Isoflurane, a Commonly Used Volatile Anesthetic, Enhances Renal Cancer Growth and Malignant Potential *via* the Hypoxia-inducible Factor Cellular Signaling Pathway *In Vitro*

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ABSTRACT

Background: Growing evidence indicates that perioperative factors, including choice of anesthetic, affect cancer recurrence after surgery although little is known about the effect of anesthetics on cancer cells themselves. Certain anesthetics are known to affect hypoxia cell signaling mechanisms in healthy cells by up-regulating hypoxia-inducible factors (HIFs). HIFs are also heavily implicated in tumorigenesis and high levels correlate with poor prognosis.

Methods: Renal cell carcinoma (RCC4) cells were exposed to isoflurane for 2 h at various concentrations (0.5–2%). HIF-1α, HIF-2α, phospho-Akt, and vascular endothelial growth factor A levels were measured by immunoblotting at various time points (0–24 h). Cell migration was measured across various components of extracellular matrix, and immunocytochemistry was used to analyze proliferation rate and cytoskeletal changes.

Results: Isoflurane up-regulated levels of HIF-1α and HIF-2α and intensified expression of vascular endothelial growth factor A. Exposed cultures contained significantly more cells (1.81 ± 0.25 *vs.* 1.00 of control; $P = 0.03$) and actively proliferating cells (89.4 ± 2.80 *vs.* 64.74 ± 7.09% of control; $P = 0.016$) than controls. These effects were abrogated when cells were pretreated with the Akt inhibitor, LY294002. Exposed cells also exhibited greater migration on tissue culture–coated ($F = 16.89$; $P = 0.0008$), collagen-coated ($F = 20.99$; $P = 0.0003$), and fibronectin-coated wells ($F = 8.21$; $P = 0.011$) as along with dramatic cytoskeletal rearrangement, with changes to both filamentous actin and α-tubulin.

Conclusions: These results provide evidence that a frequently used anesthetic can exert a protumorigenic effect on a human cancer cell line. This may represent an important

What We Already Know about This Topic

- Hypoxia-inducible factor signaling is heavily implicated in cancer growth
- Enhancing the signaling of these transcription factors may increase metastatic potential
- Whether anesthetics exert promoting effects on cancer cells *via* modulation of this pathway remains uncertain

What This Article Tells Us That Is New

- Exposure of human renal cancer cells to isoflurane (0.5–2% for 2 h) resulted in up-regulation of levels of hypoxia-inducible factor-1α and -2α along with enhanced cell migration and cytoskeleton rearrangements
- Isoflurane promotes cancer cell growth and migration *in vitro* and hence enhances malignant potential

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contributory factor to high recurrence rates observed after surgery.

SURGERY remains the first-line treatment for the majority of solid cancers and plays a vital role in staging, reconstruction, and palliation.[1] However, there is growing recognition that events within the perioperative period influence cancer recurrence, metastasis, and long-term survival.[1] Most of the current literature has focused on the impact of surgical stress on the immune and neuroendocrine systems, along with the inadvertent seeding of tumor cells during the procedure.[2–5] Anesthetic technique has also been implicated in cancer growth with a number of retrospective studies suggesting that regional anesthesia is associated with improved outcomes when compared with general anesthesia in a number of different types of cancer, including breast, colon, prostate, and ovary.[6–9] In particular, a series of 225 patients undergoing radical prostatectomy reported that the use of epidural anesthesia was associated with a 57% lower recurrence rate.[9]

More specifically, questions have been raised about the potential direct effects of certain anesthetics on cell signaling pathways in cancer cells. Over 30 yr ago, general anesthetics such as halothane and nitrous oxide were shown to strongly accelerate postoperative metastasis in murine models of lung carcinoma and melanoma and led to the development of metastases in organs in which they are not usually found,[10] whereas the microarray data demonstrated that volatile anesthetics modulated gene expression in human breast and brain tumor cell lines in a unique and time-dependent manner, including some genes that are used for predictive genetic fingerprinting.[11] To date, there has been little evidence to support the molecular basis for such findings in cancer cells.

For many years, it had been considered that general anesthetics exert a reversible effect in all body systems particularly the central nervous system, which is then returned to its erstwhile state after the agent was eliminated from the bioaction sites. However, it has been increasingly recognized that anesthetics can induce cell phenotypic changes due to their pharmacological effects *via* cellular signaling pathways. Indeed, the commonly used volatile anesthetic isoflurane (used for general anesthesia in cancer patients) and the noble gas xenon can up-regulate cellular signaling pathways including the hypoxia-inducible factor (HIF) pathway.[12,13]

HIFs are a family of transcription factors that regulate a vast array of genes involved in critical aspects of cancer activity such as cell proliferation, angiogenesis, glucose metabolism, and cell invasion.[14] High levels of both HIF-1α and HIF-2α are generally detected in the majority of primary tumors and their metastases, linking high HIF levels to poor patient outcome.[15,16] They may also affect the efficacy of therapy, with radioresistance seen in cervical cancer cells that overexpress HIF-1α[17]. Furthermore, resistance to certain chemotherapy agents such as paclitaxel disappears when HIF-1α is knocked out in cancer cells.[18] The putative up-regulation of HIFs by anesthetics in cancer patients would therefore seem to bring about highly detrimental consequences.

The aim of this study is to define the effect of the commonly used volatile anesthetic isoflurane on the HIF pathway in renal carcinoma cells and to determine its potential impact on cancer cell behavior in terms of cell proliferation and cell migration *in vitro*. It is hypothesized that isoflurane will enhance the HIF pathway, thus activating downstream effectors and increasing the cancer cells' metastatic potential.

Materials and Methods

Cell Culture

Primary renal cell carcinoma cell line (RCC4), a gift from Professor Patrick Maxwell, Ph.D. (Department of Medicine, University College London, London, United Kingdom) was cultured as monolayer at 37°C in humidified air balanced with 5% carbon dioxide. They were maintained in Roswell Park Memorial Institute: RPMI-1640 medium (GIBCO; Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Thermo Scientific, Epsom, United Kingdom), 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin (Invitrogen). The culture medium was replaced every 48 h. Cells used for immunoblotting, trypan blue assays, and cell migration assays were seeded at a density of 1×10^6/ml on 28-cm^2 Petri dishes (VWR, Leicestershire, United Kingdom) and used experimentally when become 80% confluent 24 h later. Cells used for immunofluorescence were seeded at a density of 7.5×10^4/ml on poly-L-ornithine hydrobromide (500 μg/ml; Sigma-Aldrich, Dorset, United Kingdom)–coated 13-mm circular coverslips (VWR). Cells used for 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) viability assays were plated at a density of 3×10^4/ml on 48-well plates (VWR).

Akt Inhibition

RCC4 cells were treated with 20 μM of a protein kinase B (Akt) inhibitor LY294002 in 0.02% dimethyl sulfoxide (Sigma-Aldrich), in normal media and incubated for 16 h at 37°C in a humidified atmosphere containing 5% carbon dioxide. Naive control (NC) cells where not exposed to the inhibitor.

Isoflurane Gas Exposure

Cells were placed in purpose-built 1.5-l airtight, temperature-controlled chambers equipped with inlet and outlet valves and internal electric fans, which were used to provide continuous delivery and mixture of gases as reported previously.[19] An in-line anesthetic vaporizer fed by a supply of a gas mixture containing 21% oxygen balanced with nitrogen was used to deliver isoflurane in the gas phase to the chamber at a rate of 2 l/min for a minimum of 5 min until desired isoflurane concentration (0.5–2%) was achieved. The atmosphere within the chamber was supplemented with 5% carbon dioxide. Effluent-desired concentrations of isoflurane and 21% oxygen were monitored by an anesthetic analyzer (Datex-Ohmeda, Bradford, United Kingdom) until the chamber was sealed. Cells in control

conditions defined as NC were placed in an identical gas chamber under atmospheric conditions of 21% oxygen and 5% carbon dioxide balanced with nitrogen. Once sealed, the chambers were placed in a 37°C incubator for 2 h (Galaxy R Carbon Dioxide Chamber; Wolf Laboratories, York, United Kingdom). Cells were used either immediately or up to 24 h after gas exposure.

