

cAMP-dependent protein kinase is essential for hypoxia-mediated epithelial-mesenchymal
transition, migration, and invasion in lung cancer cells

Dooniya Shaikh^{1,§}, Qiyuan Zhou^{1,§}, Tianji Chen¹, Joyce Christina F. Ibe^{1,2}, J Usha Raj^{1,2}, Guofei
Zhou^{1,3,#}

¹Department of Pediatrics, ²Children's Hospital University of Illinois, ³University of Illinois Cancer
Center, University of Illinois at Chicago, Chicago, IL, USA

[§]These authors contribute equally to this work.

[#]To whom correspondence should be addressed: Guofei Zhou, PhD, Department of Pediatrics
and University of Illinois Cancer Center, University of Illinois at Chicago, 840 S. Wood Street,
Ste 1206, Chicago, IL 60612. Phone: 312-355-0073; Fax: 312-996-8204, E-mail:
guofei@uic.edu

ABSTRACT

Lung cancer is the leading cause of cancer-related death worldwide. Hypoxia is known to increase cancer cell migration and invasion. We have previously reported that hypoxia induces epithelial-mesenchymal transition (EMT) in lung cancer cells. However, it is unknown whether hypoxia promotes lung cancer cell migration and invasion via EMT and whether cyclic AMP (cAMP) dependent protein kinase (PKA) plays a role in this process. We found that hypoxia increased PKA activity and induced mRNA and protein expression of PKA catalytic subunit α (PKACA), and regulatory subunits R1A and R1B. Knockdown of HIF-1/2 α prevented hypoxia-mediated induction of PKACA mRNA expression and PKA activity. Inhibition of PKA activity with chemical inhibitors prevented EMT induced by hypoxia and tumor growth factor β 1. However, activation of PKA by forskolin and 8-Br-cAMP did not induce EMT. Furthermore, treatment with H89 and knockdown of PKACA prevented hypoxia-mediated, EMT, cell migration, and invasion, whereas overexpression of mouse PKACA rescued hypoxia-mediated migration and invasion in PKACA deficient cancer cells. Our results suggest that hypoxia enhances PKA activity by upregulating PKA gene expression in a HIF dependent mechanism and that PKA plays a key role in hypoxia-mediated EMT, migration, and invasion in lung cancer cells.

Keywords: PKA, hypoxia inducible factor, lung cancer, migration, invasion

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide, with adenocarcinoma as the most common subtype accounting for almost half of all lung cancers [1, 2]. In the United States in 2007 alone, 203,536 people were diagnosed with lung cancer, and 158,683 people died from it (www.cdc.gov/uscs). Like other cancer cells, lung cancer cells proliferate rapidly and their growth exceeds the oxygen supply available to the cells, thus creating regions where there is low oxygen tension (hypoxia) [3, 4]. Cancer cells are able to adapt to hypoxia and gain increased potential for migration, invasion, and metastasis, which is the main cause of cancer related death [3]. Invasion and metastasis of cancer cells involves characteristic changes in the cell that are similar to those during epithelial-mesenchymal transition (EMT). EMT is a molecular and cellular process during which epithelial cells lose cell-cell contact and apico-basal polarity and acquire mesenchymal and migratory properties [5, 6]. The mechanism underlying hypoxia-induced EMT remains unknown.

cyclic AMP (cAMP) dependent protein kinase (PKA) is a hetero-tetramer consisting of two regulatory and two catalytic subunits [7], with two distinct isozymes: PKA-I and PKA-II [8, 9]. Tetrameric holoenzyme is inactive whereas the binding of cAMP to the two regulatory subunits induces a conformational change, resulting in the release of the active catalytic subunits and PKA activation [7, 8]. PKA is known to play a role in cancer cell invasion and metastasis via diverse cellular responses such as proliferation, ion transport, and regulation of metabolism and gene transcription [10, 11]. However, it is unknown whether PKA contributes to lung cancer progression by promoting EMT during hypoxia.

Previously we have reported that hypoxia induces EMT in lung cancer cells [6]. We sought to investigate whether PKA plays a role in hypoxia-mediated EMT, migration, and invasion in lung cancer cells. We have found that hypoxia increases PKA activity by upregulating gene expression of its catalytic and regulatory subunits and that the PKA activity is required for

hypoxia-mediated EMT. Furthermore, our results suggest that hypoxia-mediated upregulation of PKA requires HIF and knockdown of PKA α catalytic subunit prevents hypoxia-induced lung cancer cell migration and invasion, suggesting that PKA plays a critical role in lung cancer progression via EMT and therapies that target PKA signaling pathway may provide novel means of treatment of patients with lung cancer.

