The Apoptosis Repressor with a CARD Domain (ARC) is a Direct HIF1 Target Gene and Promotes Survival and Proliferation of VHL Deficient Renal Cancer Cells

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RUNNING TITLE: ARC IS A PRO-TUMORIGENIC EFFECTOR OF HIF1 SIGNALING

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The induction of hypoxia inducible factors (HIFs) is essential for the adaptation of tumor cells to a low oxygen environment. We found that the expression of the apoptosis inhibitor ARC was induced by hypoxia in a variety of cancer cell types and its induction is primarily HIF1 dependent. Chromatin immunoprecipitation (ChIP) and reporter assays also indicate that the ARC gene is regulated by direct binding of HIF1 to a hypoxia response element (HRE) located at -190 bp upstream of the transcription start site. HIFs play an essential role in the pathogenesis of renal cell carcinoma (RCC) under normoxic conditions, through the loss of the Von Hippel Lindau (VHL) gene. Accordingly, our results show that ARC is not expressed in normal renal tissue, but is highly expressed in 65% of RCC tumors, which also express high levels of Carbonic Anhydrase IX (CAIX), a HIF1-dependent gene. Compared to controls, ARC-deficient RCCs exhibited decreased colony formation and increased apoptosis in vitro. In addition, loss of ARC resulted in a dramatic reduction of RCC tumor growth in SCID mice in vivo. Thus, HIF-mediated increased expression of ARC in RCC can explain how loss of VHL can promote survival early in tumor formation.

Hypoxia inducible factors are oxygen sensitive transcription factors that are essential for cellular adaptation to low oxygen conditions. Increased expression of HIFs is observed in multiple cancers and have been reported to correlate with poor prognosis (1). Recent work has shown that HIF proteins regulate diverse aspects of malignancy including glucose metabolism, angiogenesis, survival, proliferation and differentiation (2). HIFs consist of an oxygen sensitive alpha subunit, HIF1α or HIF2α, and a constitutively expressed beta subunit, HIF1β, also called ARNT (aryl hydrocarbon receptor nuclear translocator). Under atmospheric conditions (21%
O₂), HIFs are hydroxylated at proline residues (HIF1α at Pro402/564 and HIF2α at Pro405/531), are recognized by the E3-ubiquitin ligase VHL and are targeted for proteasomal degradation. When oxygen levels decrease (5% O₂ or lower), HIF proteins remain non-hydroxylated, become stabilized and bind to ARNT, forming a complex that orchestrates the transcriptional response to hypoxia. Alternatively, stabilization of HIFs may occur even under normoxic conditions if VHL function is lost due to somatic mutations or epigenetic changes (e.g. promoter hypermethylation).

_VHL_ inactivation leading to increased HIFs levels and increased expression of HIF-target genes has been detected in 80-90% of sporadic clear cell renal cell carcinomas (CC-RCCs), which account for the majority of renal cancers (3), and are notoriously resistant to cytotoxic chemotherapies. In the present study, we evaluated the _ARC_ gene as a HIF target gene and investigated its contribution in renal tumorigenesis.

Over the past several years, studies have identified ARC, expressed primarily in heart, muscle and brain as a physiological mediator of apoptosis resistance. Unlike highly proliferative cells in the bloodstream, bone marrow, and gut, which have short life spans and high rates of apoptosis, differentiated post-mitotic cells in the nervous system, heart, and skeletal muscle exhibit relative resistance to apoptosis to limit excess tissue degeneration in response to stress stimuli. The anti-apoptotic action of ARC was originally attributed to its inhibition of caspases in skeletal muscle and heart through the interaction between its N-terminal caspase recruitment domain (CARD) and the homologous prodomains of apical caspases (4). ARC is implicated in the inhibition of both the intrinsic and extrinsic pathways of apoptosis (5). Recent work has shown that ARC may also promote apoptosis resistance through interactions with other proteins implicated in apoptotic pathway, including Fas and FADD (5). The extrinsic pathway is disrupted by the heterotypic binding between the CARD domain of ARC and the death domains...
of Fas and FADD, whereas the intrinsic pathway is impaired mainly by the interactions between ARC and the pro-apoptotic mediator Bax, which maintains Bax in its inactive conformation and prevents mitochondrial cytochrome C release (5, 6).

Although ARC expression is relatively low in most mammalian tissues, it is beginning to be recognized that its anti-apoptotic function may be co-opted in a variety of cancer cell types as part of an integral pro-survival mechanism during cancer development and maintenance. This hypothesis is strongly supported by the high prevalence of ARC expression in human cancers (7, 8) that increases the ability of cancer cells to inhibit apoptotic cell death in response to endoplasmic reticulum (ER) and mitochondrial stresses. For example, ARC was shown to inhibit ER stress-induced apoptosis in melanoma cells (9), and to contribute to resistance to doxorubicin-induced cancer cell death by inhibiting dynamin related protein 1 (DRP1)-mediated mitochondrial fission (10). A recent study reported the role of ARC in stimulation of cancer cell proliferation in in vivo breast cancer model (11). In addition, ARC is highly expressed in leukemia, and its expression inversely correlates with patient survival (12). ARC can also promote tumor progression by impairing p53 function in breast cancer (13), and by cooperating with the Ras oncoprotein for the maintenance of the transformed phenotype (42).

In the past five years the mechanisms governing ARC expression have started to be elucidated. Studies by Nam et al. (14) and Foo et al. (15) have proposed a role for ubiquitination and proteasomal degradation in regulation of ARC protein expression. This mechanism of decreasing ARC protein levels would limit its anti-apoptotic function in response to death stimuli. On the other hand, ARC expression is also negatively regulated at the mRNA level by p53, which blocked its expression under hydrogen peroxide treatment and anoxia, although the mechanism of this inhibition is poorly understood (16). In contrast, Ras acts as a positive
regulator of ARC expression through stimulation of transcription and increased protein stability (17).

In this paper, we demonstrate that hypoxia, through the selective induction of HIF1α, is a key regulator of ARC expression in renal cancers with wild type (WT) VHL. In addition, our data provide a mechanism for ARC over-expression in CC-RCC under normoxia: through the loss of VHL gene function and HIF1α up-regulation. Further, ARC over-expression in RCC contributes to cell survival and growth, and ARC down-regulation leads to cell death and decreased growth rate, suggesting that targeting ARC may be a useful therapeutic approach to control tumor growth.

MATERIALS AND METHODS

Cell cultures. A549, A375, MCF7, RCC4, RCC4-VHL, RCC10, RCC10-VHL, 786-0-VHL, A498-VHL, ACHN, SN12C, HeLa, TK10, HCT116, HCT116HIF1α−/−, and 293FT cell lines were grown in Dulbecco’s modified Eagle media; Caki1 cells were grown in McCoy5a media. Media were supplemented with 10% fetal bovine serum (HyClone, South Logan, UT), 2mM l-glutamine, 100u/ml penicillin, and 100μg/ml streptomycin. Cells were grown in mixed-gas CO2 water-jacketed incubators (21% O2, 5% CO2), Forma Scientific, Marietta, OH.