Immunoblotting

Cells were rinsed in ice-cold HEPES, lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 2 mM EGTA, 1% Triton, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenyl methane sulfonyl fluoride, and 1 mM dithiothreitol (Cell Signaling, Hitchin, United Kingdom); lysed by physical disruption using a cell scraper and cleared by centrifugation; concentrations were determined by the BioRad Protein Assay (BioRad Laboratories, Hercules, CA) and extracts were normalized for protein content. Whole cell extracts were taken into NuPage sample buffer (Invitrogen) and 30 μl of equal protein content samples per lane were resolved by gel electrophoresis using a NuPage 4–12% Bis-Tris gel (Invitrogen), and then transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked for 3 h in 5% non–fat-powdered milk in 0.1% Tween 20/Tris buffered saline (pH 7.4). Primary antibodies were against HIF-1α, HIF-2α, prolyl hydroxylase (PHD)-2, vascular endothelial growth factor A, β-actin (Novus Biologicals, Littleton, CO), p-Akt (ser 473), phosphorylated phosphatase and tensin homologue (Ser 380; Cell Signaling), and α-tubulin (Sigma-Aldrich). The enhanced chemiluminesence ECL-Plus system (Amersham Biosciences, Buckinghamshire, United Kingdom) and horseradish peroxidase–conjugated secondary anti-mouse or anti-rabbit antibodies (Cell Signaling) were used for detection in conjunction with the Syngene system (Syngene, Cambridge, United Kingdom). Analysis was performed by densitometry using the Syngene gene tools analysis software (Syngene), and results were normalized to levels of the housekeeping proteins α-tubulin or β-actin.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 min, washed with phosphate-buffered saline (PBS; Sigma-Aldrich) two times for 5 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and washed twice for 5 min. Blocking was carried out at room temperature for 1 h using 10% normal goat serum (Vector Laboratories, Peterborough, United Kingdom) in 0.1% Triton X-100 in PBS. Cells were then incubated overnight at 4°C in blocking solution containing one of the following antibodies: HIF-1α (Novus Biologicals), ki67 (Abcam, Cambridge, United Kingdom), tetramethylrhodamine-5-(and 6)-isothiocyanate–conjugated phalloidin (staining F-actin; Millipore, Oxford, United Kingdom), or fluorescein isothiocyanate–conjugated α-tubulin (Sigma-Aldrich). Cells were then washed three times with PBS, and in the case of the nonconjugated primary antibodies incubated for 2 h in PBS-T containing one of the following secondary antibodies: Rhodamine-conjugated donkey anti-rabbit IgG (Millipore), fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG (Millipore), cells were then rinsed in PBS and coverslipped with vectorshield fluorescence medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Cells were processed in a duplicate manner for each set of experiments. To confirm the specificity of primary antibodies, control sections were incubated with the primary antibody omitted.

Image Analysis

Images for quantification were captured under identical exposure settings. Final quantitative assessments were conducted under blinded conditions. For HIF-1α, images were captured with a Nikon E1000M (Nikon, Surrey, United Kingdom) widefield fluorescence microscope under ×20 objective. Antibody staining was then quantified by measuring mean pixel intensity using ImageJ 1.35 software (National Institutes of Health, Bethesda, MD). For cell proliferation analysis, a series of images of cells that were double labeled with DAPI and ki67 were captured using a Nikon Eclipse 80i microscope (Nikon) and tilled together using the Image-Pro Plus software (Media Cybernetics, Bethesda, MD) creating mosaic images of the whole coverslip. A region of interest that did not contain any nonspecific binding was then selected and the percentages of DAPI-stained (total number of cells) and ki67-positive cells were measured. Ki67-positive cells were then presented as a percentage of the total number of cells. For cytoskeletal rearrangement images of cells triple-labeled with tetramethylrhodamine-5-(and 6)-isothiocyanate-phalloidin (Millipore), fluorescein isothiocyanate-α-tubulin (Sigma-Aldrich), and DAPI (Vector Laboratories) were captured using a Nikon E1000M (Nikon) widefield fluorescence microscope under a ×10 and ×20 objective. For cell area analysis, 10 nondividing phalloidin-labeled cells/image were selected to measure their area (three images/n number) using ImageJ 1.35 software (National Institutes of Health). For better visualization of cytoskeletal components, z-stacks were collected under a ×63 oil objective from isoflurane-treated cells with a Leica TCS SP5, laser scanning confocal microscope (Leica, Milton Keynes, United Kingdom) and used to generate maximum projection images.

Cell Viability Measurement

Cell viability was assessed using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Merck KGaA, Darmstadt, Germany) assay. MTT was diluted with minimum essential medium + Earle L-glutamine × 1 (GIBCO Invitrogen) to a concentration of 0.5 mg/ml. Cells were incubated with 600 μl of MTT solution for 2 h followed by 1 ml of dimethyl sulfoxide (Fisher Scientific, Leicestershire, United Kingdom) to dissolve the formazan crystals and then mixed to achieve a homogeneous purple color. The absorbance of the dissolved formazan crystals was spectrophotometrically assessed at a wavelength of 595 nm using an Mrx Microplate Reader (Dynex Technologies, Chantilly, VA).