2. Material and methods

2.1 Materials

TGF- β 1 (Calbiochem, La Jolla, CA), Euk-134 (Cayman, Ann Arbor, MI), SB-431542, H89 dihydrochloride hydrate, Forskolin (FSK), 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), U0126 (Sigma-Aldrich, St. Louis, MO), and KT5720 (Santa Cruz Biotechnology, Santa Cruz, CA) were used in this study. TGF- β 1 and 8-Br-cAMP were dissolved in PBS and other chemicals were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO). Plasmid pCalphaEV encoding mouse PKA α catalytic subunit (referred as mPKACA in the figures and text) was obtained from Addgene (Cambridge, MA). Opti-MEM and lipofectamine 2000 were obtained from Life Technologies (Grand Island, NY).

2.2. Cell culture

Human lung adenocarcinoma cells (A549) and transformed rat alveolar epithelial cells (RLE-6TN) were obtained from the American Type Tissue Collection and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cell cultures were routinely split when 85–90% confluent. A549-shdHIF, A549-shHIF-1 α , and A549-shHIF-2 α are stable A549 cell lines infected with retroviral vectors

encoding small hairpin RNAs (shRNAs) against *Drosophila melanogaster* HIF (dHIF), human HIF-1 α , and human HIF-2 α , respectively. The dHIF shRNA construct does not target human HIF and has no effect on endogenous human HIF expression. These cell lines have been described and validated in our previous report [12] and were maintained in media containing 1 μ g/ml puromycin. Normoxic conditions were set at 21% O₂ and hypoxic conditions (1.5% O₂) were achieved in a humidified workstation (Invivo₂; Ruskinn Technologies, Leeds, UK), which contains an oxygen sensor to continuously monitor the chamber's oxygen tension.

2.3. Western blotting

After three washes with ice-cold phosphate-buffered saline (PBS), cells were lysed in mRIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% deoxycholate, 150 mM NaCl, and protease inhibitors) and cell lysates were cleared at 13,000 *g* for 5 min. Protein concentrations of the supernatants were determined using a D_C protein assay (Bio-Rad, Hercules, CA). After transferring to BA-S 85 nitrocellulose membrane (OPTITRAN, Middlesex, UK) using a Novex® Semi-Dry Blotter (Invitrogen, Carlsbad, CA), proteins were detected with SuperSignal West Femto kit (ThermoScientific, Rockford, IL). Gray density of Western blots was measured using ImageJ software (National Institutes of Health, Bethesda, MD). The following antibodies were used in this study: α -tubulin, actin, vimentin (Sigma-Aldrich, St. Louis, MO), PKACA (PKA α catalytic subunit), PKACB (PKA β catalytic subunit), PKAR1B (PKA regulatory subunit 1B), E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), α -smooth muscle actin (α -SMA) (R&D Systems, Minneapolis, MN), CREB, and phospho-CREB (ser133) (Cell Signaling Technology, Inc., Danvers, MA).

2.4. PKA kinase activity

PKA activity assay was carried out using PKA Kinase Activity Assay Kit (Assay Designs, Inc., Ann Arbor, MI). Briefly, after treatment, cells were washed with ice-cold PBS (1M, pH 7.4) and lysed in the lysis buffer [20mM MOPS, 50mM β -glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine, 1mM phenylmethanesulphonylfluoride (PMSF) and 10 μ g/mL leupeptin and aprotinin] for 10 minutes on ice. Cell lysates were cleared by centrifuge at 13,000 rpm for 15 minutes and protein concentrations were determined by a D_C protein assay (Bio-Rad, Hercules, CA). 30 μ L aliquots of supernatants were added to the wells of PKA Substrate Microtiter Plate, which was presoaked and washed with Kinase Assay Dilution Buffer. 10 μ L of ATP was added to initiate reaction in each well and the plate was incubated for 60 minutes at 30 °C. Reactions were stopped by emptying contents of each well and 40 μ L of Phosphospecific Substrate Antibody was added to each well and incubated at room temperature for 60 minutes. The wells then were washed 4 times with 100 μ L 1X Wash Buffer and followed with addition of 40 μ L diluted Anti-Rabbit IgG:HRP Conjugate and incubated at room temperature for 30 minutes. After washing 4 times, 60 μ L of TMB substrate was added to each well, incubated at room temperature for 30 minutes, and 20 μ L of stop solution was used to stop the reaction before reading absorbance at 450 nm. Relative PKA activity was calculated as the OD over the amount of total protein; PKA activity in normoxia group was set as 100.

2.5. Invasion assay

Invasion assay was carried out using BD Matrigel invasion chambers (BD BioCoat, Bedford, MA, USA). Transwells and inserts were warmed up with 0.5 ml DMEM in the wells and inserts. 0.5 ml cell suspension (5×10^4 /ml) was added into inserts and 0.75 ml media in wells. Cells were allowed to attach for 4 hours and exposed to normoxia or hypoxia for different periods of time.