Hypoxic and anoxic treatment. Cells were plated in glass dishes, let adhere for 16-20h in 21% O2, 5% CO2 incubator and then transferred to Invivo2 400 Hypoxic workstation 0.5% O2, or 2% O2 (Biotrace Inc., Bothell, WA) or to 0.02% O2 Bactron Anaerobic Environmental Chamber (Sheldon Manufacturing Inc., Cornelius, OR) for the indicated times. The lysis of the cells for Western blotting was performed inside the chambers.

Western blotting. Cells were lysed directly on the dishes with ice-cold RIPA buffer
(50mM Tris-HCl [pH 7.4], 150mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Na Deoxicholate) supplemented with Complete mini protease inhibitors (Roche, Indianapolis, IN), kept on ice for 15 min, vortexed and spun down at 14,000 g (Eppendorf centrifuge 5415C). Total protein in supernatant was quantified using BCA Protein Assay kit (Pierce, Rockford, IL), and 30-100μg of protein per lane were separated on 10-12% bis-acrylamide gels and then transferred onto 0.2μm nitrocellulose membranes (BioRad, Hercules, CA). The following primary antibodies were used to probe the membranes: rabbit anti-HA antibody (dilution 1:2000; Abcam Inc., Cambridge, MA), rabbit anti-ARC (dilution 1:1000; #ab2002, #ab2003, Abcam); rabbit anti-HIF2α (dilution 1:500; Novus Biological, Littleton, CO); mouse anti-HIF1α (dilution 1:250; BD Biosciences, San Jose, CA); rabbit anti-ARNT (dilution 1:1000; BD Transduction Laboratories, San Jose, CA); rabbit anti-cleaved caspase 3 (dilution 1:1000; Cell Signaling, Danvers, MA); mouse anti-α-tubulin (dilution 1:5000; Fitzgerald Industries International, Concord, MA); mouse anti-β-actin (dilution 1:5000; Sigma-Aldrich, St. Louis, MO); mouse anti-HSP-70 (mitochondrial Heat Shock Protein 70; dilution 1:2000; Thermo Scientific, Rockford, IL); and goat anti-mouse, goat anti-rabbit conjugated to horse radish peroxidase (HRP, Zymed Laboratories Inc., South San Francisco, CA). Western blot bands were quantified using ImageJ (NIH) or Image Quant (Biorad) programs.

**siRNA and plasmid transfections.** Cells were transfected according to the manufacturer’s instructions with non-targeting siControl (siCTR), siHIF1α, siHIF2α, siARNT, and siARC smart pools (Dharmacon, Chicago, IL) using Dharmafect reagent 1 (Dharmacon). 293FT cells were transfected with pcDNA3, or pcDNA3-HIF1α-CA-HA (P402A/P564A), or pcDNA3-HIF2α-CA-HA (P405A/P531A) plasmids (Addgene, deposited by Dr. Kaelin (18, 19)) using lipofectamine and plus reagents according to the manufacturer’s instructions (Invitrogen).
**Quantitative real time PCR.** QRT-PCR was performed as described previously (20). Human TATA-binding protein (hTBP) primers were chosen as an internal control. Primer sequences are available upon request.

**Chromatin Immunoprecipitation Assay.** ChIP assay was performed as described previously (21). RCC4 cells were fixed with 1% formaldehyde for 10min at RT. 70-100μg of sonicated chromatin was incubated with rabbit anti-HIF-1α antibody (Abcam), or rabbit anti-HIF-2α antibody (Abcam), followed by precipitation with protein A/G Dynabeads (Invitrogen, Grand Island, NY). Alternatively, ChIP was performed on 293FT cells transiently transfected with pcDNA3 or pcDNA3-HIF2α-CA-HA (P405A/P531A) plasmids, 24h post-transfection, using rabbit anti-HA antibody (Abcam). Relative enrichment was measured by QRT-PCR using a titration of pooled input samples as a standard curve. Normal rabbit IgG (Santa-Cruz Biothechnology, Santa Cruz, CA) was used as a non-specific IgG control; and the relative enrichment obtained for the non-specific IgG control was subtracted from the relative enrichment of the samples. The sequences of primers flanking HRE1 and HRE2 sites in ARC promoter were as follows: 5’ggggaggggaagactac3’ and 5’ctgggaatagggaggaggtgag3’. Primers flanking jumonji domain-containing protein 1A (JMJD1A) HRE (5’tccttcaaatggcggactt3’ and 5’getgctggggaatatgg3’) and primers flanking ARRDC3 HRE (22) were used as a positive control, primers upstream of ARC HRE1 and HRE2 located at -861 bp of transcription start site were used as a negative control (5’tctgaagaagggagggac3’ and 5’gcccaagtggaggcagt3’).

**Reporter plasmid construction, transient transfection and luciferase assay.** A region of DNA, 1005 bp, containing ARC promoter, exon1 and a part of intron1 (−701 bp to +304 bp, +1 corresponds to a transcription start site; sequence and intron-exon structure of ARC/Nol3 gene were obtained from UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly)
was synthetized and cloned in pUC57 by GenScript USA Inc., Piscataway, NJ. This DNA region was subsequently sub-cloned by NheI and XhoI to pGL3-basic vector upstream of firefly luciferase gene by PCR, using forward 5’agagaggctagcaaggggcttggaaccagtc3’ and reverse 5’ctctctcgagtcgactgcaacggatttc3’ primers. The reporter plasmids mutated at the HRE1 (-106 bp; CACGT) and HRE2 (-190 bp; ACGTG) sites alone or in combination were constructed by site-directed mutagenesis using QuikChange Lightning Multi Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) accordingly to manufacturer’s instructions with primers targeting HRE1 and HRE2 respectively (5’aggccctgagtgggcgctggaaaaatggggccgcgggccgaactg3’, 5’aggccgcgcggctggctggggattttcatgtagtcagctttccctc3’). All constructs were verified by sequencing. Reporter plasmids were transiently transfected to 293FT or TK10 cells using lipofectamine and plus reagents accordingly to manufacturer’s protocol (Invitrogen). 24h after transfection cells were subjected to 0.5% O2 for 17h. The luciferase activity was measured by Bright Glo Luciferase Assay Kit (Promega BioSystems, Sunnyvale, CA) on Monolight 2010 luminometer (Analytical Luminescence Laboratory). Relative luciferase units in all samples were normalized to the amount of protein in each lysate measured by BCA assay (Pierce).

shRNA and cDNA expression constructs, lentivirus packaging and infection of target cells.

