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MAGE-11 inhibits the hypoxia-inducible factor prolyl hydroxylase PHD2 and activates hypoxic response

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Abstract

Activation of hypoxia-inducible factors (HIFs), 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 (PHDs), and subsequent proteasomal degradation via the von Hippel-Lindau (VHL) ubiquitin ligase. Using a yeast-two hybrid screen, we identified an interaction between MAGE-11 cancer-testis antigen and the major HIF-α hydroxylating enzyme PHD2. The interaction was confirmed by pull-down assay, co-immunoprecipitation and co-localization in both normoxic and hypoxic conditions. Furthermore, MAGE-9, the closest homolog 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. Knock-down of MAGE-11 by siRNA results in decreased hypoxic induction of HIF-1α and its target genes. Inhibition of PHD by MAGE-11 and following activation of hypoxia-inducible factors 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.

Keywords

HIF; hypoxia; MAGE-11; PHD2

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 (HIF1-3) which activate transcription of angiogenic factors and a plethora of enzymes supporting anaerobic glycolysis and high glucose consumption. Hypoxia-inducible factors are heterodimeric complexes composed of regulated HIF-alpha subunits and constitutively expressed HIF-beta subunits or ARNT (1-3). HIF-α

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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-beta, 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 named PHD1,2,3 (also called EGLN2,1,3, or HPH3,2,1 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 402 and 564 in the ODDD of HIF-1α and the corresponding proline residues of HIF-2α (4,5,9). These proteins utilize non-heme iron for their catalytic activity and the compounds chelating iron are inhibiting PHD activity that lead 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 upregulation (9). Another dioxygenase family member, factor-inhibiting HIF (FIH), controls HIF transcriptional activity by hydroxylating asparagine 803, thereby preventing HIF interaction with transcriptional co-activators p300/CBP (13-16). This dual control mechanism provides a tight regulation of HIF activity in response to oxygen availability.

The three mammalian HIF-prolyl 4-hydroxylase 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 prolyl hydroxylases (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 3 protein turnover is regulated by the E3 ubiquitin ligases SIAH1/2, which are induced by hypoxia, and lowers availability of PHD1/3 to ensure full HIF-α induction (25). The PHD2 protein stability, however, appears to be regulated by an interaction with FK506-binding protein 38 (FKBP38), leading to destabilization of the PHD2 (26). Interestingly, PHD2 protein has a hydroxyprolyl-independent function in hypoxic cell nuclei, where it binds through HIF to hypoxia-responsive promoters and downregulates 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).

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 the melanoma antigen A11 (MAGE-A11 or MAGE-11). MAGE-11 is a member of a family of 12 MAGE-A proteins, that represent 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 re-expressed 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. Downregulation of MAGE-11 by RNA interference results in diminished HIF-1α

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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 hours. 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 co-transfected 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 (Invitrogen) and Penicillin/Streptomycin (Invitrogen) in humidified air containing 5% CO2 at 37°C. Hypoxia treatment was carried out in a modular incubator chamber (Billaps-Rothenberg) with the mixture containing different percentage of oxygen, 5% CO_2 and balanced with nitrogen. Deferoxamine mesylate (DFO), o-Phenanthroline, proteasomal inhibitor MG132 and dimethyloxallyl glycine (DMOG) were purchased from Sigma-Aldrich Inc.

Plasmids

MAGE-11 aminoacids 2-429 were PCR-amplified from a human testis cDNA library (Clontech) and subcloned into the 3xFlag-CMV10 expression vector (Sigma) or pCR2.1-TOPO (Invitrogen) for *in vitro* translation, or into pGEX5x-1 (Amersham) for expression in *E.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 C-terminal Flag tag (NCI- Frederick), 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 (UNC at Chapel Hill). GHO-plasmid was from Dr. M.C.Simon (University of Pennsylvania), the V5-PHD2 was from Dr. R.Bruick (UT Southwestern Medical Center, Dallas, TX), and Flag-tagged PHD1-3 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, 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 1-2 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 co-immunoprecipitation, cells were lysed in NP40 buffer containing 50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 0.5% NP40 and protease (Sigma) and phosphatase (Pierce) inhibitors. 300 µg of total protein were 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

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washed several times with NP40 buffer, blocked with 5% BSA for 1 hour and 30 μl were used to collect the protein complexes for 4 -16 hours at 4°C. The agarose was washed with NP40 buffer 3 times, and bound proteins were resolved in 10% SDS-PAGE. Immunoprecipitation of endogenous PHD2 and MAGE-11 from HeLa cells was performed with approximately 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 ul of Protein A/G Sepharose for 1 hour at 4°C and washed 4 times with lysis buffer supplemented with 1 mg/ml BSA and two times with the same buffer without BSA.

