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Hypoxia-inducible factor-2α regulates the expression of TRAIL receptor DR5 in renal cancer cells

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To understand the role of hypoxia-inducible factor (HIF)-2α in regulating sensitivity of renal cancer cells to tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis, we transfected wild-type and mutant von Hippel Lindau (VHL) proteins into TRAIL-sensitive, VHL-negative A498 cells. We find that wild-type VHL, but not the VHL mutants S65W and C162F that do not degrade HIF proteins, cause TRAIL resistance. Knock down of the HIF-2α protein by RNA interference (short hairpin RNA) blocked TRAIL-induced apoptosis, decreased the level of TRAIL receptor (DR5) protein and inhibited the transcription of DR5 messenger RNA. By using luciferase constructs containing the upstream region of the DR5 promoter, we demonstrate that HIF-2α stimulates the transcription of the *DR5* gene by activating the upstream region between −448 and −1188. Because HIF-2α is thought to exert its effect on gene transcription by interacting with the Max protein partner of Myc in the Myc/Max dimer, small interfering RNAs to Myc were used to lower the levels of this protein. In multiple renal cancer cell lines decreasing the levels of Myc blocked the ability of HIF-2α to stimulate *DR5* transcription. PS-341 (VELCADE, bortezomib), a proteasome inhibitor used to treat human cancer, increases the levels of both HIF-2α and c-Myc and elevates the level of DR5 in renal cancer, sensitizing renal cancer cells to TRAIL therapy. Similarly, increasing HIF-2α in prostate and lung cancer cell lines increased the levels of DR5. Thus, in renal cancer cell lines expressing HIF-2α, this protein plays a role in regulating the levels of the TRAIL receptor DR5.

Introduction

Renal cell carcinoma (RCC) affects >30 000 individuals each year and is responsible for >12 000 deaths (1). These tumors are both radio- and chemoresistant and show minimal therapeutic responses to interleukin-2, interferon γ, tumor-infiltrating lymphocytes and small molecules aimed at inhibiting growth factor receptors, including epidermal growth factor, vascular endothelial growth factor and platelet-derived growth factor receptors (2,3). New therapies are clearly needed to treat renal cancer.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a proapoptotic protein that displays minimal toxicity toward normal cells, both *in vitro* and *in vivo*, and kills a wide spectrum of tumor types including renal cancer (4–6). TRAIL binds to two receptors, DR5 (TRAIL-R2) and DR4 (TRAIL-R1) (7), as well as to two receptors that do not conduct an apoptotic signal, DcR1 and DcR2 (8,9). Cytotoxic antibodies, lexatumumab, directed at the TRAIL receptor DR5, when used in an orthotopic metastatic tumor model, decrease the size and metastatic potential of renal tumors that are known to be sensitive to TRAIL in tissue culture (10,11). Resistance to TRAIL in cancer cells, including prostate and multiple myeloma, can be overcome by the addition of VELCADE (PS-341), a proteasome inhibitor (12). This agent functions to increase the number of TRAIL receptors both transcriptionally and by preventing their degradation (12). PS-341 also enhances the ability of TRAIL to induce cell death by increasing the levels of BH3 proteins including Noxa, Bik and Bim (13–15). Thus, TRAIL treatment of renal cancer offers a novel approach to treating this disease.

Although the exact cause of renal cancer is obscure, 60–80% of RCC have biallelic loss/inactivation of von Hippel Lindau (VHL) tumor suppressor gene as a consequence of gross genetic loss, nonsense or missense point mutations and hypermethylation of the *VHL* locus (16–18). Because VHL functions as part of Skp1/Cul1/F-box-like E3 ligase for the hypoxia-inducible factor (HIF) proteins (19,20), deletion of VHL leads to increased levels of HIF-1α, HIF-2α or both proteins. VHL protein has complex functions unrelated to its regulation of HIF proteins including a role in the synthesis and degradation of extracellular cell matrix by affecting the transport of fibronectin (21), in the regulation of cytoskeletal organization and motility through focal adhesion formation and in the translocalization of fibroblast growth factor receptor (22–24). VHL also regulates RNA stability through increasing the level of RNA-binding proteins (25) and messenger RNA (mRNA) transcription, e.g. the tyrosine hydroxylase gene, by directly interacting with transcription factors, e.g. Sp1 (26). The suppression of the nuclear factor-κB pathway by VHL (27) is thought to play a role in regulating the sensitivity of renal cancer cells to varied apoptotic stimuli. However, the mechanism by which VHL and HIF proteins control the sensitivity of renal cancer cells to TRAIL is unknown.

In normoxic conditions following oxygen-dependent prolyl hydroxylation, the HIF proteins are degraded by the proteasome (28). In VHL-defective RCC cells, the HIF system is activated and a constitutively hypoxic pattern of gene expression is observed, including increases in the Bnip3, cyclin D1, transforming growth factor-α and vascular endothelial growth factor (29,30) proteins. In RCC, there is bias toward HIF-2α rather than HIF-1α expression (31), and HIF-1α actually inhibits the action of genes, i.e. β-catenin, that might enhance the growth of certain tumors (32). Overexpression of HIF-2α increases tumor growth, whereas HIF-1α appears to have the reverse effect (31). These two genes have contrasting properties on specific transcription, with HIF-1α positively and HIF-2α negatively regulating the Bcl-2-like protein Bnip3 (31). In VHL-defective renal cancer cell lines, cyclin D1 and transforming growth factor-α mRNAs are increased by HIF-2α, whereas in this model, HIF-1α had little effect on the expression of these genes (31). Opposite effects on Myc-regulated genes by these two proteins have also been demonstrated (33,34). However, it is not known how or whether HIF proteins regulate sensitivity to TRAIL-induced death.

To better understand how TRAIL might be effectively used to treat renal cancer, we have investigated the role of HIF-2α in the sensitivity of these tumor cells to TRAIL. We find A498 cells that express elevated levels of HIF-2α are sensitive to TRAIL, and overexpression of VHL or expression of a short hairpin RNA (shRNA) that decreases the level of HIF-2α mRNA and protein abrogates this sensitivity. The low or absent levels of HIF-2α decrease the levels of TRAIL receptor DR5 mRNA and protein, explaining the loss of sensitivity. Small

Abbreviations: cDNA, complementary DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HIF, hypoxia-inducible factor; mRNA, messenger RNA; PCR, polymerase chain reaction; RCC, renal cell carcinoma; shRNA, short hairpin RNA; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; VHL, von Hippel Lindau.

interfering RNAs (siRNAs) directed at Myc decrease the ability of HIF-2α to stimulate the transcription of this gene in both TRAIL-sensitive and -resistant cell lines, suggesting that these proteins may work independently or co-ordinately to regulate HIF-2α. Our data point to the importance of HIF-2α when present in renal and other cancer types in controlling the transcription of the DR5 gene.

