation, independent of PHD-VHL ubiquitin ligase pathway. When this manuscript was in preparation, the paper was published confirming that, indeed, MAGE-11 protein undergoes ubiquitinylation by yet unidentified ubiquitin ligase after an epidermal growth factor–stimulated phosphorylation (44).

How common is the MAGE-A gene expression in different malignancies? MAGE-1 has been studied most extensively. Its expression was found in almost all tumor types tested with glioma and hepatocellular carcinoma, reaching up to 86% incidence (for review, see ref. 40). We recently tested a large set of uterine cancers for the expression of cancer-testis antigens. The most represented member of this group was MAGE-9, which was expressed in 24 of 122 tumors and not expressed in any of the 10 normal tissue samples (45). In our study, we found that MAGE-9 also interacts with PHD2 (Fig. 1*D*).

Identification of MAGE-A proteins as novel activators of HIFs raises a very interesting possibility for new therapeutic approaches and diagnostic tools. This family represents an ideal target for therapeutic intervention due to the lack of their expression in normal adult tissues. However, additional research is required to fully characterize functions of MAGE-A proteins.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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