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

The antibodies used for Western blotting were: anti-Flag-HRP (Sigma), anti-V5-HRP (Invitrogen), anti-PHD2 rabbit polyclonal (Novus Biologicals), anti-HIF-1α (BD Biosciences), anti-HA (Cell Signalling), anti-MAGE-11 (MabAb94, kindly provided by Dr. Wilson, UNC at Chapel Hill), anti-VHL (BD Biosciences), anti-α-tubulin (Sigma), and anti-actin (Sigma).

siRNA transfections

SiRNAs for MAGE-11 knock-down were purchased from Dharmacon (D-017280-03) as well as Non-Silencing control siRNA #2. The duplexes were transfected into HeLa or U2OS cells as described before (29), except that HeLa cells were transfected with 100nM 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 Non-silencing shRNAmir control (Open Biosystems, RHS1703) followed by selection with 2 μg/ml of Puromycin.

VHL-binding assay

Following transfection cells were scraped in ice-cold reaction buffer containing 20mM Tris-HCl, pH 7.5; 5mM KCl, 1.5mM MgCl2 and protease inhibitor cocktail and disrupted by sonication. Synthetic peptide containing 19 HIF-1α aminoacids: DLDLEMLAPYIPMDDDFQL or DLDLEMLAP(OH)YIPMDDDFQL biotinylated on N-terminus (2 μg per reaction) were first captured with streptavidin-conjugated magnetic beads in 200 μl of NP40 buffer for 2 hours. The beads were washed twice in NP40 buffer and twice in the Reaction buffer and incubated with 50-100 μg of total protein in the presence of 0.5 mM α-ketoglutarate, 0.1 mM FeCl2 and 0.5 mM ascorbate for 30 min at 30°C. The beads were washed three times with NP40 buffer and incubated with 10 μl of *in vitro* translated HA-VHL for 1 hour at 4°C. The beads were washed three times 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). 1 μl of the cDNA was used for PCR with PCR master mix (Applied Biosystems) and 1.25 μl of 20xprimer-probe mix 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

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data were normalized to the amount of β-actin and presented as a fold change compared to control cells. MAGE-11 primers used for PCR in qRT-PCR reaction, were spanning the boundaries of exons 2-3 (Applied Biosystems, Hs00377815_m1), bHLHB2 - exons 2-3 (Applied Biosystems, Hs00186419_m1), and NDRG1 - exons 13-14 (Applied Biosystems, Hs00608389_m1).

Luciferase reporter assay

Cells were split at 0.5×10^5 into 12-well plates and transfected 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 (Roch). 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 activity of firefly and renilla luciferase with Dual Luciferase Reporter Assay System (Promega).

Confocal microscopy

HeLa cells were grown on coverslips and the next day placed in 0.5% oxygen for 16 hours or left untreated. Cells were fixed with 4% paraformaldehyde for 10 min and then treated with 0.1 M glycin for 1 hour. 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 hour at room temperature. Cell nuclei were stained with DAPI using Prolong Gold Antifade Reagent (Invitrogen). Stained cells were observed on a Zeiss Axiovert 100M microscope equipped with a 100X/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 hours. 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 aa70-420) 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 et al. (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 (aa202-301) 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 signaling pathway (30), but, neither a link to hypoxia nor a regulation of hypoxia-inducible factors 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

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(Supplementary Fig. S1A) or lysates of 293T cells overexpressing PHD2 (Supplementary Fig. S1B). We further confirmed the association between MAGE-11 and PHD2, using 293T cells co-transfected 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. 1A). Interestingly, the oxygen level had no effect on the interaction, as MAGE-11 co-precipitated with PHD2 equally well in hypoxic and in normoxic conditions. In order to co-precipitate 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 N-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. 1B).

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 paralogs 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. 1C).