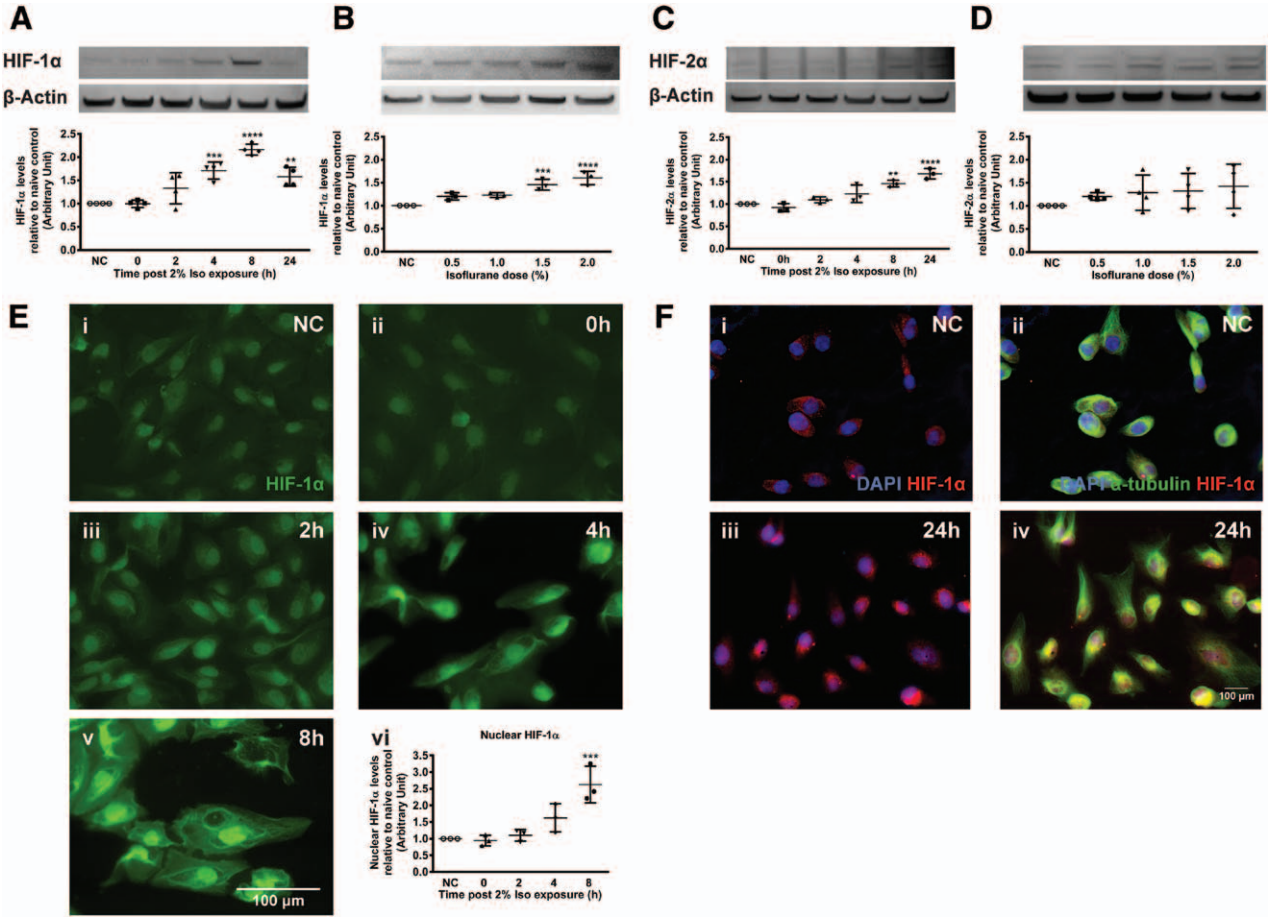

Fig. 1. Immunoblot and immunofluorescent analyses of hypoxia-inducible factor (HIF) changes after isoflurane exposure. Renal cell carcinoma cells were treated with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 h for time course experiments (*A*, *C*, *E*, and *F*) or with or without 0.5–2.0% for 2 h (*C* and *D*) for dose–response experiments. Time course experiments were assessed by immunoblotting against HIF-1α (*A*) and HIF-2α (*C*) and immunofluorescence of HIF-1α (*E* and *D*) at different time points (0, 2, 4, 8, and 24 h) postgas exposure. Dose–response experiments were assessed by immunoblotting against HIF-1α (*B*) and HIF-2α (*D*) 8-h postgas exposure. Exposure to isoflurane for 2 h induced HIF-1α in time-dependent (*A*) and dose-dependent manner (*B*). Expression of HIF-2α was also increased in a time-dependent (*C*) and dose-dependent manner (*D*). Furthermore, exposure to 2% isoflurane induced a time-dependent HIF-1α translocation from the cells' cytosol to the cells' nuclei as evident from HIF-1α/α-tubulin/4',6-diamidino-2-phenylindole (DAPI) fluorescence staining (*E* and *F*). Data were presented as mean ± SD. One-way ANOVA with Tukey corrections; ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ *versus* naive control (NC; n = 3–4). Iso = isoflurane.

Cell Death Measurement

Cell death was assessed using the trypan blue exclusion assay in double-blinded conditions. After 24-h anesthetic treatment, cells were removed from six-well plates by trypsinization and resuspended in 1 ml Dulbecco PBS (GIBCO Invitrogen). Then, 0.5 ml of the cell suspension was thoroughly mixed with 0.5 ml of 0.4% weight/volume trypan blue in PBS (Sigma-Aldrich). The mixture was placed on a hemocytometer. Under light microscopy, viable and nonviable cells in each of the four large hemocytometer squares were counted, and an average was calculated for further data analysis.

Cell Migration Assessment

Cell migration was analyzed using the Oris Cell Migration Assay (AMSbio, Oxford, United Kingdom). The cell-seeding stopper was inserted into a 96-well plate, containing well bottoms that were coated either with polylysine (tissue culture coating), collagen, or fibronectin. Fifty thousand cells were seeded into each well with a stopper to restrict cell seeding to the outer regions of the wells. Removal of the stopper resulted in the opening of a round, unseeded region, into which the seeded cells could migrate. Cells were then exposed to 21% oxygen and 5% carbon dioxide balanced with nitrogen with or without 2% isoflurane, and stained with the acetomethoxy derivative of calcein (Calcein AM; Abcam) for 30 min at the respective time points. Cell migration was assessed using FLx 800 microplate fluorescence reader (Biotek Instruments, Winooski, VT).

Statistical Analysis

Data are expressed as mean ± SD. Data were analyzed using one-way ANOVA and *post hoc* Tukey tests unless in

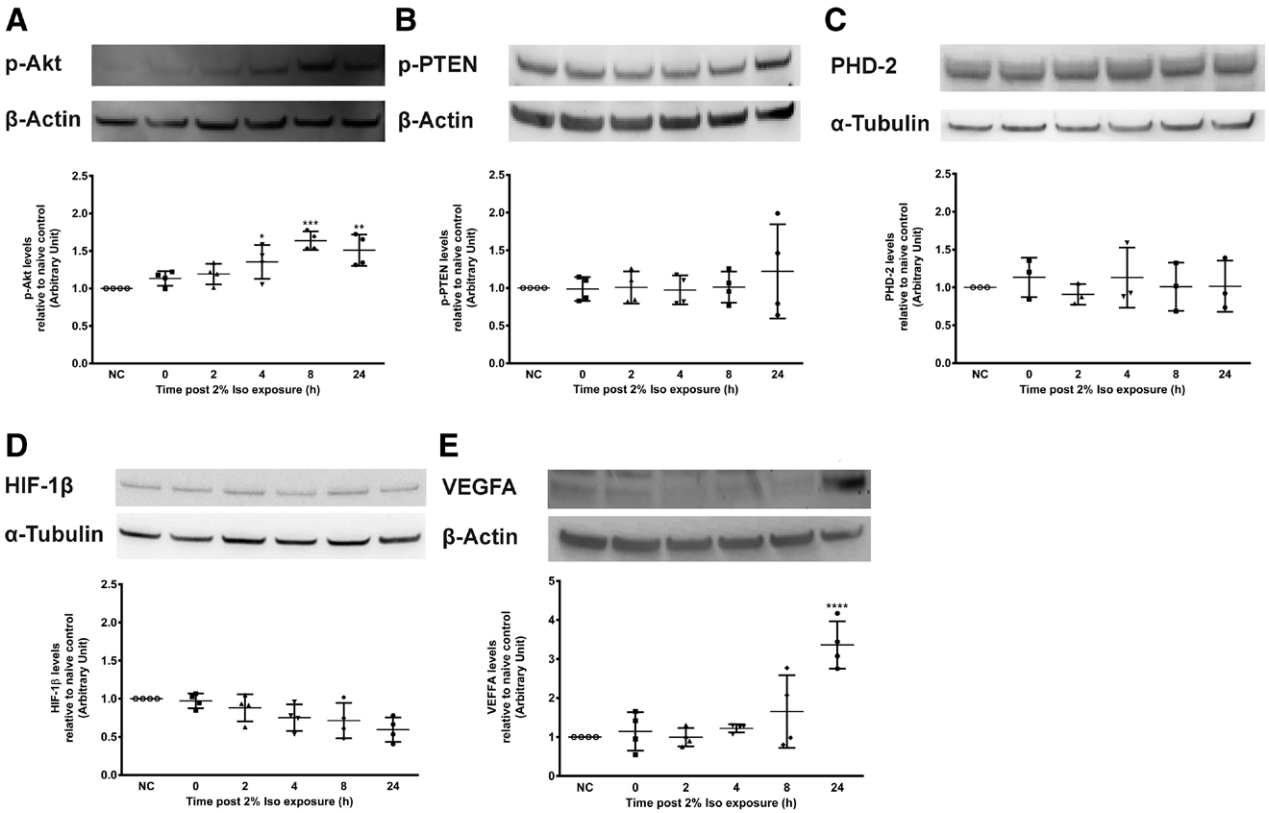

Fig. 2. Immunoblot analysis of proteins involved in isoflurane-induced hypoxia-inducible factor (HIF)-1α up-regulation after isoflurane exposure. Renal cell carcinoma cells were treated with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 h, and immunoblotting was performed at different time point (0, 2, 4, 8, and 24 h) postgas exposure. Upstream HIF-1α effector phosphorylated protein kinase B (p-Akt) was up-regulated in a time-dependent manner (*A*); amounts of the tumor suppressor and negative regulator of the PI3K pathway phosphorylated phosphatase and tensin homolog (p-PTEN) were not affected (*B*). (*C*) Prolyl hydroxylase (PHD)-2, a negative regulator of HIF-1α through the hypoxic pathway, was also unaffected. (*D*) Nuclear amounts of HIF-1β remained stable. (*E*) Furthermore, increased amounts of HIF-1α translocation to the nucleus activated the cascade of HIF downstream effectors as evident by the up-regulation of the proangiogenic factor vascular endothelial growth factor A (VEGFA). Data were presented as mean ± SD. One-way ANOVA with Tukey corrections; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ *versus* naive control (NC; n = 3–4). Iso = isoflurane; mTOR = mammalian target of rapamycin; PI3K = phosphatidylinositide 3-kinase.

the case of two groups where unpaired Student *t* test with Welch corrections was performed. Finally, in the ORIS cell migration assay, two-way ANOVA was used to compare the means of three or more unmatched groups with time postexposure as the within-subjects and treatment as the between-subjects factors. Statistical analysis was based on two-tailed hypothesis testing, and analysis was performed using GraphPad prism (version 6.0; GraphPad Software, Inc., La Jolla, CA). Differences were considered to be significant at the level of 0.05.