pLKO.1shARC no.1 and no.2 were obtained from Open Biosystems, Huntsville, AL. The target sequences in ARC mRNA are as follows: 5’gccacacacactcattgtcatt3’ for shRNA no.1 and 5’gcattggatgcactgcctgat3’ for shRNA no.2. pLKO.1shGFP plasmid was a kind gift of Dr. Silvestre Vicent (Stanford University). To conduct simultaneous expression of shRNA constructs in pLKO.1-puro vector and cDNA constructs in pLM-CMV-H4-puro-PL3 vector, we replaced the puromycin resistance in pLM-CMV-H4-puro-PL3 vector with neomycin resistance gene by PCR using forward 5’agagagaactgtgattgaacaagatgat3’ and reverse
5’ctctgctcactaagaactgtaagaa3’ primers, and subsequent cloning by SpeI and SalI sites. ShRNA insensitive (sh-in) ARC cDNA was synthetized by GenScript using a sequence template corresponding to WT human ARC (GenBank: BC012798.2) with mutations mostly in 3rd positions of codons corresponding to the region targeted by shRNA no.1 (GCATTGGATGCACTGCCTGAT \(\rightarrow\) GCccTGGAcGCcCTaCCaGAc), and with truncated 3'UTR region, which was targeted by shRNA no.2. The WT amino acid sequence was preserved. The sh-in-ARC cDNA was sub-cloned from pUC57 to pLM-CMV-H4-neo-PL3 by NheI and XhoI sites. Lentivirus productions and infections were done as described previously (23). Infected cells were selected in puromycin-containing media (1-2 \(\mu\)g/ml) or neomycin-containing media (350 \(\mu\)g/ml) for one week.

**Analysis of patients' data.** Data were obtained based on two public gene expression microarray data sets: 177 tumor tissue samples and 10 renal cortex normal tissue sample (24, 25). cDNA data from Higgins et al. (24) and Zhao et al. (25) were extracted using Stanford Microarray Database (SMD) packages, then data were transformed using Disease Specific Genomic Analysis (26) ARC: Hs.513667 UniGene build 207. Data were initially processed using SMD pre-processing: spot regression correlation greater than 0.5, signal/background intensity greater than 1.5, only genes with 80% good data were retained, and then data was collapsed by UniGene cluster ID build 207. Data were then transformed using the high-dimensional supervised method of Disease-Specific Genomic Analysis, which produces a high dimensional healthy state model, estimated from the normal tissue data, and then transforms each tumor vector to measure deviation from the healthy state. An estimate of healthy tissue values was obtained through a leave-one-out procedure performed on normal tissue data, as explained in Nicolau et al. (26). The values for each gene measure the extent to which the gene deviates from the healthy state.
Thus the measures for normal tissue – the leave-one-out values – give an estimate of significance: values in tumors are significant if they exceed the leave-one-out estimates computed on normal tissue. This estimate can be computed using the collection of values for a single gene in all normal tissue, or using all genes in all normal tissue. Since there are only 10 normal tissue samples, the latter provides a distribution based on a larger sample size for estimating significance. Specifically we compared the distribution of data from ARC (Hs.513667) in 177 tumors to two distributions: one was distribution of ARC in the normal tissue; the other was the distribution of all genes in the normal tissue. The former compares specifically the levels of ARC in normal and tumor tissues. The latter compares ARC in tumors to all genes in normal tissue.

**Immunohistochemistry.** Kidney cancer tissue arrays were purchased from US Biomax, Rockville, MD. Antigen retrieval was performed as described previously (20). The slides were incubated overnight at 4°C with a primary rabbit anti-ARC antibody (dilution 1:400, #ab2002, Abcam Inc. Cambridge MA) or mouse anti-CAIX (dilution 1:100, Bayer, Elkhart, IN), or rabbit anti-cleaved caspase3 (dilution 1:300, #9661, Cell Signaling) followed with biotinylated secondary anti-rabbit or anti-mouse (Santa Cruz Biotechnology), and finally with HRP-conjugated Streptavidin (Calbiochem, Gibbstown, NJ). The target proteins were visualized with an AEC chromogen kit (Lab vision, Fremont, CA) and counterstained in Hematoxylin solution. Slides were analyzed using Leica DM6000B microscope (Leica Microsystem Inc., Bannockburn, IL) under 40x magnification. Each spot was scored as negative or positive depending on the intensity of the staining. Since VHL loss occurs in 80% of all RCC tumors, HIF target genes get uniformly up-regulated throughout the tissue section: the majority of cancer cells stain positively, and stromal cells stain negatively.
**Cell proliferation assay.** Cells stably expressing shGFP, shARC no.1, or no.2 were counted with a Coulter particle Counter Z1 (Beckman Coulter, Fullerton, CA), and $10^5$ cells were plated in triplicate in 60mm dishes, let adhere overnight in 21% O$_2$, 5% CO$_2$ incubator, subjected to normoxic (21% O$_2$) or hypoxic (0.5% O$_2$) conditions as described above, and counted and re-plated every third day for a period of two weeks.

**Colony survival assay.** Cells were counted and seeded in 60mm dishes at 3000 cells/dish (Caki1 cells), 1000 cells/dish (RCC4 and RCC4-VHL), and 300 cells/dish (RCC10 and RCC10-VHL) in triplicates, and subjected to normoxic (21% O$_2$) or hypoxic (2% O$_2$) conditions for the duration of colony assay or to 0.5$\mu$g/ml cisplatin or vehicle control pre-treatments for 17h before colony assay as indicated in the corresponding figures. After two weeks colonies were fixed and stained with 95% ethanol and 0.1% crystal violet solution and manually counted.

**Detection of caspase 3 activity with fluorescent probes.** The detailed protocol is described in (27). In brief, Caki1 cells expressing shGFP, shARC no.1 and no.2 were either treated with caspase inhibitor Z-VAD-FMK at 20nM concentration, or left untreated for 48h. Cell lysates were labeled with caspase probe LE22 and resolved on 15% bis-acrylamide gel. Fluorescence was detected at Typhoon scanner with Cy5 laser (630/670nm excitation/emission).

**Annexin V/PI staining**

Apoptosis was assayed by staining the cells with the FITC-Annexin V apoptosis detection kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, 72h after seeding, both adherent and non-adherent cells were collected. $10^6$ cells were incubated with FITC-Annexin V and PI and analyzed by flow cytometry using a FACScan (Becton Dickinson). The percentage of FITC-Annexin V positive and PI negative cells (marking early apoptotic cells)
was used to compare apoptotic rates in the cell lines analyzed. The analysis of the percentage of apoptotic cells was performed using CellQuest software (BD Biosciences).

**In vivo experiments.** Male SCID (B6.CB17) mice supplied by Charles River Laboratories International Inc. (San Diego, CA) were housed in APLAC-approved facility at Stanford. Caki1 cells stably expressing shGFP, shARC no.1 or no.2 were detached by trypsin, washed in 1xPBS, counted, and 3x10^6 trypan blue negative cells in 100μl 1xPBS were injected subcutaneously into the right and left dorsal flanks of the mice. All mice were sacrificed 13 weeks after cell implantations; primary tumors were excised and measured with calipers. Tumor volume was calculated using the formula: width^2 x length x 0.5.