The fragment of MAGE-11 identified in the yeast two-hybrid screen spanned the region of aa212-421. 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 co-immunoprecipitation, while MAGE-4 was not (Fig. 1D). 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 co-localizes 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 MAGEA-11 (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 co-localization of MAGE-11 with PHD2 proteins in cytoplasm and less, but still obvious, co-localization 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

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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 following 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. S2A). This phenomenon was independent of whether or not PHD2 was overexpressed together with MAGE-11 (Supplementary Fig. S2A). The inhibition of endogenous PHD activity by hypoxia or by the iron-chelating agents deferoxamine mesylate and o-phenanthroline, did not affect MAGE-11 accumulation either (Supplementary Fig. S2B). 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. S2C). 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. S2D), but were unresponsive to hypoxia or PHD inhibition (Supplementary Fig. S2E).

MAGE-11 inhibits prolyl hydroxylase activity

Several proteins have been identified that modulate PHD activity either by bridging PHDs with HIF-α (OS-9) (24), destabilizing the PHD2 protein (FKBP8) (26), or causing suppression of HIF transcriptional activity under hypoxia (ING4) (28). 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 co-expressed with the increasing amounts of MAGE-11 in HeLa or Hep3B cells (Fig. 3A and B, bottom panels). Similarly, there was no change in endogenous PHD2 protein levels in normoxic or hypoxic MDA-MB435 (Fig. 3C) or 293 cells overexpressing MAGE-11 (data not shown). We then tested the effect of MAGE-11 on prolyl hydroxylase activity by using a VHL-binding assay. This *in vitro* reaction utilizes a 19-amino acid peptide derived from the ODDD of HIF-1α, containing Pro-564 that 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. The top panels in Fig. 3A show that an increase in MAGE-11 in cell extracts results in decreased binding of VHL. This effect was specific because MAGE-4 did not change PHD activity (Fig. 3B). Because pVHL binds only to the hydroxylated peptide, these results indicate that PHD activity is suppressed by MAGE-11 protein.

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 of a GAL4 DNA binding domain and HA-tagged HIF-1α ODDD fragment. This recombinant protein is hydroxylated by all three prolyl hydroxylases and undergoes a proteasomal degradation after ubiquitination by pVHL and previously served as a useful model to study HIF-α proline hydroxylation (38). Fig. 4A shows that when overexpressed in 293T cells, GHO protein abundance was greatly increased by co-expression with MAGE-11. This effect was further magnified by treatment of cells with 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.4B). The increase in GHO stability was specific because co-expression of PHDs together with GHO and MAGE-11 diminished the ability of MAGE-11 to stabilize GHO

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(Fig. 4C). The degree to which each PHD abrogates the effect of MAGE-11 seems to be different, with PHD2 protein being the most potent (Fig.4C). 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, stable form of HIF-1α, mutated at both Pro402 and Pro564, was unaffected by MAGE-11 (Fig. 4D).

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 (39), MAGE-11 overexpression resulted in increasing the amounts of HIF-1α protein (Fig. 5A). 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. 5B). Transfection with MAGE-4, that did not interact with PHD2 (Fig.1D), did not affect the levels of HIF-1α induction by DFO in A549 cells (Fig.5B). To estimate if ectopic levels of MAGE-11 were in the average range of endogenous levels observed in tumor cells, we performed an 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 to untransfected HeLa cells (data not shown).

We also tested the effect of MAGE-11 on HIF-1α transcriptional activity by using a 3xHRE-luciferase construct in the reporter assay. The reporter luciferase activity was increased several fold by HIF-1α and decreased almost back to the control levels by PHD2 (Fig. 5C). MAGE-11 partially reversed this downregulation 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), that can not be hydroxylated by PHDs, the luciferase reporter activation by HIF-1αPP was not affected by PHD2 or MAGE-11 (Fig.5D).

Knock-down 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 downregulating MAGE-11. We first identified a siRNA oligonucleotide that suppresses MAGE-11 mRNA by 90% in U2OS cells and by 60% in HeLa cells (Fig. 6A). Western blot analysis confirmed that this siRNA downregulated 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. 6A, top panels) and a decrease in hypoxic induction of two HIF-regulated genes, NDRG1 and bHLHB2 (Fig. 6A, lower panels) or HRE-driven luciferase reporter (Supplementary Fig.3B).