Results

Isoflurane Induces HIF Expression in a Time- and Concentration-dependent Manner

To evaluate the effects of isoflurane exposure on HIF protein levels, renal cell carcinoma cells (RCC4) were exposed to 0.5–2% isoflurane for 2 h and then harvested for immunoblotting and immunocytochemistry at different time points (0, 2, 4, 8, and 24 h) after anesthetic exposure. Immunoblotting data revealed significant increases in HIF-1α protein levels in samples exposed to 2% isoflurane (fig. 1A). Specifically at 4 h (1.71 ± 0.18 *vs.* 1.00 of NC; $F = 23.42$; $P = 0.0005$), 8 h (2.16 ± 0.12 *vs.* 1.00 of NC; $F = 23.42$; $P < 0.0001$), and 24 h (1.58 ± 0.2 *vs.* 1.00 of NC; $F = 23.42$; $P = 0.0040$) postexposure with a maximal increase relative to the NC observed at 8 h (fig. 1A). Isoflurane also induced HIF-1α production in a dose-dependent manner with significant increases observed at 8-h postgas exposure with 1.5% (1.45 ± 0.11 *vs.* 1.00 of NC; $F = 19.34$; $P = 0.0009$) and 2% isoflurane (1.60 ± 0.15 *vs.* 1.00 of NC; $F = 19.34$; $P < 0.0001$; fig. 1B). Similar effects are observed in HIF-2α induction with significant increases observed at 8 h (1.46 ± 0.07 *vs.* 1.00 of NC; $F = 21.43$; $P = 0.0026$) and 24 h (1.68 ± 0.12 *vs.* 1.00 of NC; $F = 21.43$; $P < 0.0001$) postgas exposure (fig. 1C). Dose–response tests on HIF-2α revealed slight increases, but no significant differences ($F = 0.95$;

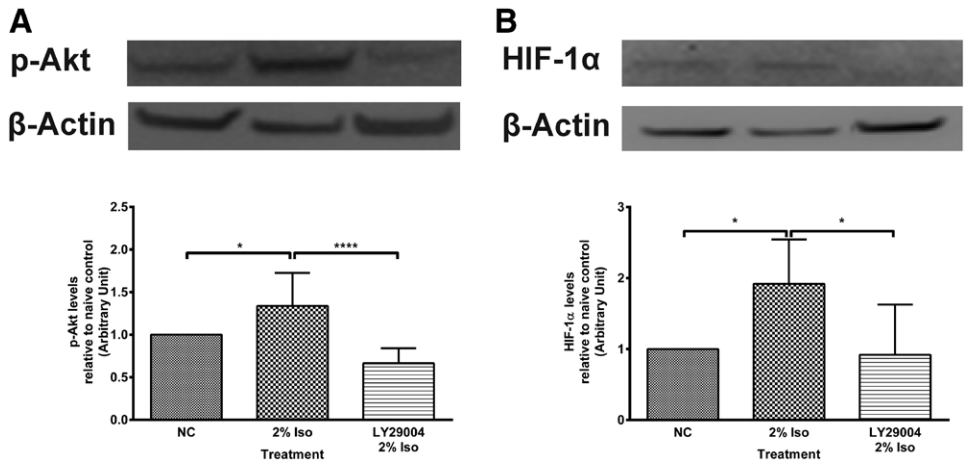

Fig. 3. Immunoblot analysis of phosphorylated protein kinase B (p-Akt) and hypoxia-inducible factor (HIF)-1α after Akt inhibition and isoflurane treatment. Renal cell carcinoma cells were pretreated with an Akt inhibitor (LY294004) for 16 h and then with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 h, and immunoblotting was performed at different time point 8-h postgas exposure. Isoflurane-induced increases of p-Akt (*A*) and HIF-1α (*B*) were abolished by an Akt inhibitor LY294002. Data were presented as mean ± SD. One-way ANOVA with Tukey corrections; * $P < 0.05$, **** $P < 0.0001$ as presented (n = 8). Iso = isoflurane; NC = naive control.

$P = 0.64$ for 1.5%; $P = 0.39$ for 2%) at 8-h postanesthetic exposure (fig. 1D). HIF-1α up-regulation induced by isoflurane exposure was confirmed by immunocytochemistry where analysis of nuclear HIF-1α fluorescence intensity revealed a time-depended increase (fig. 1E i–v) with a marked increase at 8 h when compared with the NC (2.63 ± 0.55 *vs.* 1.00 of NC; $F = 13.84$; $P = 0.0009$; fig. 1E v, vi). Furthermore, there is a distinct translocation of HIF-1α to the nuclei of RCC4 cells starting at 4 h (fig. 1E iv) and showing most prominently at 8 h (fig. 1E v). This effect is still present at 24 h and can be observed clearly when comparing the NC (fig. 1F i, ii) with the treatment group (fig. 1F iii, iv).

Isoflurane Induces HIF Expression through the PI3K/Akt Pathway

HIF levels may be increased by two distinct mechanisms: by the enhancement of HIF generation through molecular pathways such as the phosphatidylinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway or by the inhibition of HIF degradation facilitated by PHDs. We investigated this by measuring the amount of phosphorylated Akt (p-Akt) by immunoblotting; Akt is phosphorylated by kinases in the PI3K pathway when it is activated. There was an increase in the levels of p-Akt in RCC4 cells at 4 h (1.35 ± 0.22 *vs.* 1.00 of NC; $F = 10.09$; $P = 0.039$), 8 h (1.64 ± 0.12 *vs.* 1.00 of NC; $F = 10.09$; $P = 0.0002$), and 24 h (1.51 ± 0.21 *vs.* 1.00 of NC; $F = 10.09$; $P = 0.0019$; fig. 2A). One of the key regulators of the PI3K/Akt/mTOR signaling pathway is the phosphatase and tensin homolog, a tumor suppressor that is known to negatively regulate the PI3K pathway; no significant effects ($F = 0.38$; $P > 0.99$ at 8 h; $P = 0.90$ at 24-h posttreatment) were observed at the levels of phosphorylated phosphatase and tensin homolog postisoflurane exposure (fig. 2B). To determine whether or not the rate of HIF degradation was affected, we performed immunoblotting to quantify levels of PHD-2, one of the key enzymes that hydroxylates HIFs before being recognized and ubiquitinated by the Von Hippel–Lindau E3 ubiquitin ligase which labels them for rapid degradation by the proteasome.[20] This pathway is inhibited at hypoxic conditions as PHDs use oxygen as a cosubstrate.[21] Levels of PHD-2 remained stable after treatment ($F = 0.29$; $P > 0.99$ at 8 h; $P > 0.99$ at 24-h posttreatment; fig. 2C). Levels of HIF-1β also remained relatively stable after treatment ($F = 4.02$; $P = 0.083$ at 8 h; $P = 0.074$ at 24-h posttreatment; fig. 2D). In addition, it was important to examine whether this anesthetic affected downstream effectors of HIF-1α. It was observed that there was a significant increase in vascular endothelial growth factor A, a potent angiogenic factor, 24-h postanesthetic exposure (3.36 ± 0.61 *vs.* 1.00 of NC; $F = 12.94$; $P < 0.0001$; fig. 2E). To further investigate whether HIF up-regulation was directly linked to the activation of the PI3K/Akt/mTOR cell signaling pathway, an Akt inhibitor LY294002 was used before isoflurane exposure. Results revealed a statistically significant decrease in p-Akt in the group of cells that treated with both the inhibitor and 2% isoflurane when compared with the group of cells that had been treated only with 2% isoflurane (0.66 ± 0.18 *vs.* 1.34 ± 0.39 of isoflurane alone; $F = 16.79$; $P < 0.0001$; fig. 3A). A statistically significant decrease in HIF-1α was also observed when comparing cells treated with both the inhibitor and 2% isoflurane with the group of cells that had been treated only with 2% isoflurane (0.92 ± 0.71 *vs.* 1.92 ± 0.63 of isoflurane alone; $F = 6.63$; $P = 0.11$; fig. 3B).