Materials and methods

Cell lines and reagents

A498, 786-O, Caki-1 and HK-2 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle (high glucose) medium supplemented with 10% fetal bovine serum. Human VHL and HIF-2α genes were polymerase chain reaction (PCR) cloned into pEGFP-C1 (Clontech, Mountain View, CA) and pCDNA3.0 (Invitrogen, Carlsbad, CA), respectively. The pCDNA3.1 plasmid containing shRNAs-targeting HIF-2α (shRNA I sense:5′-GCGACAGCTGGAGTATGAA-3′ and shRNA II sense:5′-GAACAGCAAGAGCAGGTTC-3′) was constructed as described previously (23). Cells were transfected with complementary DNAs (cDNAs) using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions, and then FACS sorted for enhanced green fluorescent protein or Ds-Red. For stable expression of HIF-2α shRNA, A498 cells were selected with 1.5 μg/ml of G418 (Invitrogen). Recombinant His-tagged human TRAIL was purified from bacteria, as described previously (12).

SiRNAs

All siRNAs were purchased from Dharmacon (Lafayette, CO). HIF-2α and Myc siRNAs were a pool of four siRNAs of the following sequence—siHIF-2α: GGCAGCACCUCACAUUUGAUU, GAGCGCAAAUGUACCCAAUUU, GACAAGGUCUGCAAAGGGUUU and GCAAAGACAUGUCCACAGAUU; siMyc: ACGGAACUCUUGUGCGUAAUU, GAACACACAACGUCUUGGAUU, AACGUUAGCUUCACCAACAUU and CGAUGUUGUUUC-UGUGGAAUU. HIF-1α siRNA was a pool of two siRNAs of the following sequence—GAACAAAUACAUGGGAUUAUU and AGAAUGAAGUGUACCCUAAUU. The sequence of the control siRNA was UUCUCCGAACGUGUCACGUdTdT.

Apoptosis assay

To examine apoptosis, cells were treated with TRAIL for 16 h in growth medium. Flow cytometric analysis was done to detect and quantify apoptosis. Cells were fixed in 70% ethanol and stained with 50 μg/ml propidium iodide in hypotonic lysis buffer (0.1% sodium citrate and 0.1% Triton X-100) containing DNase-free RNase A for 30 min. Acquisition and analysis were performed by FACScan using Cell Quest Alias software (BD Biosciences, San Jose, CA). The sub-G_1 peak was assumed to contain dead or apoptotic cells. The colorimetric acid phosphatase assay was performed to determine the extent of survival of cells undergoing varied treatments, as follows: a 100 μl aliquot of buffer A [0.1 M sodium acetate (pH 5.5), 0.1% (vol/vol) Triton X-100 and 10 mM p-nitrophenyl phosphate (Sigma–Aldrich Chemicals, St Louis, MO)] was added to 100 μl of cell suspension in a 96-well plate. The plates were placed in the incubator at 37°C for 2 h. The reaction was stopped by the addition of 10 μl 1 M NaOH to each well, and the absorbance of each well was measured at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Chromatin immunoprecipitation assays

Using the chromatin immunoprecipitation assay kit from Millipore according to the manufacturer's recommendations direct binding of c-Myc to the *DR5* promoter was assessed. In brief, 1×10^7 A498 cells were fixed in 1% formaldehyde for 20 min to cross-link protein–DNA and protein–protein complexes. Chromatin was sheared to an average DNA size of 200–800 bp by sonication using Cole Palmer ultrasonic cell disrupter (10 times of 10 s pulses at 30% output using 2 mm tip). The chromatin fragments were immunoprecipitated with 4 μg of anti-Myc (sc-768; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit IgG (Sigma-Aldrich Chemicals, St Louis, MO) antibodies. Immunoprecipitates were washed with the recommended buffers, incubated overnight at 65°C to remove cross-linking and then treated with proteinase K for 2 h. DNA was purified by phenol–chloroform extraction and ethanol precipitation. DNA samples were analyzed by PCR by using the following pairs of primers: Myc-BS1, CCGAATGACGCCTGCCCG (forward) and GGAACGCTCTTATAGTCT (reverse); Myc-BS2, GCGGAGGATTGCGTTGAC (forward) and GCGGCTGTACTTTCACTGCC (reverse); Myc-BS3, GGACCCAGAAACAAACC (forward) and CACCACAGGTTGGTGAC (reverse); Myc-BS4, GCGGACTCTGAACCTCAAG (forward) and GGCTGTGGTTTGTTTCTGG (reserve); Myc-BS5, GCAGGAAGGAAGGAAAG (forward) and CTTGAGGTTCAGAGTCCG (reverse); Myc-BS6, GCTAAGTGTAGCAAGGGTG (forward) and GGACTACAGGCCTGCACC (reverse); Myc-BS7, GACACAGTACCATGAAGG (forward) and CTGTGTCCCTGCACCCTTG (reverse) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (control), TACTAGCGGTTTTACGGGCG (forward) and TCGAACAGGAGGAGCAGAGAGCGA (reverse).

PCR was performed at an annealing temperature of 55°C for 30 cycles. The products were analyzed in 1.5% agarose gel stained with ethidium bromide.

Site-directed mutagenesis

The pGVB2-DR5/−1188 construct was used as a template for site-directed mutagenesis to inactivate the c-Myc-binding site 5 (Myc-BS5), CAGGTG by PCR. The following nested primers were used to generate a mutant site (GGATCC): GAGCTCCCACCAGAAGGAAGAAACTCC (wild-type forward), CTTCCCAGGGGCGGATCCCCCTTCTGTTC (mutant reverse), GAACAGAAGGGGGATCCGCCCCTGGGAAG (mutant forward) and CCATGGCGGTAGGGAACGCTCTTATAG (wild-type reverse). The PCR product was cloned into pGVB2 vector via SacI and NcoI sites and the insert verified by sequencing.