**RESULTS**

ARC is induced by hypoxia in tumor cells. ARC is expressed in cancer cell lines of different tissue origins even though it is not represented in their normal counterparts (7, 8). We confirmed the presence of ARC in a collection of lysates coming from melanoma (A375), lung (A549), cervix (HeLa), breast (MCF7) and renal (Caki1, SN12C, ACHN, and RCC4) carcinoma cell lines (data not shown). Moreover, while systematically analyzing a microarray of hypoxia induced genes in renal cancer cells, previously published by our group (21), we found the expression of ARC to be induced by exposure to 0.5% O\(_2\). To confirm the induction of ARC by hypoxia in renal cancer we used Caki1 cells, which express a WT VHL gene at a lower level compared to other WT VHL RCC cell lines (28) and respond to hypoxia by inducing HIF stabilization and activation. We subjected Caki1 cells to hypoxia (0.5% O\(_2\), 16h) and normoxia (21% O\(_2\)) and assessed ARC expression at the mRNA level by quantitative real time polymerase chain reaction (QRT-PCR). ARC mRNA exhibited similar hypoxic induction as a well-known
HIF1α target gene phosphoglycerate kinase 1 (PGK1, (29), Fig. 1A). Moreover, we found ARC protein to be induced by hypoxia in cell lines of diverse histological origins, such as A549 (adenocarcinoma of the lung, NSCLC), A375 (melanoma), HeLa (cervical cancer, Fig. 1B), Caki1, SN12C, ACHN (renal cancer, Fig. 1B), and MCF7 (breast cancer, Fig. 1C) cell lines. Furthermore, hypoxic inducibility of ARC protein could be detected at oxygen levels ranging from 2% to 0.02% (anoxia, Fig. 1C).

The hypoxic induction of ARC in CC-RCC is dependent on HIF1α activation. Since hypoxia induced ARC expression at the mRNA level, we investigated whether ARC was regulated through HIF transcription factors (2). We used RCC4-VHL, 786-0-VHL, and A498-VHL cells, derivatives of VHL deficient RCC4, 786-0 and A498 cells, which were stably transduced with WT VHL. Stable transduction of VHL resulted in destabilization of HIFs α in normoxia and restoration of their inducibility in hypoxia. We exposed these cell lines to normoxia (21% O2) or hypoxia (2% O2) for 16h and found that, while RCC4-VHL cells (expressing both HIF1α and HIF2α) showed induction of ARC protein by hypoxia, 786-0-VHL or A498-VHL cells (expressing HIF2α, but not HIF1α) failed to induce ARC (Fig. 2A). These results suggested that ARC was regulated by HIF1α under hypoxic conditions. To confirm these findings, we used QRT-PCR to quantify changes in ARC expression in RCC4-VHL cells transiently transfected with siRNA duplexes targeting HIF1α, HIF2α and ARNT, or non-targeting scrambled siRNA, and exposed to normoxia (21% O2) and hypoxia (2% O2) for 16h. ARNT is recruited by both HIF1α and HIF2α to form heterodimers that directly bind hypoxia-response elements (HREs) in the regulatory sequences of hypoxia dependent genes, and inhibition of ARNT results in decreased transcriptional activity of both HIF1α and HIF2α. Inhibition of HIF1α or ARNT expression repressed the induction of ARC by hypoxia, while the
knock-down of HIF2α had little effect (Fig. 2B). We also confirmed that the induction of the known HIF1α target PGK1 (29) by hypoxia mirrored ARC induction, and was also decreased in cells expressing HIF1α or ARNT siRNAs (Fig. 2C). To complement these data we confirmed that each specific target gene was down-regulated by the relative siRNA (Fig. 2D). Next, we monitored the induction of ARC by hypoxia at the protein level in response to HIF1α, HIF2α, and ARNT inhibition by siRNAs, and confirmed that ARC hypoxic induction is specifically dependent on HIF1α (Fig. 2E). Finally, we showed that ARC is up-regulated by hypoxia in HCT116 cells, but not in HCT116HIF1α-/- cells (Fig. 2F).

HIF1α directly binds to HRE in ARC promoter located at -190 bp from transcription start site.

In order to determine if ARC was a direct HIF1 target gene, we conducted chromatin immunoprecipitation assays (ChIP). We used anti-HIF1α, anti-HIF2α antibodies and normal IgG (negative control) and chromatin derived from the RCC4 cells, where HIFs α are constitutively expressed under normoxia due to VHL loss. The strong HIF1 and HIF2 binding region was flanked with primers located adjacent to the transcription start site, and contained two HREs: HRE1 CACGT at -106 bp and HRE2 ACGTG at -190 bp from transcription start site (Fig. 3A). Primers flanking JMJD1A HRE and ARRDC3 HRE were used as positive controls (21, 22), and primers flanking an HRE located at -861 bp upstream of ARC transcription start site were used as a negative control. Interestingly, both HIF1α and HIF2α are capable of binding HRE1-2 region in ARC promoter, but only in case of HIF1 does this binding lead to ARC up-regulation by hypoxia (see Fig. 2). We further assessed if HIF2 can contribute to ARC regulation by over-expressing the stable P405A/P531A mutant of HIF2α in 293FT cells. Figure 3B confirms that HIF2α binds to the promoter region of ARC (HRE1-2) in 293FT cells. Also, ARC expression is
induced in 293FT cells over-expressing HIF1α or HIF2α (Fig. 3C). Since the two HREs identified are located close to each other, the resolution of ChIP was not enough to identify if HIF bound one or both sites.