Isoflurane Increases Cell Proliferation of RCC4 Cells

The effect of isoflurane exposure on the proliferative activity of RCC4 cells was assessed by DAPI/ki67 immunostaining and MTT assays. Increased proliferation was demonstrated by DAPI/ki67 double staining (fig. 4, A–C). Results revealed

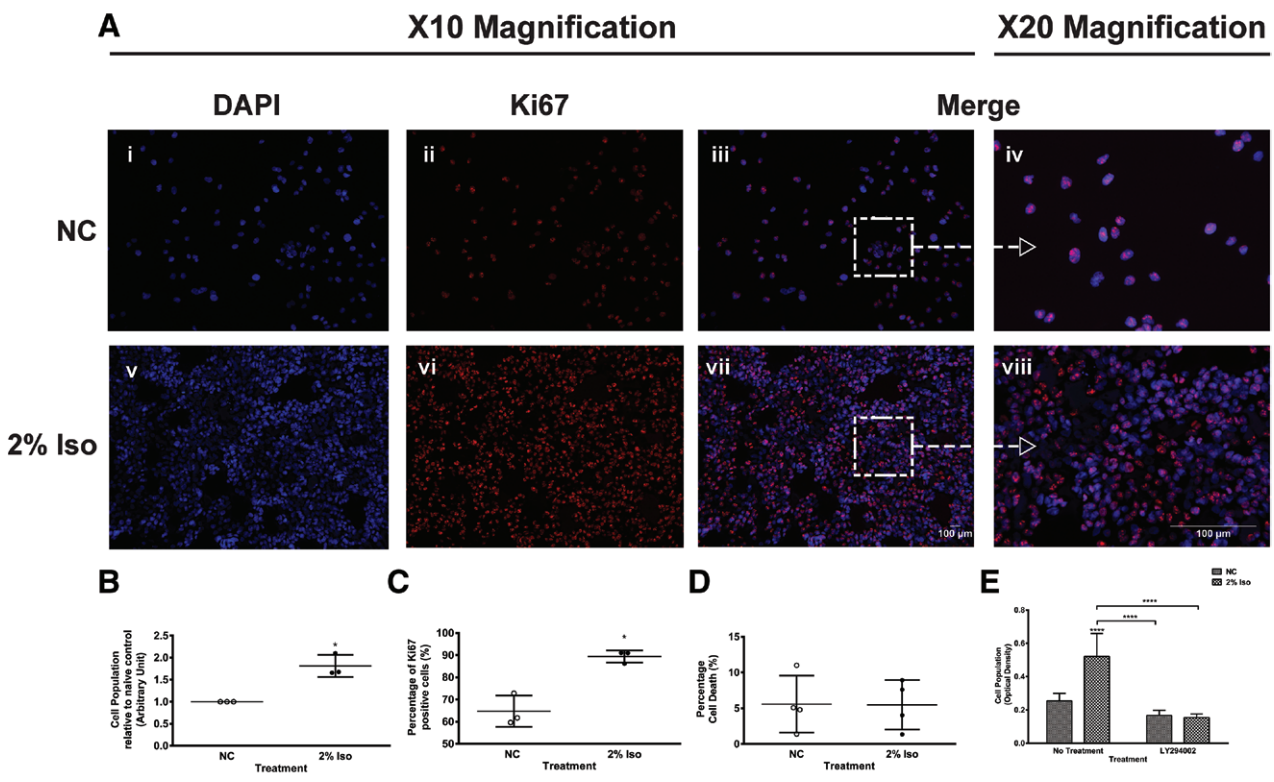

Fig. 4. Analysis of cell proliferation after isoflurane exposure. Renal cell carcinoma cells were treated with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 h, and immunofluorescent analysis of ki67/4',6-diamidino-2-phenylindole (DAPI)-stained cells (*A–C*) and trypan blue assays were performed 8-h postgas exposure. Cells were also pretreated with an Akt inhibitor (LY294004) for 16 h and then with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 h, and a 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed 8-h postgas exposure. Two percent of isoflurane induced cell division 8-h posttreatment evident by nuclear DAPI (*A* and *B*) and ki67 staining (*A* and *C*), without affecting cell death (*D*). (*E*) These effects were negated when the cells were treated with LY294002 as evident by an MTT assay. Data were presented as mean ± SD. Unpaired *t* test with Welch corrections (ki67, trypan blue); One-way ANOVA with Tukey corrections (MTT); **P* < 0.05, *****P* < 0.0001 *versus* naive control (NC) or as presented (n = 3 for ki67; n = 4 for trypan blue; n = 12 for MTT). Iso = isoflurane.

a significant increase in cell numbers postisoflurane exposure, as evident by DAPI nuclear staining (1.81 ± 0.25 *vs.* 1.00 of NC; $t = 5.61$; degrees of freedom (*df*) = 2; $P = 0.03$; fig. 4, A i, v and B). The amount of actively proliferating cells was also significantly increased as presented by ki67 staining (89.4 ± 2.8 *vs.* 64.74 ± 7.09% of NC; $t = 5.60$; $df = 2.6$; $P = 0.016$; fig. 4, A ii–iv, vi–viii and C). A trypan blue exclusion assay revealed no changes in cell death at this time point (5.48 ± 3.44 *vs.* 5.58 ± 3.99 of NC; $t = 0.04$; $df = 6$; $P = 0.97$; fig. 4D). This effect was negated when cells were treated with LY294002, and proliferation was measured *via* an MTT assay. There was significantly lower cell proliferation in the groups treated with the inhibitor with and without isoflurane (0.17 ± 0.031 LY29004 *vs.* 0.52 ± 0.14 of 2% isoflurane alone; $F = 35.67$; $P < 0.0001$; 0.15 ± 0.022 LY29004 with 2% isoflurane *vs.* 0.52 ± 0.14 of 2% isoflurane alone; $F = 35.67$; $P < 0.0001$; fig. 4E) suggesting that increased cell proliferation is partly linked to the activation of PI3K/Akt/mTOR pathway.