The pcDNA3.1/HIF-2α plasmid was used as a template for PCR to mutate the HIF-2α shRNA target site (nucleotides 2127–2145, GCGACAGCTGGAGTATGAA) using the following nested primers: GAATTCTACCATGCGCTAG (wild-type forward), GCTTGCTCCTCGTATTCCAGCTGTCGC (mutant reverse), GCGACAGCTGGAATACGAGGAGCAAG (mutant forward) and GAATTCGCGGCCGCTCAGGTGGCCTGGTCCAGG (wild-type reverse). The mutant PCR fragment with EcoR I and Not I ends was cloned into pcDNA3.1/HIF-2α plasmid and cut with the same restriction enzymes to yield a full-length HIF-2α cDNA clone.

Flow cytometric detection for TRAIL receptor expression

The expression levels of DR4 and DR5 were evaluated by flow cytometry. Cells were seeded in six-well plates at 3×10^5 per well for 24 h prior to treatment. After 16 h of incubation with PS-341 (500 nM), cells were collected, washed twice with phosphate-buffered saline, incubated at 4°C with phycoerythrin-conjugated antibodies recognizing DR4 and DR5 (eBioscience, San Diego, CA) and then analyzed by flow cytometry.

Quantitative real-time mRNA analysis

Total RNA was isolated from A498 cells using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The first-strand cDNA was synthesized using Superscript first-strand synthesis kit and Oligo (dT) primer (Invitrogen). Relative quantification of DR5 expression was achieved by quantitative real-time PCR (iQ5 Multicolor Real-Time PCR detection system, Bio-Rad Laboratories, Hercules, CA) using iQ5 optical system software. The expression level of DR5 was normalized to GAPDH. The primers used for real-time PCR were as follows: *DR5* forward ATCACCCAACAAGACCTAGC and reverse TTCTGAGATATGGTGTCCAGG and *GAPDH* forward CAGCCTCAAGATCATCAGCA and reverse GTCTTCTGGGTGGCAGTGAT.

Luciferase assay

Portions of the *DR5* promoter were cloned into the pGVB2 vector that contains a luciferase reporter (Toyo ink, Tokyo, Japan), a gift of Dr Toshyuki Sakai (35). Twenty-four hours prior to transfection, A498 or A498-containing HIF-2α shRNA (0.7×10^6) was seeded onto 60 mm dishes. Five micrograms of each plasmid was transfected into these cells using Lipofectamine 2000 (Invitrogen); and after 48 h, the cells were harvested and assays performed using luciferase assay reagents (Promega, Madison, WI) and a luminometer (Turner Biosystems, Sunnyvale, CA). Transfection efficiency was normalized by cotransfection of the pSV-β-galactosidase plasmid (Promega).

Western blotting and antibodies

For western blotting, cells were harvested by scraping, washed with phosphate-buffered saline and lysed using 1× sodium dodecyl sulfate sample buffer. To probe western blots, the following antibodies were used according to the manufacturer's instructions: mouse monoclonal antibodies to VHL (BD Biosciences), enhanced green fluorescent protein (Clontech), HA (Covance, Berkeley, CA), HIF-1α (BD Biosciences), HIF-2α (Novus Biologicals, Littleton, CO), CXCR4 (EMD Biosciences San Diego, CA), caspase 8 (BD Biosciences), caspase 3 (BD Biosciences), poly ADP ribose polymerase (PARP) (BD Biosciences), anti-polyhistidine agarose (Sigma), GAPDH (Santa Cruz Biotechnology) and c-Myc 9E10 (Santa Cruz Biotechnology). The following rabbit polyclonal antibodies were used: DR5 (Sigma), p21 (Santa Cruz), cyclin D2 (Santa Cruz Biotechnology) and c-Myc (Santa Cruz Biotechnology).

Results

A498 renal cancer cells, which express high levels of HIF-2α and no HIF-1α or VHL, are sensitive to TRAIL-induced apoptosis (11). As

shown by the appearance of a sub-G_1 peak on FACS analysis, the addition of TRAIL to these cells induces apoptosis as early as 4 h, and 100% of cells are dead by 20 h (Figure 1B). To examine the ability of HIF-2α to modulate the sensitivity of A498 renal cancer cells to TRAIL, A498 cells were first transiently transfected with a construct expressing a green fluorescent protein (GFP)–VHL fusion protein and then FACS sorted for the expression of GFP. The overexpression of VHL drives down the level of HIF-2α protein (Figure 2A). The GFP-positive cells were then treated with TRAIL overnight. In comparison with the wild-type cells (Figure 1A), there was no cleavage of caspases, PARP or appearance of a sub-G_1 peak on FACS analysis (Figure 2B and C). Transfection of enhanced green fluorescent protein alone did not inhibit the ability of TRAIL to induce apoptosis (supplementary Figure 1A is available at *Carcinogenesis* Online). In these VHL-transfected cells, we see an increase in the number of cells in the G_1 phase of the cell cycle. This result might be secondary to the induction of p27kip1 or to stabilization of p53 (36,37). Specific VHL mutants are expressed in varied tumor types and may or may not function to regulate the HIF proteins. For example, the S65W and C162F mutants are common in RCC, but rarely occur in pheochromocytoma, and these proteins do not induce the degradation of the HIF protein. In comparison, the Y98H and P154L mutant proteins rarely occur in RCC and induce the degradation of HIF (23). As above, these cDNAs were transiently transfected as GFP fusions into

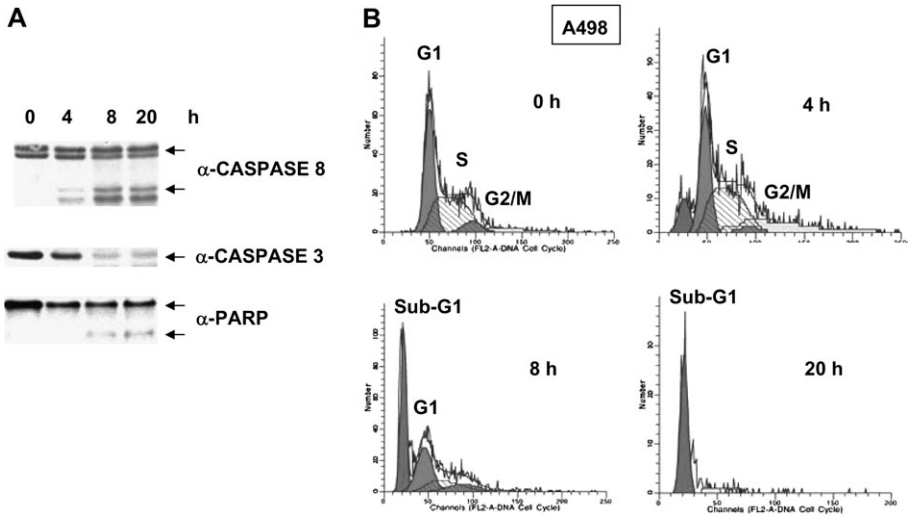

Fig. 1. Induction of apoptosis in A498 cells by TRAIL. (**A**) A498 cells were treated with TRAIL (1 μg/ml) at 37°C for 4, 8 and 20 h. Cell lysates collected at each time point were subjected to western blotting using the indicated antibodies. (**B**) A498 cells treated with TRAIL were harvested at the indicated time points, washed three times with phosphate-buffered saline and fixed with 70% ethanol in phosphate-buffered saline for 2 h at 4°C. Following fixation, cells were stained with propidium iodide as described in Materials and Methods to detect the presence of hypodiploid cells (sub-G_1 peak) by flow cytometry.