To determine which HRE site was the HIF binding site, we conducted an ARC promoter reporter assay. 1005 bp DNA region, containing ARC promoter, exon1 and a part of intron1 (−701 bp to +304 bp, +1 corresponds to a transcription start site) was cloned to pGL3-basic vector upstream of firefly luciferase gene (Fig. 3D). We also generated the reporter plasmids mutated at the HRE1 (CACGT to TTTTT) and HRE2 (ACGTG to ATTTT) sites alone or in combination (sequencing data not shown). To conduct the reporter assays, we used 293FT cell line, which is established from human embryonic kidney immortalized cells. The choice of 293FT cells over RCC cell lines was based on their high transfection efficiency: RCC cell lines are notoriously hard to transfect. First, we confirmed the ARC induction by hypoxia in 293FT cells (Fig 3E; the average induction over normoxia is 1.76 times, as assessed by Western blot densitometry, p=0.023). 293FT cells are known to have mild hypoxic up-regulation of HIF target genes, where 5xHRE reporter construct (positive control containing 5 consecutive hypoxia response elements fused to luciferase) consistently displayed an average 6-fold induction in comparison to other cancer cell lines which displayed up to a 170-fold induction of the same construct. Next, reporter plasmids were transiently transfected into 293FT cells, which were subsequently subjected to 0.5% O₂ for 17h or kept in 21% O₂. Comparison of luciferase activity in normoxia and in hypoxia for all those reporter constructs clearly shows that HRE2 site (ACGTG at -190 bp) is the HIF binding site, since mutations in this site completely disrupted the induction of ARC promoter by hypoxia (Fig. 3F). These results were confirmed in TK10 renal cancer cell line, characterized by WT VHL expression (Fig. 3G).
ARC is highly expressed in human renal cell cancer and its expression overlaps with the HIF1α target carbonic anhydrase IX (CAIX). Given the HIF1α dependence of ARC expression in cancer cell lines, we hypothesized that ARC expression should be elevated in CC-RCC, where HIFs are stabilized in about 80% of cases as a consequence of VHL loss (3). The analysis of ARC expression in the Oncomine database revealed three studies (30-32) showing the elevation of ARC expression in renal cancer when compared to normal kidney tissue (Fig. 4A-C). In addition, we analyzed microarray data to compare expression levels of ARC in RCC tumors and in normal kidney tissue. Data were obtained from two public gene expression microarray data sets containing 177 tumor tissue samples and 10 renal cortex normal tissue samples (24, 25). The distribution of data for ARC in tumors was compared to two distributions: one was the distribution of ARC in normal tissue; the other was the distribution of all genes in normal tissue (see materials and methods for the detailed procedure). Box plots of ARC distribution are displayed in Fig. 4D and show that ARC is significantly prevalent in tumor tissue relative to normal tissue.

To assess ARC protein levels in renal tumors, we stained a human kidney cancer tissue microarray (TMA), and compared the expression of ARC with the HIF1α regulated enzyme CAIX (33) on a serial section from the same TMA. We found that, while the normal renal tissue samples were all negative for both ARC and CAIX staining, among 57 cases of RCCs, 37 stained positive for ARC and 47 stained positive for CAIX with 78.7% of overlap between the two stainings (Fig. 4E-F).

ARC knock-down decreases cell survival and retards growth of renal cancer cells, especially under hypoxia. The high prevalence of ARC in RCC prompted us to investigate whether ARC was acting in an anti-apoptotic role in this type of cancer. To directly test the role
of ARC in cell survival, we infected Caki1 cells with lentiviral constructs expressing two different shRNAs targeting ARC, and with a lentiviral construct expressing shRNA targeting green fluorescent protein (shGFP) as a control. After puromycin selection, we obtained two cell lines with a stable down-regulation of ARC protein of 53% and 77% respectively as compared to shGFP expressing control cells (assessed by Western blot densitometry, p<0.0001 for both). This down-regulation was accompanied by an increase in caspase 3 cleavage (34) (Fig. 5A, the average induction of caspase 3 cleavage over shGFP infected cells is 10.31 fold for shARC no.1 and 22.54 fold for shARC no.2, as assessed by Western blot densitometry, p=0.036 and 0.021 respectively). We also assessed caspase 3 activity using LE22 fluorescent probe (27), which specifically binds to caspase 3 in an active conformation. We observed the probe binding to caspase 3 in cells where ARC was knocked-down, but to a much lesser extent in shGFP-expressing cells (Fig. 5B). The binding was no longer detected when cells were incubated with caspase inhibitor Z-VAD-FMK. We also conducted the AnnexinV/Propidium Iodide (PI) staining of shGFP, shARC no.1 and no.2 expressing cells, and observed a difference in the percentage of AnnexinV/PI positive cells (Fig. 5C). Those results suggest that ARC plays an anti-apoptotic role in Caki1 cells.

While Caki1 cells express a low level of WT VHL and possess detectable levels of HIF1α and ARC expression under normoxia, Caki1 cells can also induce HIF1α and ARC under hypoxia (Fig. 1B). Thus, we tested the colony forming capacity of Caki1 cells expressing either a shGFP knock-down construct, or two shARC knock-down constructs in normoxia (21% O₂) or hypoxia (2% O₂). In response to ARC knock-down, we observed a marked decrease of Caki1 survival under normoxia and hypoxia when compared to GFP knock-down (Fig. 5D). In parallel experiments, we compared the growth rate of the same cells under normoxia (21% O₂) and
hypoxia (0.5% O₂) for two weeks and we found a substantial decrease in the growth of Caki1 cells expressing shARC, particularly under hypoxia (Fig. 5E). Importantly, Caki1 shARC no.2 cells retained the least amount of ARC compared to Caki1 shARC no.1, had more cleaved caspase 3 (Fig. 5A), more active caspase (Fig. 5B), more AnnexinV/PI positive cells (Fig. 5C), formed the least amount of colonies (Fig. 5D), and had the slowest growth rate (Fig. 5E) among the three groups analyzed.

Next, we assessed the role of ARC in RCC chemoresistance. Figure 5F shows that ARC knock-down contributes to chemo-sensitization of Caki1 cells, and the effect can be rescued by over-expression of shRNA insensitive ARC cDNA (sh-in-ARC), confirming that the effects are on-target (see Materials and Methods for details on construct generation). This result is of particular interest since RCC type of cancer is notoriously resistant to chemotherapy.

We also assessed ARC and cleaved caspase 3 staining on the TMA to evaluate if there is a correlation between the two stainings confirming the role of ARC as inhibitor of caspase 3 cleavage in human tumors. Although the TMA contained just 3 out of 35 spots positive for cleaved caspase 3, all of them were ARC negative (Fig. 5G). Further studies involving large number of tumor samples with different tumor grades and stages are needed to confirm this finding and to draw more definitive conclusions.

**Expression of ARC in RCC cell lines confers cell survival and protects them from cisplatin-induced cell death.** To evaluate the effect of ARC knockdown on survival of matched RCC cell lines with or without VHL, we included four more cell lines in the study. Genetically matched RCC4 and RCC4-VHL cells, and RCC10 and RCC10-VHL cells were infected with viruses encoding shRNA targeting GFP (control), and two shRNAs targeting ARC. After selection on puromycin, twelve acquired cell lines were subjected to colony assays. Cells were
pre-treated with 0.5μg/ml cisplatin or vehicle treated (control) as indicated. Figure 6 shows that ARC knockdown leads to suppression of colony formation in RCC4±VHL and RCC10±VHL cells, especially when combined with cisplatin treatment, and the effect of knockdown on cell survival is more pronounced in VHL-deficient cells.