Isoflurane Increases Cell Migration and Induces Cytoskeletal Rearrangement in RCC4 Cells

In order to determine the effects of isoflurane on cell migration, RCC4 cells were allowed to migrate on different components of extracellular matrix (tissue culture, collagen, and fibronectin coatings). Two-way ANOVA was performed with isoflurane treatment and time posttreatment as the independent variables (fig. 5, A–C). Cells that allowed to migrate on tissue culture (polylysine)–coated wells were migrated significantly more when treated with isoflurane ($F = 16.89$; $P = 0.0008$), with migration significantly increased with time in a time-dependent manner ($F = 10.62$; $P = 0.0004$) showing that there was no significant interaction between time and treatment ($F = 0.19$; $P = 0.90$; fig. 5A). Similar results were observed with migration on collagen-coated wells with treatment ($F = 20.99$; $P = 0.0003$) and time ($F = 9.44$; $P = 0.0008$) showing significant differences and no interaction between variables ($F = 0.12$; $P = 0.95$; fig. 5B). Finally, there were further significant effects on fibronectin-coated wells with treatment ($F = 8.21$;

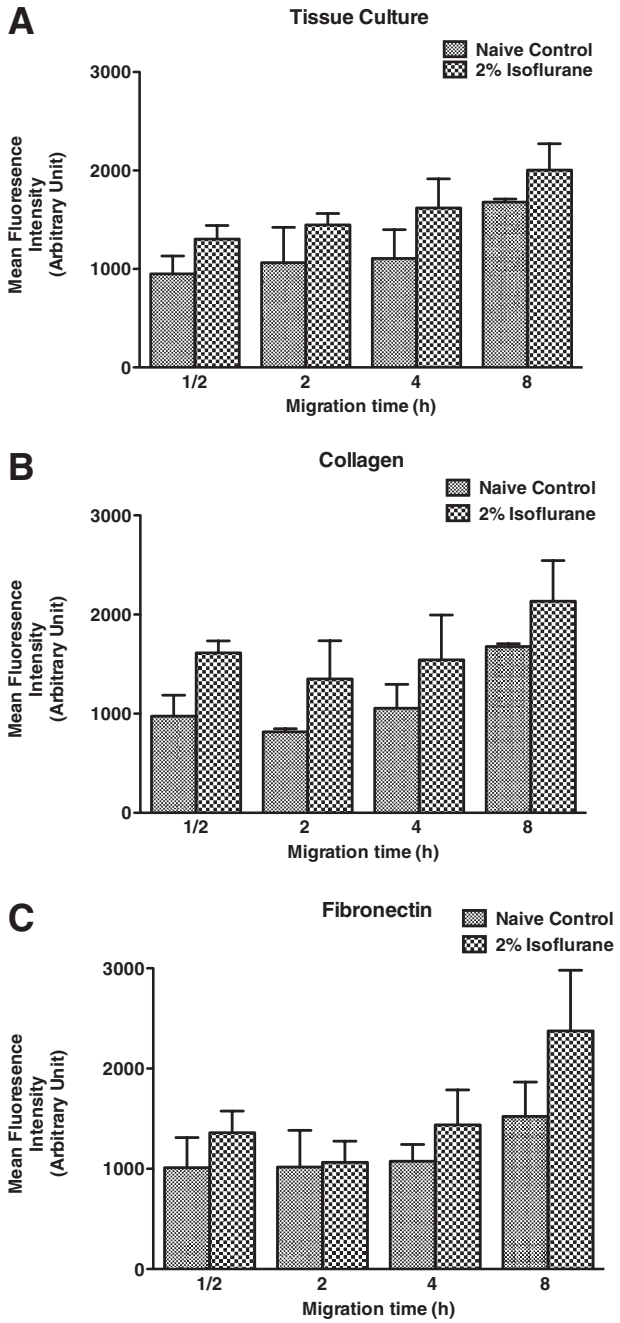

Fig. 5. Analysis of cell migration after isoflurane exposure. Renal cell carcinoma cells were treated with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 h, were allowed to migrate on tissue culture–coated (*A*), collagen-coated (*B*), or fibronectin-coated (*C*) wells and were stained with Calcein AM at different time points (1/2, 2, 4, and 8 h) at which point fluorescence intensity was measured. Increase in cell migration of renal cell carcinoma 4 cells tagged was observed on tissue culture–coated (*A*), collagen-coated (*B*), or fibronectin-coated (*C*) wells. Data were presented as mean ± SD. Two-way ANOVA (n = 3). AM = acetomethoxy.

$P = 0.011$) and time ($F = 8.26$; $P = 0.0015$) showing significant differences and no interaction between variables ($F = 1.40$; $P = 0.28$; fig. 5C). Cell migration was thus shown to be increased in all three conditions by 2% isoflurane in a time-dependent manner.

Cytoskeletal rearrangement was investigated by immunocytochemistry staining for microtubules (fluorescein isothiocyanate-α-tubulin) and F-actin (tetramethylrhodamine-5-(and 6)-isothiocyanate-phalloidin; fig. 6 and 7). Isoflurane exposure induced extensive cytoskeletal rearrangement resulting in more spread out cells appearing larger in size (fig. 6A). Indeed, when the cell area was measured, there was a significant increase after isoflurane exposure (250.9 ± 129.9 μm *vs.* 162.0 ± 79.38 μm of NC; $t = 6.36$; $df = 195.3$; $P < 0.0001$; fig. 6B). Furthermore, F-actin staining revealed migratory and invasive structures such as stress fibers (fig. 7, D and F), filopodia (fig. 7, D and F), and lammellipodia (fig. 7, D and F), whereas α-tubulin staining showed a much denser network of microtubules (fig. 7, E and F) postisoflurane exposure when compared with the NC (fig. 7, B and C) as evident by confocal microscopy.

Discussion

We present evidence for the first time that isoflurane, a volatile general anesthetic agent commonly used in cancer surgery, stimulates a cell signaling pathway involving HIFs, which have been heavily implicated in tumorigenesis, and enhances several cellular activities associated with a malignant phenotype. This takes place in a time- and dose-dependent manner independent of oxygen *via* the PI3K/Akt/mTOR pathway, resulting in HIF neo-synthesis, nuclear translocation, and transcription of target genes (fig. 8). Having exposed RCC4 cells to clinically relevant concentrations of isoflurane, we found evidence of increased proliferation, cytoskeletal rearrangement, and migration of cells across different components of the extracellular matrix. We also detected statistically significantly higher levels of the proangiogenic vascular endothelial growth factor A. Taken together, our data reveals that isoflurane enhances renal cancer cell growth and has noteworthy effects on the cells' malignant potential.

HIFs are ubiquitously expressed transcription factors that directly coordinate the expression of more than 800 genes acting to compensate for changes in physiological conditions, allowing the cell to adapt and survive.[22] Previous work has explored how this endogenous mechanism may be used to help protect tissues that are exposed to ischemic insults.[12,23] However, HIFs and many of its target genes are also heavily involved in a whole host of tumorigenic activities,[22] further up-regulation in patients undergoing cancer surgery could be detrimental and may explain the higher recurrence rates that have been reported in patients receiving general compared with regional anesthesia. Our data simultaneously demonstrate that exposure to clinically relevant concentrations of isoflurane up-regulates HIFs and enhances the cells' hallmark malignant behaviors. We also found these