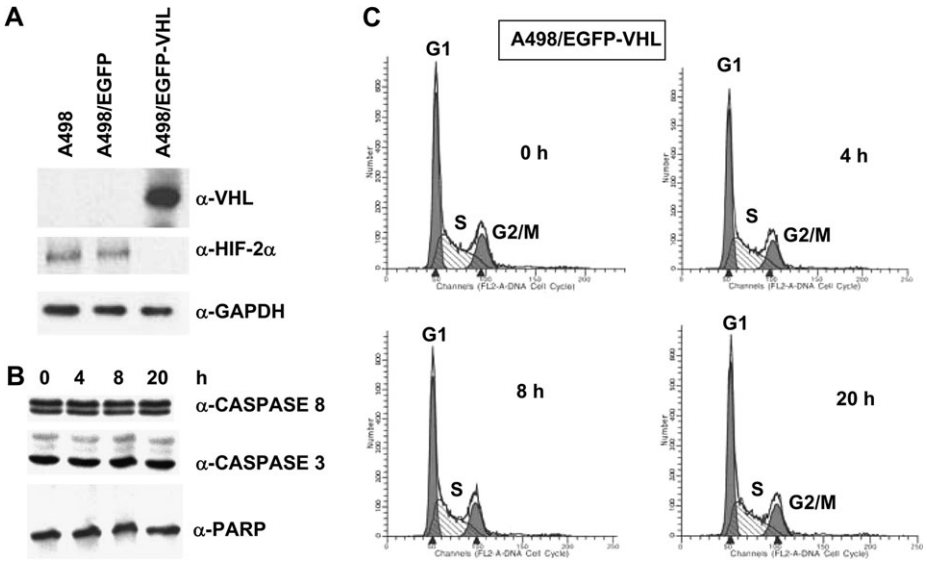

Fig. 2. Inhibition of A498 cell death induced by VHL. (**A**) A498 cells were transfected with pEGFP-C1 (empty vector) or pEGFP-VHL and (wild-type) vectors. Forty-eight hours after transfection, cells were washed with phosphate-buffered saline, FACS sorted and enhanced green fluorescent protein (EGFP)-positive cells retained. These cells were lysed and the extract was western blotted with the indicated antibodies. (**B**) The enhanced green fluorescent protein-sorted cells were grown for 24 h at 37°C before adding TRAIL (1 μg/ml). At the indicated hours of treatment, cells were harvested, lysed in sodium dodecyl sulfate sample buffer and the cleavage of caspase 8, caspase 3 and PARP determined by western blot. (**C**) A portion of the cells were harvested, washed with phosphate-buffered saline and cell cycle analysis (see legend Figure 1B) done by flow cytometry to detect the presence of a sub-G_1 peak.

A498 cells, the cells FACS sorted and then incubated overnight with TRAIL. The transfection of S65W and C162F respond to TRAIL with cell death (supplementary Figure 1B and C is available at *Carcinogenesis* Online), whereas those VHL mutants that lead to HIF-2α degradation function similarly to wild-type VHL and make these cells insensitive to TRAIL killing (supplementary Figure 1D and E is available at *Carcinogenesis* Online). Since the level of cell death regulated by the C162F is more extensive than the S65W mutant, it is possible that the latter mutant has a slightly increased ability to degrade HIF-2α protein when compared with C162F, as suggested by the western blot. These experiments suggest that HIF-2α plays a role in regulating TRAIL sensitivity in A498 cells.

To examine the ability of HIF-2α to regulate TRAIL-induced cell death, A498 cells were transfected with a cDNA expression vector encoding HIF-2α shRNA, and five clones were generated that contained markedly decreased levels of HIF-2α (Figure 3A). These clones also expressed decreased levels of the HIF-2α-regulated gene *CXCR4*. All the clones expressed decreased levels of DR5 protein on western blot (Figure 3A). When incubated with TRAIL 1 μg/ml, these clones showed resistance to TRAIL-induced killing, whereas the parental cells remained highly sensitive (Figure 3B). To verify that this was not specific to a single shRNA, the experiment was repeated with a second shRNA (see Materials and Methods) with identical results (supplementary Figure 2A and B is available at *Carcinogenesis* Online). To examine the mechanism of HIF-2α action, we carried out quantitative real-time PCR for DR5 expression on the RNA from wild-type and cells expressing the HIF-2α shRNA. Results from these five clones demonstrated that in the absence of HIF, the levels of DR5 mRNA were markedly decreased (Figure 3C). A498 cells do not express the second TRAIL receptor DR4.

To attempt to determine whether HIF-2α could regulate the transcription of the *DR5* gene, promoter constructs containing different regions of the *DR5* upstream region in front of a luciferase reporter were transfected into either A498 cells or A498 cells containing a HIF-2α shRNA. We find that in wild-type A498 cells, the region between −448 and −1188 contains sequences that are essential for regulation of the transcription of the *DR5* promoter (Figure 4). In comparison, in A498 cells expressing the HIF-2α shRNA, the expression of these identical promoter constructs was markedly diminished (Figure 4). Transfection of *HIF-2a* into cells with decreased HIF-2α levels overcame the shRNA and stimulated these promoter constructs (Figure 4), but had little effect when transfected into wild-type cells (data not shown). Thus, HIF-2α is capable of regulating transcription of the DR5 promoter.