Caki1 cells with down-regulated ARC expression show decreased tumorigenic potential in SCID mice. In light of the effects the down-regulation of ARC expression had on survival and growth of renal cancer cell lines in vitro, we decided to investigate the effect of ARC inhibition on tumor forming potential in immuno-deficient mice. It is important to mention that currently there is no robust mouse model to study RCC primary tumor formation and metastasis. Caki1 cells are a low VHL expressing cell line and form subcutaneous (sc) tumors with long periods of latency and do not metastasize. We used Caki1 cells, which we also used in our in vitro work, for in vivo studies, since they are low VHL expressing cell line, and are frequently used by others for animal studies of RCC (35-37). We injected Caki1 cells stably transduced with shGFP, shARC no.1 and shARC no.2 sc into the dorsal flanks of SCID mice. After 13 weeks, tumor dimensions were measured with calipers and tumor volume was calculated. The results clearly showed that knock-down of ARC expression lead to impairment of tumor initiation ability in Caki1 cells (Fig. 7). Nine out of ten injections of shGFP Caki1 cells grew into tumors. In contrast, only one out of fourteen injections of shARC Caki1 cells formed a tumor. These results indicate that elevated levels of ARC in CC-RCC are essential for initiating tumor growth.

DISCUSSION
In this study we showed that the anti-apoptotic protein ARC confers RCC the capacity to maintain their neoplastic phenotype in vitro and in vivo: ARC down-regulation is accompanied by decreased cell survival and impairment of growth in vitro, and by inhibition of tumor forming ability in vivo. Although ARC regulation at the posttranslational level through ubiquitination and proteasomal degradation was rigorously studied (13, 14), actual factors binding to its promoter and regulating its expression just start to be elucidated. The indirect effects of Ras/MEK/ERK pathway on ARC promoter stimulation, and of p53 pathway on ARC promoter repression were previously reported (16, 17), along with a recent report of HIF-dependent ARC regulation ((38), see discussion below). In the present study, we have shown that increased ARC expression is stimulated by direct HIF1 binding to ARC promoter: under hypoxia in cells with WT VHL, or under normoxia in cells with mutated/lost VHL. Solid tumors are known to be invariably less well oxygenated in comparison to their normal tissue of origin, making low oxygen tension, or hypoxia, a critical hallmark of cancer development (39). Our findings of ARC regulation by hypoxia and HIF1 in CC-RCC deserve further investigation in solid tumors of other tissue origin, aiming at better defining the contribution of ARC to tumor cell survival and possibly therapeutic resistance.

The anti-apoptotic function of ARC appears to be well conserved in different cell types, including cancer cells as well as cells in normal tissues (40-42). In agreement with our data, ARC was shown to inhibit cell death potently in cancer cells (HeLa, MCF7, HCT116) (42, 43) and in cardiomyocytes (40, 41). However, the regulation of ARC expression by hypoxia or other injuries (ischemia/reperfusion) shows cell type and/or species specificity. Unlike our observations in a variety of human cancer cell types, hypoxia treatment of rat H9C2 cardiomyocytes and ventricular myocytes led to decreased ARC levels in a manner that
correlated with the induction of apoptosis and/or necrosis (40). On the other hand, in the recent report by Zaiman et al., ARC was shown to be induced by hypoxia in isolated rat pulmonary arterial smooth muscle cells (44). Interestingly, the putative HIF1α binding site identified in our study appears to be conservative in primates, but is not present in the mouse or rat ARC promoter (Fig. 8). Importantly, we could not detect ARC expression in mouse embryonic fibroblasts (MEFs) under normoxia and hypoxia by Western blot, which is in line with the fact that MEFs are highly susceptible to apoptotic death under hypoxia (45). Thus, the mechanism of ARC regulation by hypoxia might be complex and tissue specificity and/or species-specific regulation should be taken into consideration. Studies by Nam et al. and Foo et al. have shown the role of ARC ubiquitination and proteasomal degradation in regulation of its expression at the protein level in cells of rat and mouse origin. Further studies are needed to clarify the contribution of proteasomal degradation and transcriptional up-regulation by hypoxia in ARC expression, and its subsequent impact on cell survival/proliferation in human cancer.

A recent paper by Ao et al. identified ARC as a hypoxia inducible and HIF1 regulated gene (38), although the study did not investigate if ARC is a target gene of HIF1, HIF2 or both. These authors identified the GCGTG as a HIF1 binding site, which is different from the ACGTG site identified in our study. The GCGTG site might represent an alternative HIF binding site, and it would be important to assess its conservation in different species.

Our ChIP data clearly showed that HIF1α and HIF2α are capable of binding to HRE2 in the ARC promoter. At the same time, just endogenous HIF1 regulates ARC transcription (Fig. 2), although HIF2 can regulate ARC transcription when exogenously over-expressed (Fig. 3C). This finding one more time strengthens the notion that binding to the promoter does not equal activation.
The HIF1 dependence of ARC raises the question as to how a HIF1 regulated gene can have such an essential role in CC-RCC, considering the largely accepted view that it is rather the silencing of HIF2α that is sufficient to impair growth of VHL deficient tumors in vivo (46). There is a current debate in the field as to what are the roles of each family member HIF1α and HIF2α in initiation and/or progression of RCC, with an extreme view of HIF1α being a tumor-suppressor gene and HIF2α – an oncogene. Indeed, several lines of evidence support this hypothesis, including loss HIF1α locus in a number of RCCs, supported with functional studies suggesting that over-expression of HIF1α in VHL WT cells restrains tumor growth, whereas suppression of HIF1α in VHL-deficient cells enhances tumor growth (reviewed in (47)). It is important to note that HIFs are transcription factors regulating a large list of target genes, including genes with opposite functions. Thus, tumor suppressive and oncogenic functions are intrinsic to HIFs, and the shift in their balance will depend on the stage of tumor evolution, as well as a genetic context of a given tumor. We predict that HIF1-mediated anti-apoptotic function is going to be important at early stages of tumor development, when just a few anti-apoptotic mechanisms are in play. Later during tumor evolution more genetic mutations accumulate and more signaling pathways get reprogrammed, allowing multiple mechanisms of apoptosis evasion. At these later stages HIF1 anti-apoptotic function gets dispensable, and balance shifts to more tumor suppressive functions, leading to selective pressure for HIF1 elimination. A recent and exhaustive study analyzing VHL genotype, and HIF1α and HIF2α expression in 160 primary renal tumors, identified three distinct molecular patterns for human CC-RCCs: those characterized by WT VHL, and two subtypes with VHL deficiencies. Among the latter were tumors typified by both HIF1α and HIF2α induction (H1H2 tumors), and those with exclusive over-production of HIF2α (H2 tumors) (48). In our study we found that ARC is a
HIF1α regulated gene, which promotes CC-RCC cell survival. Thus, we suggest that ARC might play an essential role in survival of renal cancer cells in some tumors from the first two groups: WT VHL, and VHL deficient tumors (H1H2).

Since CC-RCC type of cancer is highly aggressive and unresponsive to radiation and chemotherapy, there is a large interest in the field concerning the mechanism of this resistance, especially because p53 mutations are rarely detected in this type of cancer (49) (IARC p53 database [http://www-p53.iarc.fr/]). There were several mechanisms of resistance to therapy proposed in the literature in relation to p53: disrupted USP10-mediated de-ubiquitination of p53 (50), NFκB-dependent suppression of p53 (51, 52), HDM2-mediated suppression of p53 (53, 54). Our results suggest an additional mechanism of cell survival in CC-RCC through increased ARC expression. Importantly, ARC was shown to be a negative regulator of p53 function, which interferes with p53 tetramerization and stimulates p53 nuclear export (13). Additional research is needed to directly test the role of ARC in the blockade of p53-dependent apoptosis and cell cycle arrest in CC-RCC.