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 following reoxygenation of hypoxic cells. To test this hypothesis, we created MDA-MB435 cell line, stably expressing shMAGE-11 hairpin under U6 promoter. Fig. 6B 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 low induction of HIF target gene NDRG1 in hypoxic cells (Fig. 6C). In order 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 following reoxygenation every 5 min during 20 min time period (Fig.6D). The results show that first, shMAGE expression decreased

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the protein levels of HIF-1α in the nuclei of normoxic cells (Fig.6D, compare lanes 1 and 7), second, the magnitude of HIF-1α induction by hypoxia was significantly decreased (Fig. 6D, 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 have been identified that control stability and transcriptional activity of HIF in normal and pathological conditions. 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 strong inhibition of p53 transcriptional activity and p53-dependent resistance of cells to etoposide (41). In agreement with this 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 the androgen receptor (AR) (30). Further study has shown that MAGE-11 interacts with an N-terminal FXXLF motif in AR that increases the ability of AR to interact with transcriptional co-activators 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 and HIF-1 cooperation with AR (43). Therefore, in addition of AR stimulation, activation of the hypoxia pathway by MAGE-11 could account for the induction of 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 EGF-stimulated phosphorylation (44).

How common is MAGEA 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 (40). We recently tested a large set of uterine cancers for the expression of cancer-testis antigens (CTA). The most represented member of this group was MAGE-9, which was expressed in 24 of 122 tumors and not expressed in any of 10 normal tissue samples (45). In our study we found that MAGE-9 also interacts with PHD2 (Fig. 1D).

Identification of MAGEA proteins as novel activators of hypoxia-inducible factors raises a very interesting possibility for new therapeutic approaches and diagnostic tools. This family

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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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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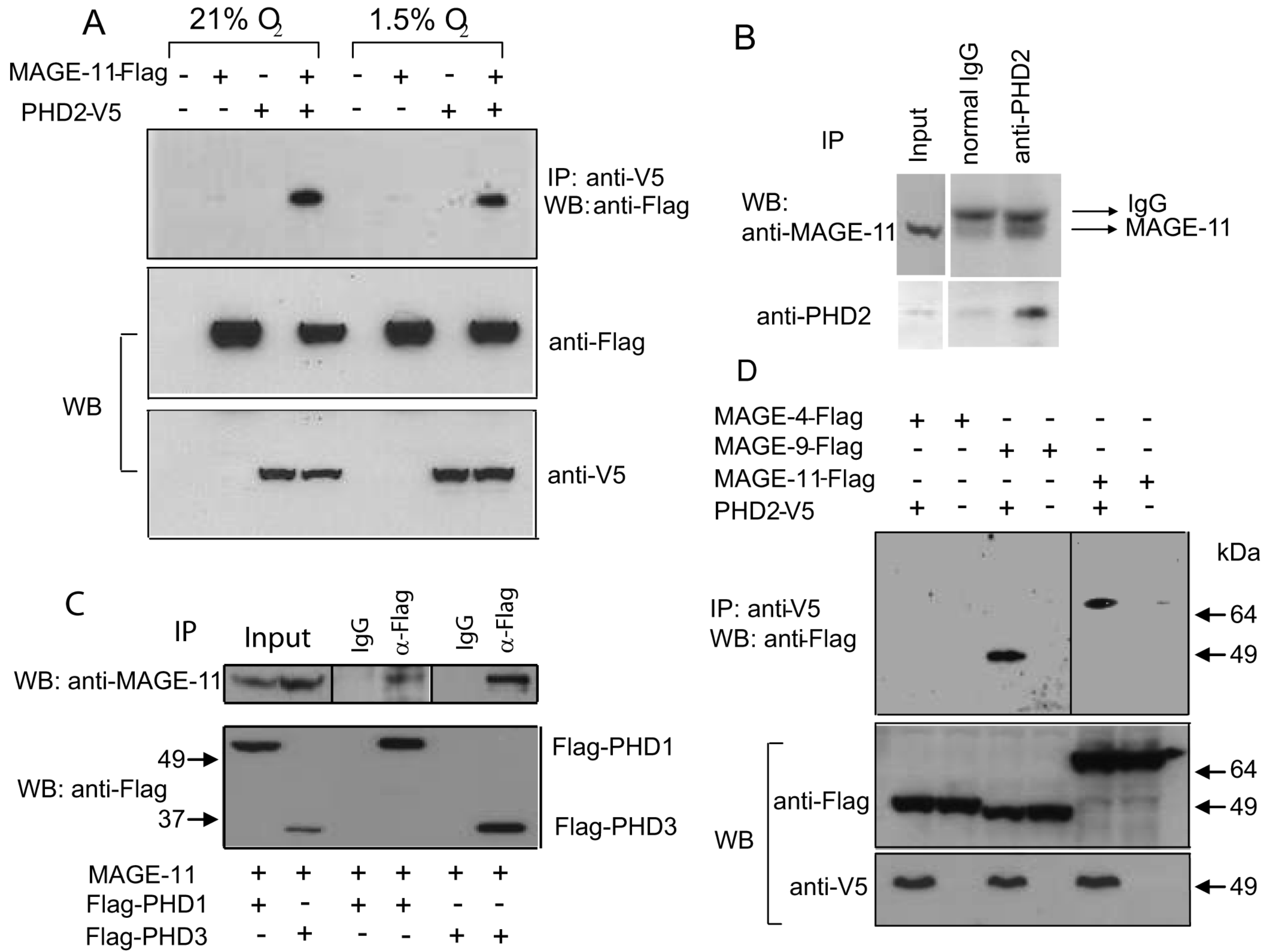