Recent results from other laboratories demonstrate that HIF-2α functions by stimulating the activity of the Myc protein through interactions with Myc partner proteins, for example Max (33). This observation coupled with the existence of multiple potential E-box-like elements in the upstream region of the *DR5* promoter (38), suggested that the HIF-2α effects on the *DR5* promoter might be tied to its interaction with Myc and its partners. To examine this possibility, we used both siRNA-targeting Myc and a cDNA chimeric construct that expresses a MadMyc chimera that acts as a dominant-negative protein (39). As predicted by the transcriptional activity of Myc (33), a decrease in this protein (Figure 5A) led to an increase in p21 levels and a decrease in cyclin D2 (Figure 5A). Overexpression of HIF-2α (Figure 5B) in one of the A498 clones expressing the shRNA-targeting HIF-2α increased HIF-2α expression and induced transcription from the *DR5* (−1188) promoter (Figure 5B). To carry out these experiments (Figure 5A and B), a mutation was placed in the HIF-2α coding sequence (HIF-2α Mut) so that this mRNA could not be recognized by the shRNA. This stimulation was reversed both by the transfection of Myc siRNA and the dominant-negative MadMyc cDNA construct (Figure 5B), suggesting that the transcriptional regulation of DR5 involved both HIF-2α and Myc proteins. To determine the exact site of c-Myc binding to *DR5* promoter, chromatin immunoprecipitation analysis was done focused on seven E-box-like sites identified previously (38), using the known c-Myc-binding site in *GAPDH* as a control. Results from this experiment demonstrate that the sequence

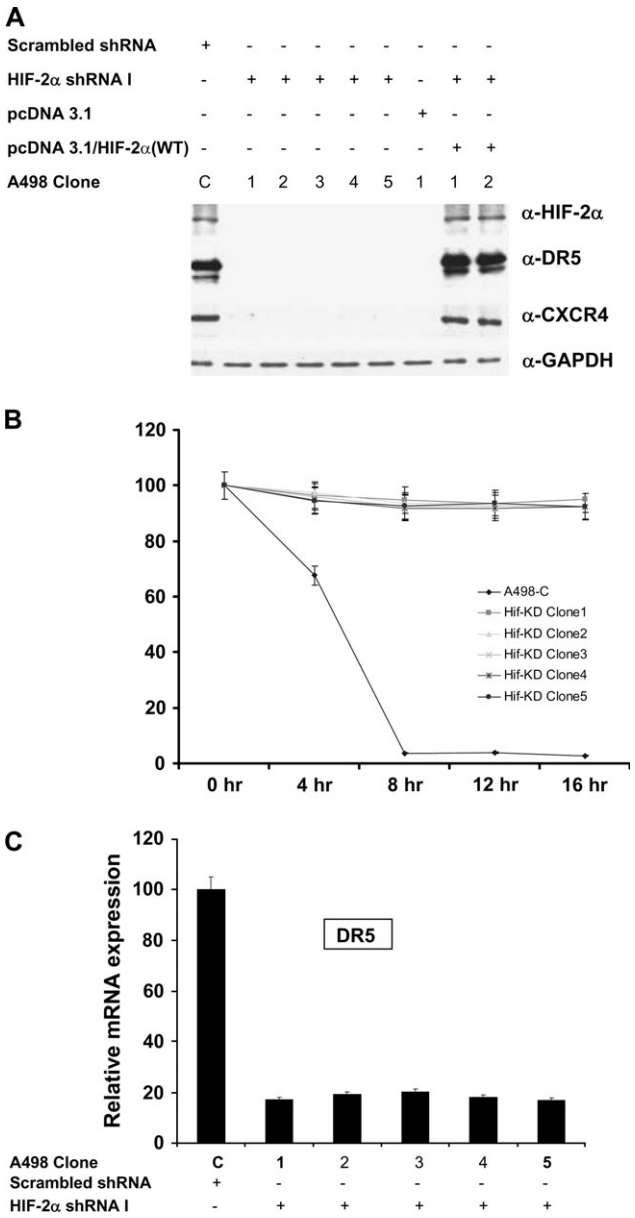

Fig. 3. HIF-2α shRNA decreases the level of DR5 mRNA and protein. (**A**) A498 cell lines containing scrambled shRNA (A498 clone C), HIF-2α shRNA I (clones 1–5), pcDNA3.1 vector alone or HIF-2α shRNA I-containing cells transfected with the wild-type HIF-2α cDNA were homogenized and extracts subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotted with antibodies to HIF-2α, DR5 and CXCR4 and GAPDH as a loading control. (**B**) A498 clones containing scrambled shRNA or HIF-2α shRNA I were treated with TRAIL (1 μg/ml) for varying time periods and percent viability measured by the acid phosphatase assay as described in Materials and Methods. Each assay is performed in triplicate and the standard error of the mean is shown. (**C**) RNA was extracted from these A498 clones and subjected to quantitative real-time PCR to measure the levels of the DR5 mRNA using GAPDH as an internal control. The experiment was performed in triplicate and the standard error of the mean is shown.

GTGGAA from −532 to −537 is capable of binding c-Myc when compared with the IgG control. All other sites demonstrated similar binding to the control. To examine whether this site regulated transcriptional activation of the *DR5* promoter by c-Myc, it was mutated in the *DR5* promoter luciferase construct and this construct was transfected into A498 cells. Mutation of this sequence markedly decreased the baseline activity of the promoter (Figure 5D).

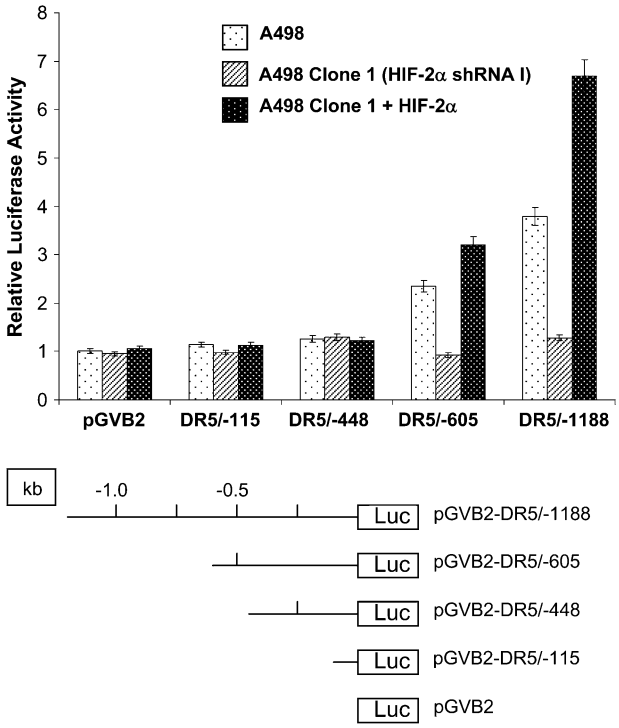

Fig. 4. *DR5* promoter activity detected by luciferase assay. A498 clones were transfected with the pGVB2-based reporter plasmids (5 μg DNA per 60 mm dish) containing various lengths of 5′-human *DR5* promoter gene (see reporter plasmid structure). Ten microliters of each cell lysate (protein concentration: 5 μg/μl) was used to determine luciferase activity that the values obtained were normalized by assaying the activity of a cotransfected pSV-β-galactosidase plasmid. The data shown are the mean with the standard error from the mean of three independent transfections.