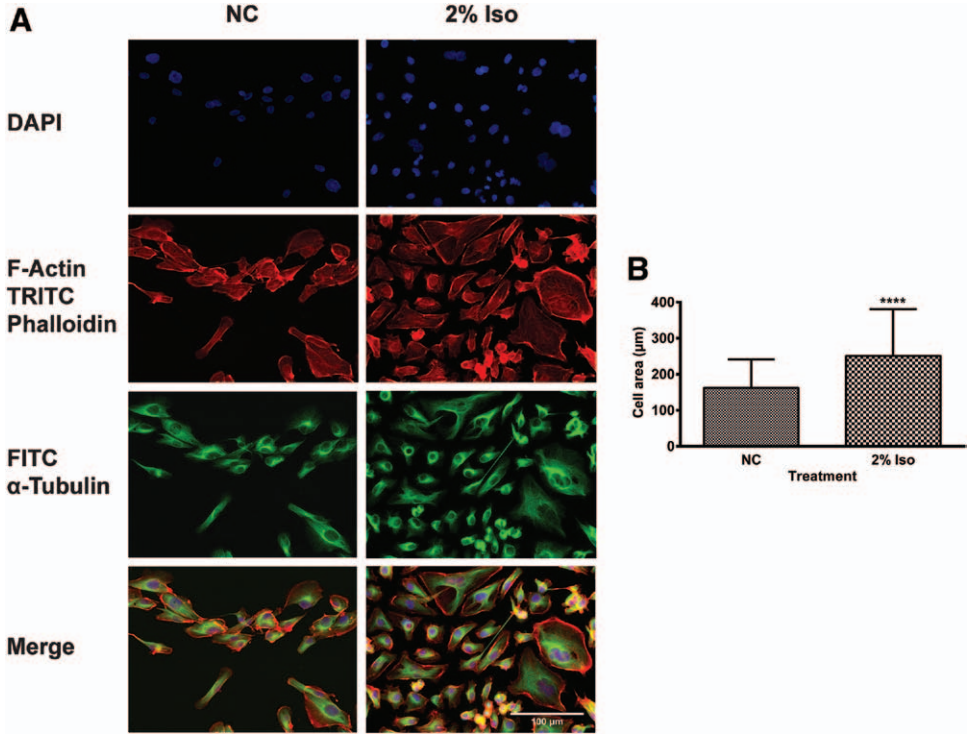

Fig. 6. Immunofluorescent analysis of cellular microstructural changes after isoflurane exposure. Renal cell carcinoma cells were treated with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 and 8 h after exposure, they were then stained with tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)-phalloidin for visualization of F-actin and fluorescein isothiocyanate (FITC)-α-tubulin for visualization of the microtubular network. Renal cell carcinoma 4 cells exposed to 2% isoflurane exhibited dramatic cytoskeletal rearrangement with both F-actin and α-tubulin being (*A*). There is a significant increase in the area of 2% isoflurane-exposed cells (*B*). Data were presented as mean ± SD. Unpaired *t* test with Welch corrections, ****$P < 0.0001$ *versus* naive control (NC; n = 4). DAPI = 4',6-diamidino-2-phenylindole; Iso = isoflurane.

phenomena (data not shown) in other human cancer types including prostate and ovarian cancer cell lines.

In contrast to hypoxia, which leads to almost immediate rises of HIF-α, isoflurane increases levels of HIF-1α and HIF-2α steadily over a period of hours after exposure, suggesting that the increase is induced through neosynthesis rather than by inhibition of its degradation by the 26S proteasome. The finding that p-Akt levels also rise in response to isoflurane supports this, as the PI3K/Akt/mTOR pathway is one mechanism by which the rate of translation of HIF-1α and HIF-2α messenger RNA is regulated.[24,25] Importantly, levels of the PI3K/Akt/mTOR pathway inhibitor, phosphatase and tensin homolog, and levels of PHD enzymes remained constant. Furthermore, the rise in levels of HIF-1α was abolished when cells were pretreated with the Akt inhibitor LY294002. These data, obtained under normoxic conditions, confidently indicate that the rise in HIF levels occurred independently from the oxygen-sensing mechanism. However, given that the majority of tumor environments are hypoxic and that blood oxygen levels in the perioperative period may vary, future work may wish to contemplate the combination of isoflurane-induced HIF neo-synthesis with its hypoxic stabilization and whether this enhances tumorigenic processes yet further.

HIF-1α and HIF-2α have distinct properties and are not functionally redundant.[26,27] It has been reported that high levels of HIF-2α within tumors confer a more aggressive phenotype[28] and that it is this isoform that plays the more important role in RCC4 tumorigenesis.[29] This study demonstrates that isoflurane up-regulates HIF-2α as well as HIF-1α—a finding that strengthens the claim that this anesthetic could promote undesirable consequences in tumors by transcriptionally activating both sets of HIF-responsive genes. However, the two isoforms are maximally induced at different time points: although it appears that HIF-1α levels return toward baseline by 24 h having reached peak levels at 8 h, HIF-2α levels rise steadily and are greatest at 24 h implying that there is variation in the way that isoflurane up-regulates each isoform. Indeed, there is already evidence for minor differences between HIF-1α and HIF-2α in terms of their regulation: cell-specific expression[30] and alternative splice variants[31] of PHD enzymes have been postulated as contributory factors toward differences in the hypoxic regulation of HIF-1α and HIF-2α.[32]

One of the features that distinguish neoplastic from healthy cells is the rate of proliferation. To appreciate how isoflurane could affect cell proliferation, it is important to consider the PI3K/Akt/mTOR pathway itself. The overzealous activity of this pathway has additional important functions besides up-regulating HIFs, namely influencing

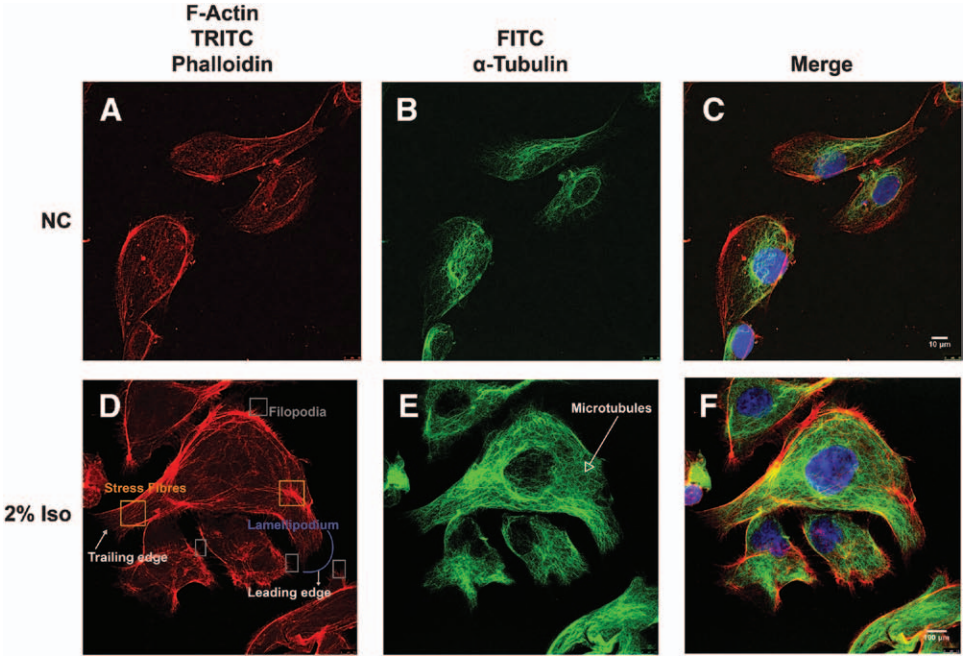

Fig. 7. Confocal immunofluorescent analysis of cellular microstructural changes after isoflurane exposure. Renal cell carcinoma cells were treated with or without 2% isoflurane in 21% oxygen and 5% carbon dioxide balanced with nitrogen for 2 and 8 h after exposure, they were then stained with tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)-phalloidin (*A* and *D*) for visualization of F-actin and fluorescein isothiocyanate (FITC)-α-tubulin for visualization of the microtubular network (*B* and *E*). Merged images correspond to an overlay of 4',6-diamidino-2-phenylindole, F-actin, and α-tubulin images (*C* and *F*). *A–C* correspond to the naive control (NC), whereas *D*, *E*, and *F* are corresponding micrographs relative to NC of 2% isoflurane-treated cells. In *D*, structures such as F-actin stress fibers are marked by *orange boxes*, filopodia are marked by *gray boxes*, lamellipodia marked by *blue line*, and the leading and trailing edge of the migrating cell is denoted by a *white arrow*, as is the microtubular network in *C*. All images taken at ×63, however, cells in the NCs were zoomed in for better visualization of structures as isoflurane treatment enlarged cell size remarkably. Iso = isoflurane.

protein synthesis and cell growth through mTOR. mTOR promotes ribosome biogenesis and messenger RNA translation as well as stimulating entry into the G1 phase of the cell cycle by activating c-Myc and Cyclin D1.[33] In addition, Akt overexpression has been linked directly to growth signal autonomy and resistance to antiproliferative stimuli *via* its interaction with $p21^{CIP1/WAF1}$ and $p27^{KIP1}$.[34] Activation of the PI3K/Akt/mTOR pathway is therefore a likely explanation for the increased rate of proliferation seen in cell cultures that had been exposed to isoflurane. The effect was lost when cells were treated with the Akt inhibitor LY294002.