In conclusion, our data suggest that ARC is a promising therapeutic target for WT VHL and VHL deficient (H1H2) renal cancers and possibly other types of cancer associated with increased HIF1α activity.

ACKNOWLEDGEMENTS

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The authors would like to thank Giovambattista Pani (Universita’ Cattolica del Sacro Cuore, Roma, Italy) for critical reading of the manuscript, Quynh-Thu Le (Stanford) for anti-CAIX antibodies, Alejandro Sweet-Cordero (Stanford) for A549 cells, Marianne Broome-Powell (Stanford) for A375 cells, Silvestre Vicent (Stanford) for pLKO.1shGFP plasmid, Scott Lowe for pLPC plasmid, Aaron Puri (Stanford) for Z-VAD-FMK caspase inhibitor.

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**TITLES AND LEGENDS TO FIGURES**

FIG. 1. ARC expression is induced by hypoxia. (A) QRT-PCR showing induction of ARC and PGK1 expression in Caki1 cells exposed to hypoxia for 16h compared to normoxia. The TBP expression was used as an internal control. The data represent the mean relative expression ±SEM of four independent experiments, each performed in triplicate. P-values: * < 0.001, ** < 0.01, comparison to normoxia. (B) Western blots showing the ARC and HIF1α protein expression under normoxia or hypoxia in the indicated cancer cell lines. (C) Western blots, showing ARC and HIF1α protein levels in A549 and MCF7 cancer cell lines, under different oxygen tensions. In B and C α-tubulin is shown as a loading control.

FIG. 2. Hypoxic induction of ARC is HIF1α-dependent. (A) Western blot, showing hypoxic induction of ARC and HIF1α in RCC4-VHL cells, but not in 786-0-VHL or A498-VHL cells, which lack HIF1α expression. (B) QRT-PCR showing the expression of ARC mRNA in RCC4-VHL cells transiently transfected with siRNAs targeting HIF1α, HIF2α, ARNT, or non-targeting control siRNA (siCTR) and exposed to normoxia or hypoxia. ARC induction by hypoxia gets ablated when either the siRNA to HIF1α, or ARNT was used, but is unaffected by siRNA to HIF2α. P-values: * < 0.05, **, †< 0.01, ‡< 0.001. (C) QRT-PCR showing the induction of a known HIF1α target gene (PGK1) by hypoxia, and abrogation of its induction in
response to HIF1α down-regulation by siRNA transfection. P-values: *, # < 0.0001, **, ## < 0.001. For B-C: *, # - comparison to mock transduced cells at 2% O₂, **, ### - comparison to siCTR transduced cells at 2% O₂. (D) QRT-PCR showing down-regulation of HIF1α (i), HIF2α (ii), and ARNT (iii) expression in RCC4-VHL cells 40h after appropriate siRNA transfection (as indicated) under normoxia and hypoxia. (E) Western blot, showing the impact of knocking down HIF1α, HIF2α, and ARNT in RCC4-VHL cells on ARC induction by hypoxia. (F) Western blot, showing hypoxic induction of ARC and HIF1α in HCT116 cells, but not in HCT116HIF1α-/- cells, which lack HIF1α expression. In A, E-F α-tubulin is shown as a loading control. In A-F hypoxic exposure was for 16h. In B-E mock-transfected refers to Dharmafect1 transfection reagent only sample; siCTR is a non-targeting Control siRNA. In B-D the TBP expression was used as an internal control, and the data represent the mean relative expression ±SEM of three independent experiments, each performed in triplicate.

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FIG. 5. ARC knock-down decreases cell survival and retards cell growth under normoxia and hypoxia.  (A) Western blot showing down-regulation of ARC expression in Caki1 cells after infection with two shRNA expressing lentiviral constructs, leading to cleavage of caspase 3. (B) Caspase 3 activity was assessed using LE22 fluorescent probe. We observed the probe binding to caspase 3 in cells where ARC was knocked-down, but not in shGFP-infected cells, which was abrogated by treatment with caspase inhibitor Z-VAD-FMK. Coomassie stained gel controls for equal protein loading. (C) Caki1 cells stably expressing either shGFP or two different shRNAs targeting ARC were analyzed for apoptosis by flow cytometry. The percentages of Annexin V positive/PI negative cells are indicated. A representative experiment is shown in i). Results in ii) represent the average percent of AnnexinV positive/PI negative cells ±SEM of three independent experiments. P-value: * = 0.027. (D) ARC knock-down reduces colony forming capacity of Caki1 cells under normoxic and hypoxic conditions. Graphs represent the average colony number per plate in two independent experiments ±SEM. P-values: * < 0.05, **: 0 < 0.01, ## < 0.001. *, ** - comparison to Caki1 shGFP in normoxia; , # - comparison to Caki1 shGFP in hypoxia. (E) ARC knock-down retards cell growth of Caki1 cells under normoxic and especially hypoxic conditions. Cell number per plate (Y axis) is plotted against days after initial plating at day 0 (X axis). Error bars represent SD. (F) ARC knock-down contributes to chemo-sensitization of Caki1 cells, and the effect can be rescued by over-expression of shRNA insensitive ARC cDNA. Cells were treated with 10μg/ml cisplatin for 17h or left untreated, and caspase 3 cleavage was assessed by Western blot. (G) Examples of ARC negative and cleaved caspase 3 positive, as well as ARC positive and cleaved caspase 3 negative IHC staining on the RCC TMA. TMA contained just 3 out of 35 spots positive for cleaved caspase 3, all of them were ARC negative. In A and F experiments were performed under normoxia; shRNA targeting green
fluorescent protein (shGFP), or pLM-CMV-H4-neo-PL3 vector were used as controls; Hsp-70 (mitochondrial Heat Shock Protein 70) in A and α-tubulin in F are shown as loading controls.

FIG. 6. ARC knockdown leads to suppression of colony formation in RCC4±VHL and RCC10±VHL cells, and the effect of knockdown on cell survival is more pronounced in VHL-deficient cells. RCC4 and matched RCC4-VHL cells, as well as RCC10 and matched RCC10-VHL cells were infected with viruses encoding shRNA targeting GFP (control), and two shRNAs targeting ARC. After selection on puromycin, twelve acquired cell lines were subjected to colony assays. Cells were pre-treated with 0.5μg/ml cisplatin or vehicle treated. This experiment was repeated two times in triplicate.