Previously, we have shown that the proteasome inhibitor PS-341, which is used to treat patients with cancer, sensitizes prostate cancer cells to TRAIL-induced cell death by regulating the level of the DR5 receptor mRNA and protein (12). To examine whether the HIF shRNA-containing A498 cells would respond similarly, these cells were treated overnight with TRAIL (1 μg/ml) or PS-341 (0.5 μM) alone or in combination. PS-341 treatment greatly sensitized these cells with low levels of HIF-2α to TRAIL-induced cell death (supplementary Figure 3A is available at *Carcinogenesis* Online) and, as demonstrated by FACS analysis of two different HIF-2α shRNA-containing clones, this affect is correlated with an increase in TRAIL receptors (supplementary Figure 3B is available at *Carcinogenesis* Online). Similar results were obtained with MG-132, another proteasome inhibitor (data not shown). As demonstrated by western blots (supplementary Figure 3C is available at *Carcinogenesis* Online), the increase in DR5 receptors induced by PS-341 is correlated with an increase in both Myc and HIF-2α proteins in both the control and two clones that were examined. This proteasome-induced increase in HIF-2α was reversed when additional siRNA to HIF-2α was transfected into these cells, suggesting that regulation of the level of HIF-2α mRNA might be important to the PS-341 activity.

To examine whether regulation of *DR5* transcription by HIF-2α can be generalized to other renal cell cancer cell lines, we studied the affect of siRNA to HIF-2α and Myc on the level of DR5 in PV-10 and RCC4 renal cancer cell lines. Both of these cell lines contain HIF-1α and -2α but no VHL, and in comparison with A498, are relatively insensitive to TRAIL-induced apoptosis. Transfection of HIF-2α siRNA decreased the level of HIF-2α in RCC4 (Figure 6A) and PV-10 (data not shown). Even in the presence of unchanged levels of HIF-1α, a decrease in HIF-2α inhibited the transcription of the DR5 (−1188)-Luc plasmid in both cell lines. Likewise, both Myc siRNA and the MadMyc cDNA transfections decrease the levels of DR5 protein and transcription (Figure 6A and B). To attempt to determine whether HIF-2α was important in other tumor cell lines in controlling DR5 levels, A549 lung and PC3 prostate cancer cell lines were placed in hypoxia to induce an increase in HIF-2α levels (Figure 6C). Western blots demonstrate that this increase in HIF-2α levels was accompanied by a parallel increase in DR5 protein. Knock down of HIF-2α using two different siRNAs inhibited this increase in hypoxia-driven changes in DR5. To evaluate the importance of HIF-1α levels to the control of *DR5* transcription, this protein was decreased by two different siRNAs (Figure 6D) and the −1188 *DR5* promoter construct was transfected into these cells. In contrast to HIF-2α, lowering the level of HIF-1α did not decrease the luciferase output from the upstream elements of the *DR5* promoter (Figure 6E).

Discussion

Our data suggest that HIF-2α protein plays an important role in regulating the levels of DR5 receptors in renal carcinoma cells that express this protein. The ability of HIF-2α to control the level of the TRAIL receptor DR5 does not appear to be limited to TRAIL-sensitive cell lines. siRNAs targeted at HIF-2α decrease the expression of the TRAIL receptor mRNA and protein when transfected into the TRAIL-resistant PV-10 and RCC4 cell lines as well as the TRAIL-sensitive A498 cells. Nor is this effect limited to renal cancer cells since we see similar results in A549 lung and PC3 prostate cancer cell lines when they are placed in hypoxia. In contrast, lowering the level of HIF-1α did not decrease either the level of the DR5 protein or transcription from the DR5 promoter. Additionally, renal cancer Caki cells, which express only VHL, when placed in relative hypoxia induce increased levels of HIF-1α but do not increase either *DR5* transcription or protein levels (data not shown). Although HIF-2α plays an important role in regulating the level of DR5, renal cancer cells containing this protein are both sensitive and resistant to TRAIL-induced apoptosis, suggesting that sensitivity to TRAIL is controlled by a complex set of proteins other than the receptor.

Previously, we have shown that PS-341 increases DR5 levels both by inhibiting protein destruction and by increasing DR5 transcription. In renal cancer cells with low HIF-2α levels, we find that PS-341 treatment increases both the level of DR5 protein and sensitivity to TRAIL-induced apoptosis. The affect of this drug is correlated with its ability to increase the levels of both HIF-2α and Myc, two proteins that appear to regulate *DR5* transcription. It is possible that PS-341 increases HIF-2α mRNA levels since transfection of siRNA to HIF-2α blocks this compound's ability to increase protein levels. Trials of PS-341 in humans with renal cancer have yielded varying results. In one study (40), there was little single-agent activity, whereas in a second trial, stable disease was seen in 14 patients with 4 patients having a partial response (41). The combination of PS-341 and TRAIL may have utility in treating renal cancer.

The clear differences between HIF-1α and -2α appear to be found in multiple cells models. Most recently, HIF-1α has been shown to inhibit the function of the Myc protein (34,42). In keeping with the antagonism of Myc, HIF-1α decreases Myc-activated genes including *hTERT* and *BRCA1* (42). HIF-2α has the exact opposite effect (33) enhancing Myc-induced transformation of fibroblasts and promoting cell cycle progression of hypoxic renal carcinoma cells. HIF-1α has been shown to retard tumor growth, whereas HIF-2α stimulates the growth of tumors (31). If in embryonic stem cells the *HIF-1a* gene is replaced by *HIF-2a*, the resulting teratomas have increased expression of transforming growth factor-α, vascular endothelial growth factor and cyclin D1 (43). Using microarray analysis, HIF-1α but not HIF-2α has been shown to regulate the levels of glycolytic genes (44). Thus, it is not surprising that HIF-1α and HIF-2α appear to regulate DR5 levels differently. Although there is not a consensus-binding site for HIF-1 (5′G/ACGTG3′) (45) in the *DR5* promoter, complex regulation of this promoter by HIF-2α and Myc/Max is possible.