Uninvaded cells in the upper chamber were removed with cotton swabs and invaded cells were fixed with 100% methanol for 2 minutes, followed with staining with 0.5% crystal violet in 2% methanol for 2 minutes. After rinsing with water, five random microscopic fields at x200 magnification were counted in each filter using a calibrated ocular grid. Experiments were carried out in triplicates. Data are expressed as the average number of cells/field \pm S.E.

2.6. Establishment of a stable cell line with suppression of PKA α catalytic subunit (A549-shPKACA)

PKA α catalytic subunit (PKACA) shRNA lentiviral particles and Control shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A549 cells were plated in a 12-well plate and incubated overnight. A mixture of complete medium with polybrene (final concentration 5 μ g/ml) and lentiviral particles were added to the culture and incubated overnight. Stable clones expressing the shRNA were selected by incubating cells in media containing puromycin dihydrochloride (1 μ g/ml). Resulting puromycin-resistant single clones were collected and expanded and the expression of PKA α was analyzed by Western blot analysis.

2.7. Quantitative real-time reverse transcription PCR (qRT-PCR)

The mRNA amount of PKA catalytic and regulatory subunits was determined by quantitative real time RT-PCR (qRT-PCR) and the mitochondrial ribosomal protein L19 (RPL19) gene was used as internal control. Total RNA of cells was isolated using the RNeasy Micro Kit (Qiagen, Valencia CA) and transcribed to complementary DNA (cDNA) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California). PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) on StepOnePlus Real-Time PCR system

(Applied Biosystems). Cycle threshold values were normalized to amplification of RPL19. Sequences of primers used for qRT-PCR were as follows: human PRKACA, ACATTCAGGTGACAGACTTC (sense), TTGCTCAGGATAATCTCAGG (antisense); human PRKACB, CGTCCTTGTTGAAGCAGTTAC (sense), AGCGGTGCCTTGTTACATAG (antisense); human PRKAR1A, GCAGGCACTCGTACAGACTC (sense), CCGCATCTTCCTCCGTGTAG (antisense); human PRKAR2A, TGGGCAACCTCGGGCAGTAG (sense), GCGGCAACGGCAGGAACAG (antisense); human PRKAR1B, GTGAGTGCCGAGGTGTAC (sense), CATCCAGGTGAGCGAAGAG (antisense); human PRKAR2B, TCAGAAGTGGAAGAGAATGGTG (sense), CAATGGCGTGGGCAGAAG (antisense); human RPL19, ATCATCCGCAAGCCTGTG (sense), TGACCTTCTCTGGCATTTCG (antisense).

2.8. Scratch assay

0.8 Million A549 cells were cultured on a 35 mm dish and incubated overnight. A wound was created by scratching with 250 μ l microtips and cells were washed once with complete media to remove detached cells, followed by the acquisition of the original image of the wound under a microscope. Dishes were then put back in an incubator and incubated for 24 hours, and the second image of the wound was obtained. The width of the wounds was measured with AxioVision LE software (Zeiss, Oberkochen, Germany). The difference between the widths is taken as the migration distance.

2.9. Cell proliferation assay

Cells were plated at the density of 200,000 cells per 60mm dish and were cultured for 1, 3, 5, and 7 days. At each time point, cells were trypsinized, spun down, and resuspended in PBS.

The number of cells per plate was counted with TC10 automated cell counter (Bio-Rad, Hercules, CA).

2.10. Transfection of a plasmid encoding mouse PKACA (mPKACA) in A549-shPKACA cells

A549-shPKACA1 cells were plated in a 100 mm dish, incubated overnight, and transfected with a mixture of 3 ml Opti-MEM, 45 μ l lipofectamine 2000, and 12 μ g of mPKACA plasmid or vector. After 4 hours of incubation, cells were replaced with complete media and incubate overnight. Cells were trypsinized, counted, and replated for the PKA kinase assay, invasion assay, scratch assay and Western blot analysis as described above.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 (GraphPad, San Diego, CA, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA) when applicable; *t* tests were performed and significant difference values were set at 0.05.

3. Results

3.1. Hypoxia stimulates PKA activity and increases gene expression of PKA α catalytic subunit via HIF

To determine whether exposure to hypoxia affects PKA activity, we exposed A549 to normoxia or hypoxia (1.5% O₂) for 1, 5, 24, and 48 hours and measured PKA activity in the whole cell lysates. As shown in Fig 1A, hypoxia increased PKA activity in a time dependent manner and PKA activity was significantly increased after 24 hours of exposure. Hypoxia also

induced phosphorylation of CREB (Fig 1B), a downstream target of PKA [13]. These results suggest that hypoxia may increase PKA activity via induced expression of PKA. Therefore, we determined the mRNA levels of PKA catalytic and regulatory subunits after exposure to normoxia or hypoxia for 24 and 48 hours using quantitative real time PCR. We found that hypoxia induced mRNA levels of PKACA, PKAR1A, and PKAR1B, whereas mRNA levels of PKACB, PKAR2A and PKAR2B remained unchanged (Fig 1C). Furthermore, we measured the protein levels of PKA subunits and as shown in Fig 1D, hypoxia increased protein levels of PKACA and PKAR1B but not PKACB. Together, these results suggest that hypoxia selectively induces PKA-I in A549 cells.