Several reports, predominantly from studies investigating mechanisms of β-amyloid protein–induced neurotoxicity, indicate that isoflurane induces apoptosis.[35–37] For instance, H4 human neuroglioma cells exposed to 2% isoflurane for 6 h showed a 249% increase in caspase-3 activation and a 25% decrease in cell viability.[35] Nevertheless, others have reported that isoflurane inhibits apoptosis and confers tissue-protective properties.[38–40] Interestingly, these studies attribute these protective effects on cellular-mediated pathways such as the extracellular signal-regulated kinase pathway,[38] or the enhancement of Akt[40] and HIFs.[39] The conflicting data on isoflurane and apoptosis serves to illustrate the heterogeneity of responses to the anesthetic depending on cell type, as well as the likely importance of anesthetic exposure duration and concentration.[41] Interestingly, local anesthetics, such as bupivacaine and licocaine, increased tumor cell apoptosis[42–44] although local and regional anesthesia associated with lower cancer recurrence has been largely considered to be due to patients' immunity being less unaffected when compared with general anesthesia.[1]

Finally, we investigated the effect of isoflurane on the ability of cancer cells to migrate and metastasize to distant sites. Again, HIFs activate transcription of a number of genes involved in these processes, such as proteases that degrade and remodel the extracellular matrix and motility factors. Cytoskeletal components influence cell mechanics, locomotion, differentiation, and neoplastic transformation, and thus changes in these structures will affect cell mechanics and the disease state.[45] In order to migrate, cells require a spatial asymmetry allowing them to turn intracellularly generated forces into net body translocation.[46] During early polarization, there are changes in the actin cytoskeleton, namely monomeric G-actin molecules polymerize to form F-actin, forming distinct membrane protrusions such as filopodia, lamellipodia, and stress fibers within the cells.[46] Filopodia, found in highly motile cancer cells are long and thin transient plasma membrane protrusions that extend out from the cell surface and are implicated in cellular processes such as cell adhesion, migration, and the formation of cell–cell contacts.[46] Lamellipodia form as protrusive sheets that have a ruffling appearance,

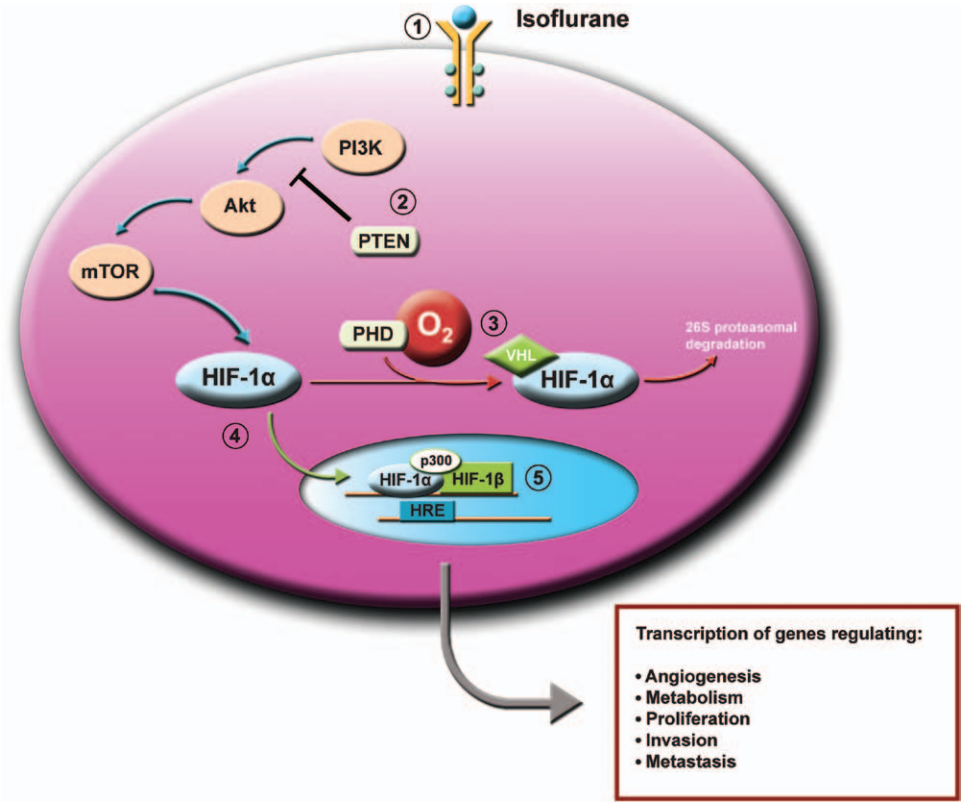

Fig. 8. Hypoxia-inducible factor (HIF)-1α up-regulation/degradation pathways and its downstream effects in the cancer cell. (1) Isoflurane used as an anesthetic up-regulates the neo-synthesis of HIF-1α *via* the PI3K/Akt/mTOR pathway. (2) Phosphatase and tensin homolog (PTEN) tumor suppressor inhibits this pathway, but its levels are not affected by isoflurane. (3) HIF-1α has a very short half-life under normal conditions. Prolyl hydroxylas (PHD) enzymes use oxygen in a reaction that permits binding of the Von Hippel–Lindau (VHL) tumor suppressor. This complex is then targeted for ubiquitination and degradation by the 26S proteasome. In solid tumors, oxygen availability is usually low, resulting in greater stability of HIF-1α within the cytoplasm. (4) HIF-1α neo-synthesis by isoflurane results in its rapid accumulation within the cytoplasm. It subsequently translocates to the nucleus. (5) Here, it forms a complex with HIF-1β and cofactor p300, allowing it to bind to the hypoxia response elements (HRE) and promote transcription. Akt = protein kinase B; mTOR = mammalian target of rapamycin.

containing branched F-actin polymers.[47,48] Actin stress fibers are contractile actomyosin bundles that have a central role in cell adhesion and morphogenesis as well as cell retraction.[49] In highly motile cells, the lamellipodia are formed in the leading edge of the cell, whereas the trailing edge is often rich in parallel F-actin bundles forming stress fibers.

Cells exposed to 2% isoflurane exhibited an impressive degree of spreading with actin being organized into stress fibers and filopodia and developed long microtubules. Moreover, we tested migration of the cells on normal tissue culture coating and different components of the extracellular matrix: collagen and fibronectin. Collagen is a major constituent of the extracellular matrix, during migration and invasion cancer cells will produce proteases, including the urokinase-type plasminogen-activator receptor and matrix metalloproteinase-2, which digest the basement membrane/extracellular matrix.[14] The degraded extracellular matrix is then replaced by fibronectin and other extracellular matrix proteins that are recognized by integrins expressed on the cancer cells.[14] The results showed that cell migration is higher in the isoflurane-treated cells in all three conditions.

Our study has certain limitations. We reported the effect of one anesthetic on a single cell line and, given the array of agents and combinations currently in use and the huge heterogeneity amongst individual tumor types and their genetics, it will be an essential next step to compare the effects of different agents on different tumor cell lines. Although our experiments were designed with clinically relevant details in mind, ultimately only *in vivo* research can accurately model the tumor environment within its host and the delivery of the anesthetic to those cells. All these proposed studies are currently under investigation in our laboratory.

In conclusion, isoflurane is responsible for a significant increase in cell proliferation and cell migration caused by the up-regulation of HIFs *via* the PI3K/Akt/mTOR pathway in RCC4 cells. This provides novel preclinical evidence for a direct effect of an anesthetic on cancer cell biology and thus represents an important avenue down which to explore the impact of anesthesia on postoperative cancer recurrence. The clinical implication is that the use of such an anesthetic may, amongst other factors, promote cancer cell survival and progression in patients undergoing surgery. Furthermore,

because many studies use isoflurane for anesthesia as *in vivo* models of metastasis, it may be necessary to review the influence of this confounding variable on such work.

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