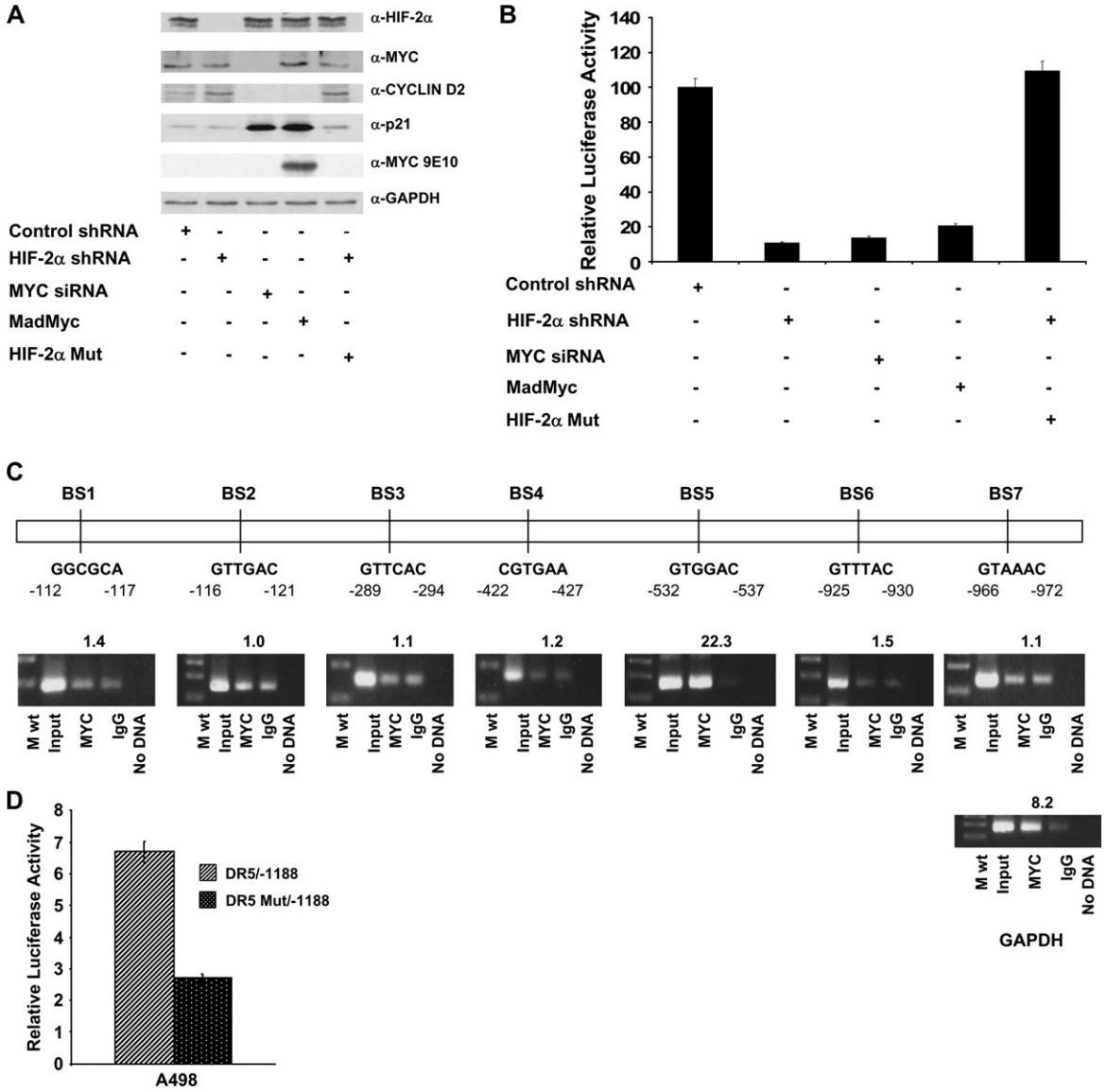

Fig. 5. Decreasing Myc protein levels regulates HIF-2α control of DR5 transcription. (**A**) Western blot of extracts from A498 cells studied in (**B**) were probed with antibodies to Myc, HIF-2α, p21, cyclin D2 or GAPDH as a loading control. (**B**) Regulation of DR5 promoter activity in A498 HIF-2α-knockdown cells. A498 cells containing HIF-2α shRNA were transfected with DR5 promoter construct (−1188) with or without the cDNA expressing HIF-2α Mut, MYC siRNA or MadMyc protein were assayed after 48 h. HIF-2α Mut contains a mutation (see Materials and Methods) in the coding sequence so it cannot be recognized by the shRNA. Luciferase activity was obtained using 5 μg of cell protein lysate that was normalized to cotransfected pSV-β-galactosidase plasmid. The luciferase values shown are the mean ± SD from three independent observations. (**C**) Schematic representation of the human DR5 promoter with seven non-canonical E-box elements is shown. The specific primers for each element used in the PCR in chromatin immunoprecipitation assay are described in the Materials and Methods. The sizes (bp) of the amplified regions for BS1–BS7 are 182, 130, 143, 145, 141, 154 and 112, respectively. A control reaction shown demonstrates c-Myc binding to a known site in GAPDH. The specificity of binding of c-Myc to each site is shown as the fold difference between c-Myc immunoprecipitation and rabbit IgG control (background) as demonstrated by densitometry. (**D**) The effect of mutation in the BS5 region on DR5 baseline promoter activity. A498 cells were transfected for 48 h with wild-type or E-box mutant DR5/−1188 promoter constructs. The luciferase activity was measured by using 5 μg of cell protein lysate and values normalized to cotransfected pSV-β-galactosidase plasmid. The luciferase values shown are the mean ± SD from three independent observations.