FIG. 7. ARC knock-down decreases tumor forming ability of Caki1 cells. Caki1 cells stably expressing shGFP, shARC no.1, or shARC no.2 shRNA lentiviral constructs were injected sc into right and left flanks of SCID mice. In this analysis mice of shARC no.1 and shARC no.2 groups are pooled together and designated as a shARC group. Representative pictures of mice are shown for each group. Average tumor volumes calculated at the end of the study (Y axis) are plotted (X axis). P-value: * = 0.0153.

FIG. 8. Genomic DNA alignment of regions containing HRE2 HIF binding site in ARC promoter among different species, as indicated. The numbers above the sequences correspond to coordinates in UCSC Genome Browser, Human Feb. 2009 (GRCh37/hg19) Assembly.
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FIG. 2.

A

786-OVHL RCC4-VHL A498VHL

ARC
HIF1α
HIF2α
α-tubulin

B

21% 2% [O₂]

Relative ARC expression

Mock siCTR siHIF1α siHIF2α siARNT

C

21% 2% [O₂]

Relative PGK1 expression

Mock siCTR siHIF1α siHIF2α siARNT

D

i) [O₂]

Relative HIF1α expression

ii) [O₂]

Relative HIF2α expression

iii) [O₂]

Relative ARNT expression

E

RCC4-VHL

ARC
HIF1α
HIF2α
ARNT
α-tubulin

F

HCT116 HCT116 HIF1α-/-

ARC
HIF1α
HIF2α
α-tubulin

α-tubulin

Downloaded from http://mcb.asm.org on December 28, 2017 by guest
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FIG. 3.

A. Graph showing relative enrichment of various antibodies against HIF1α and HIF2α.

B. Graph showing relative luciferase activity with different O2 concentrations and promoter mutations.

C. Western blot analysis for different proteins: ARC, HIF1α, HIF2α, HA-tag, and α-tubulin.

D. Diagram of luciferase promoter construct with HRE1 and HRE2.

E. Western blot analysis under different O2 conditions.

F. Graph showing relative luciferase activity with different O2 concentrations and promoter mutations.

G. Graph showing relative luciferase activity with different O2 concentrations and promoter mutations.

-0.02 0 0.02 0.04 0.06 0.08 0.1 0.12 0.14
0 0.1 0.2 0.3
21% O2 0.5% O2

-0.05 0 0.05 0.1 0.15 0.2 0.25 0.3

-0.05 0 0.05 0.1 0.15 0.2 0.25 0.3
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FIG. 5.

**A**

<table>
<thead>
<tr>
<th></th>
<th>shGFP</th>
<th>shARC no.1</th>
<th>shARC no.2</th>
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<tbody>
<tr>
<td>ARC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp-70</td>
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<tr>
<td>Cleaved Caspase 3</td>
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</table>

**B**

Z-VAD-FMK
LE22-labeled caspase 3
coomassie staining

**C**

Propidium iodide
Annexin V-FITC

<table>
<thead>
<tr>
<th></th>
<th>shGFP</th>
<th>shARC no.1</th>
<th>shARC no.2</th>
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<tbody>
<tr>
<td>3.0%</td>
<td>5.9%</td>
<td>8.2%</td>
<td></td>
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**D**

21% O₂
Caki1
2% O₂

![Graph showing colony number per plate](image)

**E**

![Graph showing cell number per plate](image)

**F**

Caki1

![Graph showing untreated and Cisplatin-treated samples](image)

**G**

Cleaved Caspase 3
Renal Cell Carcinoma

α-tubulin
Cleaved Caspase 3

![Images of protein staining](image)
FIG. 5. ARC knock-down decreases cell survival and retards cell growth under normoxia and hypoxia. (A) Western blot showing down-regulation of ARC expression in Caki1 cells after infection with two shRNA expressing lentiviral constructs, leading to cleavage of caspase 3. (B) Caspase 3 activity was assessed using LE22 fluorescent probe. We observed the probe binding to caspase 3 in cells where ARC was knocked-down, but not in shGFP-infected cells, which was abrogated by treatment with caspase inhibitor Z-VAD-FMK. Coomassie stained gel controls for equal protein loading. (C) Caki1 cells stably expressing either shGFP or two different shRNAs targeting ARC were analyzed for apoptosis by flow cytometry. The percentages of Annexin V positive/PI negative cells are indicated. A representative experiment is shown in i). Results in ii) represent the average percent of AnnexinV positive/PI negative cells ±SEM of three independent experiments. P-value: * = 0.027. (D) ARC knock-down reduces colony forming capacity of Caki1 cells under normoxic and hypoxic conditions. Graphs represent the average colony number per plate in two independent experiments ±SEM. P-values: * < 0.05, ** < 0.01, ### < 0.001. *, ** - comparison to Caki1 shGFP in normoxia; #, ### - comparison to Caki1 shGFP in hypoxia. (E) ARC knock-down retards cell growth of Caki1 cells under normoxic and especially hypoxic conditions. Cell number per plate (Y axis) is plotted against days after initial plating at day 0 (X axis). Error bars represent SD. (F) ARC knock-down contributes to chemo-sensitization of Caki1 cells, and the effect can be rescued by ARC over-expression. Cells were treated with 10ug/ml cisplatin for 17h or left untreated, and caspase 3 cleavage was assessed by Western blot. (G) Examples of ARC negative and cleaved caspase 3 positive, as well as ARC positive and cleaved caspase 3 negative IHC staining on the RCC TMA. TMA contained just 3 out of 35 spots positive for cleaved caspase 3, all of them were ARC negative. In A and F experiments were performed under normoxia; shRNA targeting green fluorescent protein (shGFP), or pLPC vector were used as controls; Hsp-70 (mitochondrial Heat Shock Protein 70) in A and α-tubulin in F are shown as loading controls.
FIG. 6. ARC knockdown leads to suppression of colony formation in RCC4±VHL and RCC10±VHL cells, and the effect of knockdown on cell survival is more pronounced in VHL-deficient cells. RCC4 and matched RCC4-VHL cells, as well as RCC10 and matched RCC10-VHL cells were infected with viruses encoding shRNA targeting GFP (control), and two shRNAs targeting ARC. After selection on puromycin, twelve acquired cell lines were subjected to colony assays. Cells were pre-treated with 0.5ug/ml cisplatin or vehicle treated. This experiment was repeated two times in triplicate.
FIG. 7. ARC knock-down decreases tumor forming ability of Caki1 cells. Caki1 cells stably expressing shGFP, shARC no.1, or shARC no.2 shRNA lentiviral constructs were injected sc into right and left flanks of SCID mice. In this analysis mice of shARC no.1 and shARC no.2 groups are pooled together and designated as a shARC group. Representative pictures of mice are shown for each group. Average tumor volumes calculated at the end of the study (Y axis) are plotted (X axis). P-value: * = 0.0153.
FIG. 8. Genomic DNA alignment of regions containing HRE2 HIF binding site in ARC promoter among different species, as indicated. The numbers above the sequences correspond to coordinates in UCSC Genome Browser, Human Feb. 2009 (GRCh37/hg19) Assembly.