Figure 1.

MAGE-11 interaction with PHD2. (A) Flag-MAGE-11 and V5-PHD2 were cotransfected into 293T cells and two days later treated with 1.5% oxygen or left untreated for 16 hours. Immunoprecipitation was performed with anti-V5 agarose, followed by Western blotting with anti-Flag antibodies. (B) Co-immunoprecipitation 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 panel) or anti-PHD2 antibody (lower panel). (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 MAGEA 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.

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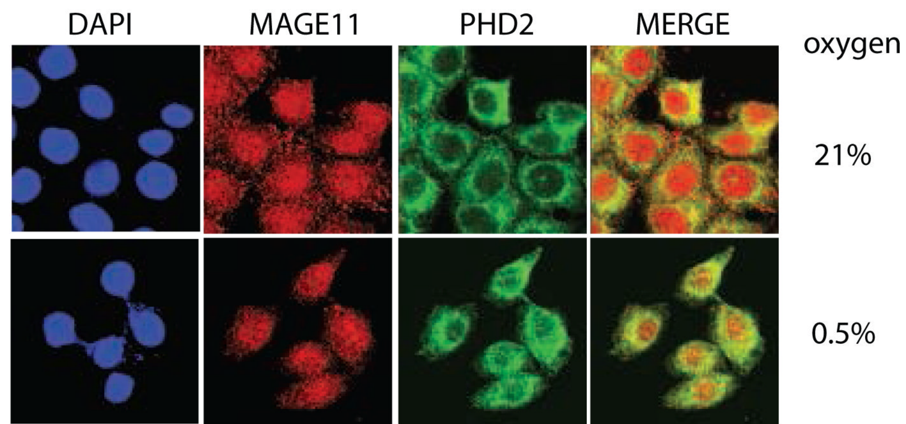

Figure 2.

PHD2 co-localizes 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.

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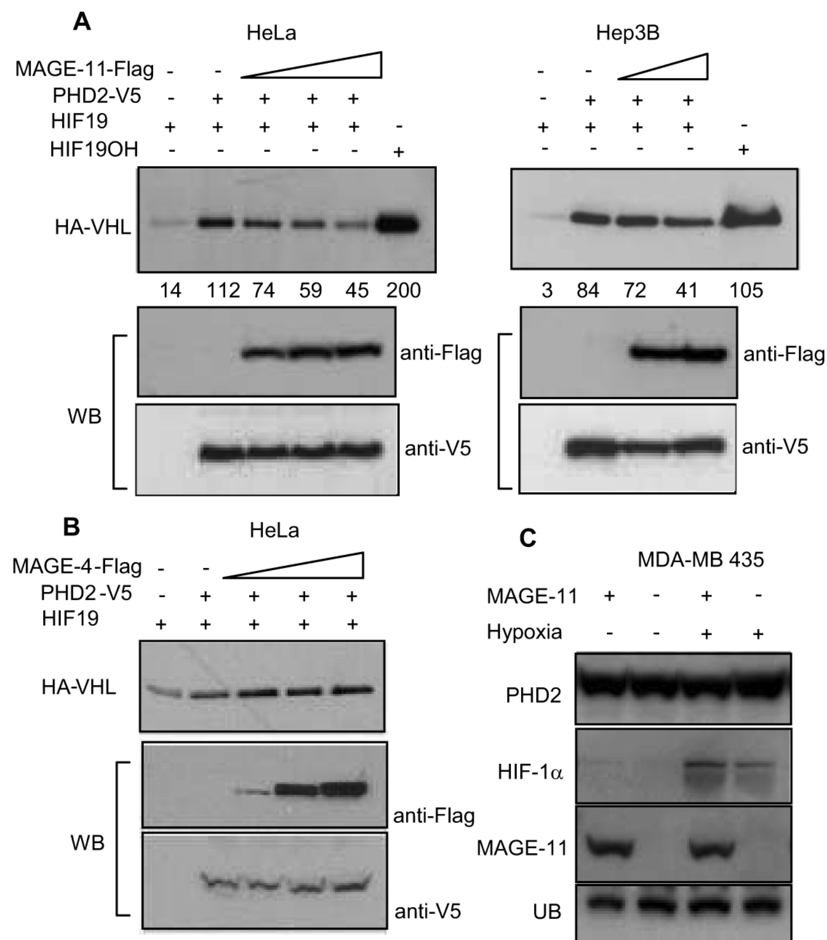