Myc protein has been shown to regulate cellular sensitivity to TRAIL through multiple mechanisms. For example, Nieminen *et al.* (46) found that Myc primed mitochondria by a mechanism involving activation of Bak, enabling weak TRAIL signals to stimulate the mitochondrial pathway. Myc has also been found to decrease the TRAIL-induced increases (47) in Mcl-1 and cIAP2 by inhibition of nuclear factor-κB thus sensitizing the cell to the intrinsic pathway. Increasing the cellular levels of Myc has been shown to upregulate the level of DR5 protein whereas transfection of the dominant-negative MadMyc protein decreases the level of this protein (39). Myc appears to control the transcription of *DR5* (39) and there is data to either support an indirect control of transcription (39) or direct control by binding to non-canonical E-boxes (38). We have demonstrated that Myc is capable of binding to the E-box between −532 and −537 in the *DR5* promoter and mutation of this site decreases the activity of the *DR5* promoter in A498 cells. HIF-2α has been shown to enhance the binding of Myc to gene promoters by increasing the levels of Myc/Max (33) or by directly binding to the E-Box as a HIF-2α–Max complex. Indirect regulation of *DR5* transcription could arise from the ability of Myc to control protein synthesis through regulation of ribosomal proteins that may lead to the production of transcription factors that bind to the *DR5* promoter [for review see ref. (48)]. HIF is also capable of

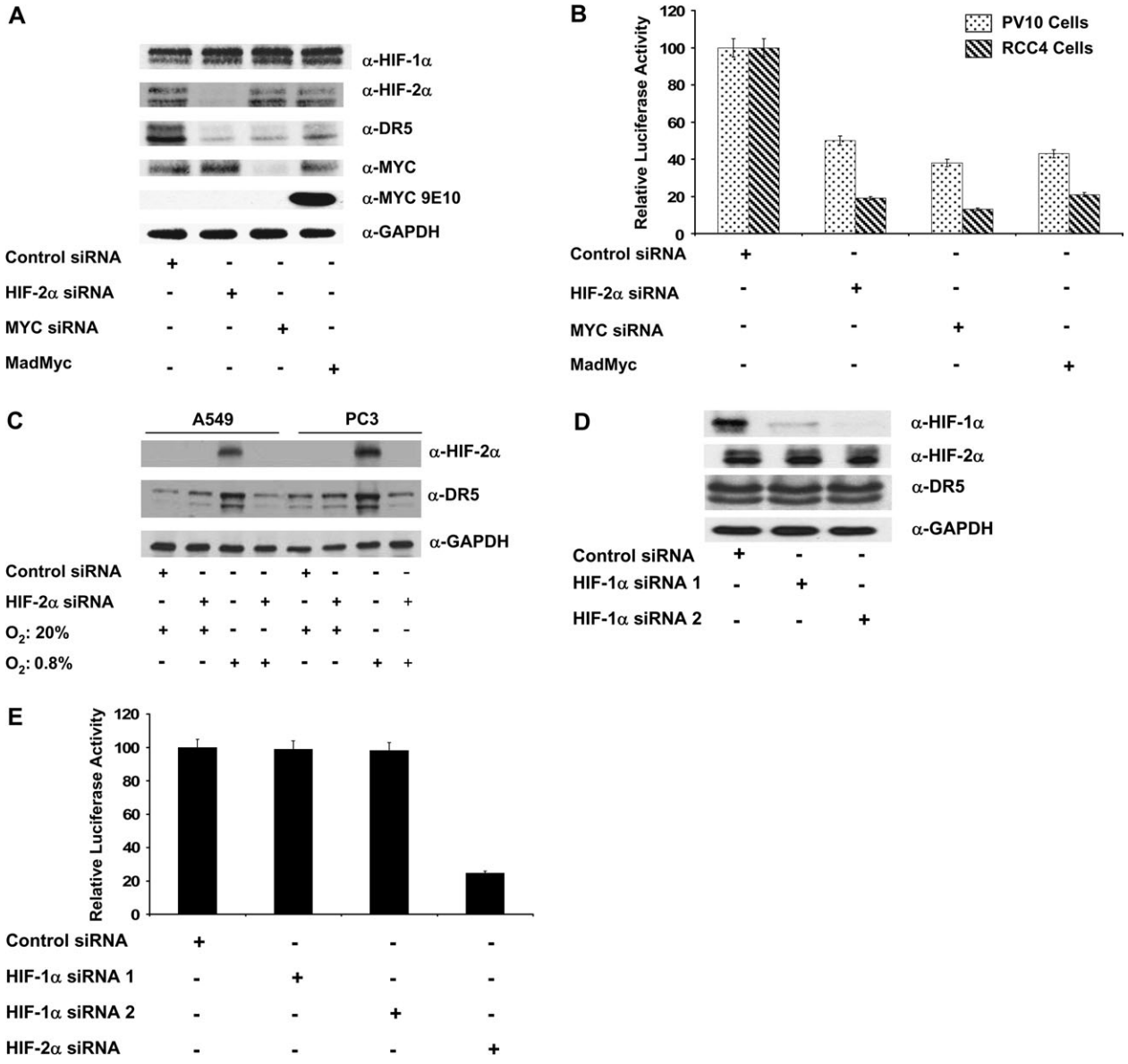

Fig. 6. Regulation of DR5 transcription in PV-10 and RCC4 cells. (**A**) Levels of HIF-2α and Myc, MadMyc after siRNA and pCMVMadMyc transfections into RCC4 cells. RCC4 cells were transfected with siRNA or plasmids. Forty-eight hours later, the cells were homogenized and western blots were carried out. (**B**) The effect of lowering HIF-2α and Myc levels on DR5 transcription. RCC4 and PV-10 cells were transfected with DR5 (−1188)-Luc and HIF-2α siRNA, Myc siRNA or the Mad/Myc plasmid DNA. Forty-eight hours after transfection, luciferase assays were carried out. The values shown are the mean ± SD from three independent observations. (**C**) Induction of HIF-2α regulates DR5 levels. Following transfection with the indicated siRNAs for 48 h, A549 lung and PC3 prostate cancer cells were grown at 20 or 0.8% O_2. Cells were lysed and subjected to western blot analysis with HIF-2α, DR5 and GAPDH antibodies. (**D**) siRNA to HIF-1α does not decrease DR5 protein. RCC4 cells were transfected with siRNA to HIF-1α and 48 h later, homogenates were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotted for DR5, HIF-2α and HIF-1α. (**E**) The effect of knock down of HIF-1α on DR5 promoter transcription. RCC4 cells were transfected with DR5 (−1188)-Luc and HIF-1α or HIF-2α siRNA and 48 h after transfection, luciferase assays were carried out. The values shown are the mean ± SD from three independent observations.

regulating protein synthesis. Thus, it is also possible that HIF-2α, Myc or both could regulate the *DR5* promoter by indirectly controlling the production of proteins that in turn stimulate this promoter. In summary, these data are the first to demonstrate the role of HIF-2α and Myc together in regulating the levels of the TRAIL receptor DR5.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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