Figure 3.

MAGE-11 suppresses enzymatic activity of PHD2. (A) V5-PHD2 and increasing amounts of Flag-MAGE-11 were co-expressed in HeLa and Hep3B cells, and 48 hours later, 100 μg of protein were used to hydroxylate HIF-1α-derived 19 amino acid peptide (HIF19), followed by binding with *in vitro* translated HA-VHL (top panels). The last lanes in both panels show VHL binding to the same peptide that was chemically hydroxylated on Pro-564 and served as a positive control. The numbers under the top panels 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.

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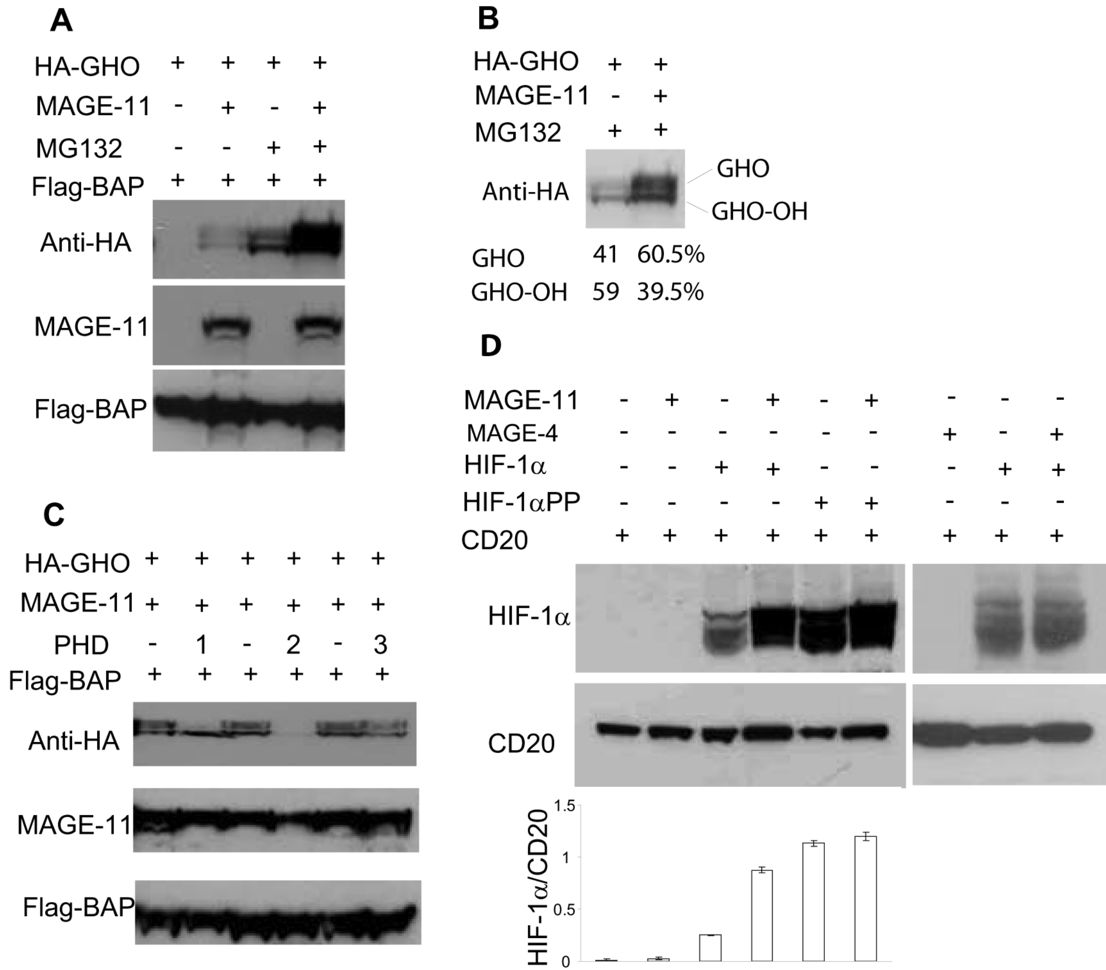

Figure 4.

MAGE-11 increases the stability and transcriptional activity of HIF-1α. (A) A plasmid containing Gal4 DNA binding domain and HA-tagged HIF-1α ODD (amino acids 496-626) domain (HA-GHO) was co-transfected with pSG5-MAGE-11, and 2 days later, cells were treated with 10 μM MG132 for 4 hours. 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 Fig.4A, 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) Co-expression with PHDs completely (PHD2) or partially (PHD1 and 3) eliminates the ability of MAGE-11 to stabilize GHO protein. MAGE-11 and HA-GHO were co-transfected into 293T cells with or without Flag-tagged PHD1, 2 or 3. (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 co-transfected with the full-length wild-type HIF-1α or HIF-1α with two proline residues (P402 and P564) mutated to alanine (HIF-1αPP), and the stability of HIF-1α was analyzed with Western blotting. Equal amounts of pCMV-CD20 plasmid were co-transfected in each condition, and served as a transfection efficiency control. The bottom panel in Fig. 4D shows the amounts of HIF-1α protein normalized to CD20.

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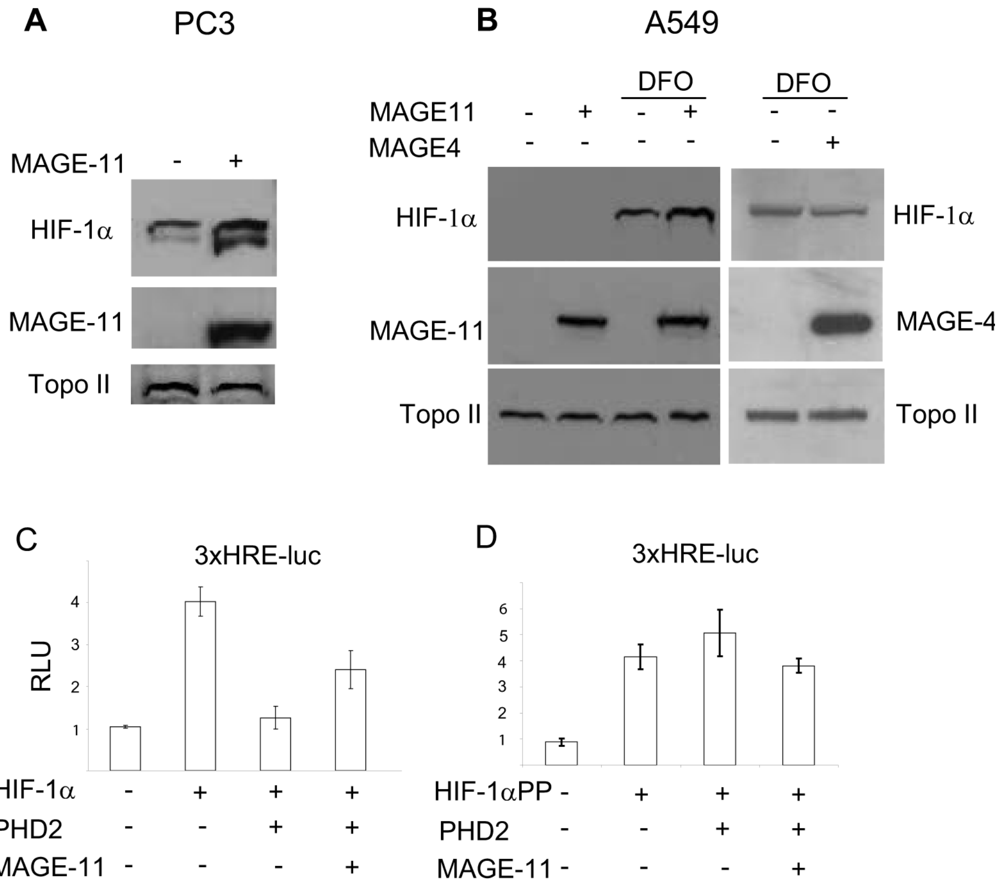

Figure 5.

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 μM for 16 hours (B). (C and D) HIF-1α (C) or HIF-1αPP (D) were co-transfected into 293T cells with or without PHD2 and MAGE-11 and equal amounts of 3xHRE-luciferase and pCMV-RL as an internal control. The experiments were performed in triplicate and repeated twice. The data show the mean values ± s.e.m.

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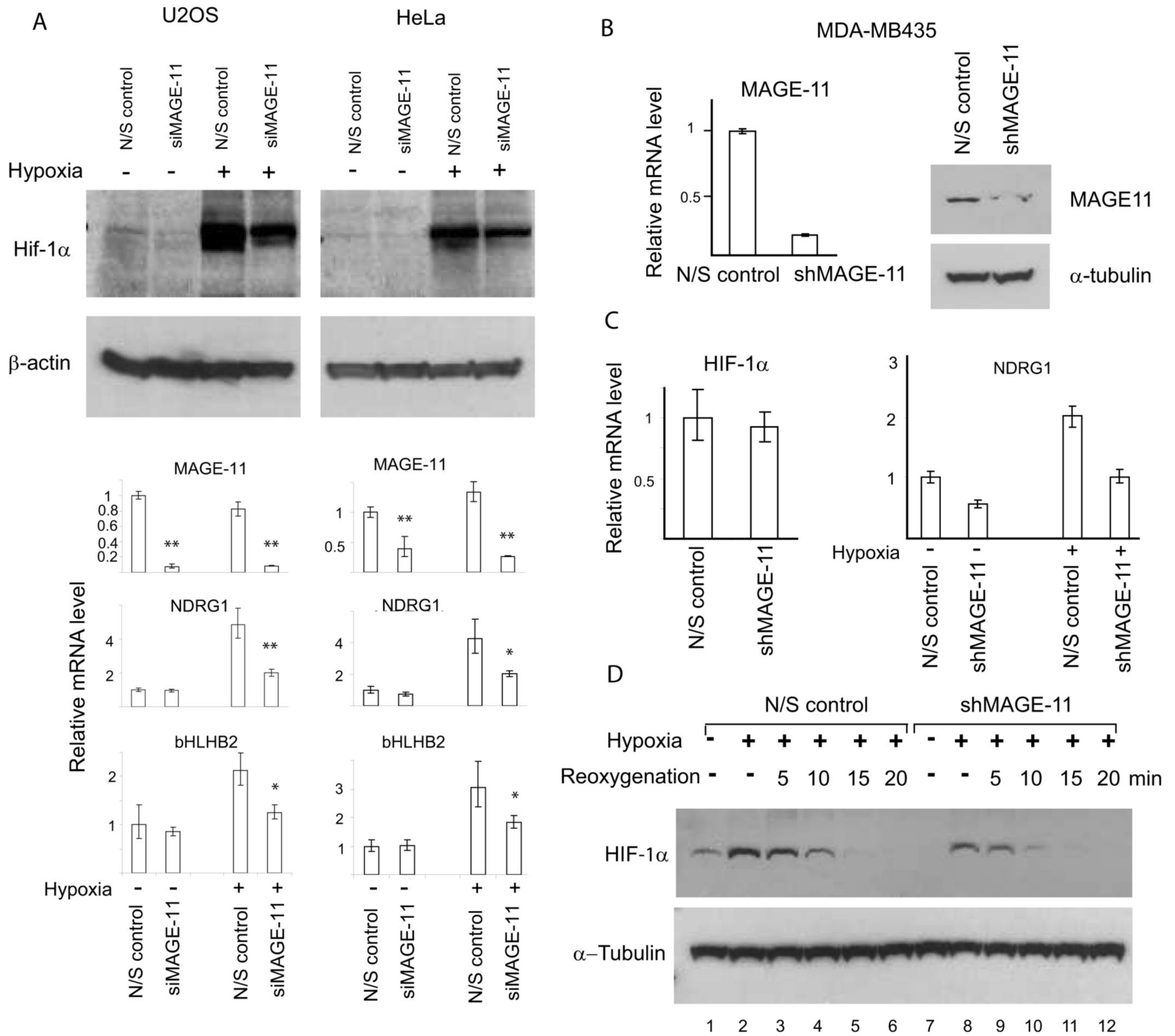

Figure 6.

Downregulation 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 hours samples were collected for Western blotting (top panels) or quantitative RT-PCR analysis (bottom panels). The results are mean values of relative mRNA induction normalized to beta-actin plus standard errors of the mean of at least three experiments. 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 downregulation of MAGE-11 mRNA (left panel) and protein (right panel). (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 three times. The representative experiment is shown.