HIF is a master transcription factor regulating many downstream targets to facilitate the adaptation to hypoxia. To address whether hypoxia induces PKA through HIF, we established A549 cells with knockdown of HIF-1 α or HIF-2 α [12] and exposed them to normoxia or hypoxia, followed with the measurement of mRNA levels of PKACA, PKAR1A, PKAR1B. As shown in Fig 2A, 2B, and 2C, knockdown of either HIF-1 α or HIF-2 α prevented hypoxia-induced expression of PKACA; however, suppression of HIF-1 α or HIF-2 α had little effect on hypoxia-mediated induction of PKAR1A and PKAR1B mRNA levels, suggesting that hypoxia-induced PKACA expression requires HIF, whereas hypoxia-induced PKAR1A and PKAR1B are HIF independent. To further investigate the role of HIF on PKA activity, we compared the PKA activity of these cells exposed to normoxia and hypoxia for 24 hours. As shown in Fig 2D, knockdown of HIF-1 α and HIF-2 α did not alter protein expression levels of PKACA in normoxic conditions, but abolished hypoxia-induced PKA activity, confirming that HIF- α is critical for hypoxia-induced PKA activity. During hypoxia, mitochondria generate ROS, which are essential for the stabilization of HIF- α [14]. To determine whether ROS are required for the increase in PKA activity during hypoxia, we pre-treated cells with Euk-134 (Euk), a synthetic superoxide dismutase and catalase mimetic, prior to exposure to hypoxia or normoxia. We found that Euk-

134 diminished hypoxia-induced PKA activity in a dose dependent manner (Fig 2E). Together, these results suggest that elevated PKA activity during hypoxia is ROS/HIF dependent.

3.2. PKA activity is necessary for hypoxia-induced EMT, migration, and invasion

Previously we have shown that hypoxia induces epithelial mesenchymal transition (EMT) in A549 cells and EMT has been implicated in the promotion of cancer cell migration and invasion [5, 6]. Therefore we investigated whether PKA participates in hypoxia-mediated EMT, migration, and invasion in lung cancer cells. We treated cells with a PKA inhibitor H89 (10 μ M) and exposed cells to hypoxia or normoxia for 4 days, followed by the measurement of the expression of α smooth muscle actin (α -SMA), an EMT marker. As shown in Fig 3A, H89 prevented hypoxia-mediated α -SMA expression. We validated these results using another PKA inhibitor KT5720 (20 μ M) [15] and as shown in Fig 3B, KT5720 also prevented hypoxia-mediated induction of EMT. In contrast, U0126, an MEK inhibitor, did not affect hypoxia-mediated α -SMA expression (Fig 3C), suggesting that PKA is critical for hypoxia-mediated EMT phenotype. Since hypoxia is known to stimulate cell migration and invasion [3, 4], we next determined whether PKA activity is required for cancer cell migration and invasion. As shown in Fig 3D, we found that H89 prevented hypoxia-induced migration in A549 cells. We determined cell invasion using invasion chambers coated with matrigel. As shown in Fig 3E, H89 prevented hypoxia-mediated cell invasion. In addition, we determined whether PKA activation is sufficient to induce EMT. As shown in Fig 3F, after activation of PKA by two PKA agonists (Forskolin (FSK) and 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP)), at various doses for 4 days, the levels of α -SMA and vimentin (another EMT marker), did not increase, suggesting that PKA is necessary for hypoxia-mediated EMT, but PKA activation alone is not sufficient to induce EMT.

3.3. PKA activity is necessary for the induction of EMT by TGF- β 1

TGF- β 1 is a potent inducer of EMT. To address whether PKA plays a universal role in EMT or whether it is specific for hypoxia-mediated EMT, we measured the expression of PKACA and PKA activity after the treatment of TGF- β 1. We found that exposure to TGF- β 1 for 2 days also induced the expression levels of PKACA (Fig 4A), whereas transient exposure to TGF- β 1 for up to 1 hour did not induce PKA activation (Fig 4B). Furthermore, we pre-treated cells with H89 and induced EMT by adding TGF- β 1, followed by the measurement of EMT markers. As shown in Fig 4C, SB-431542, an inhibitor of ALK5 (a TGF- β 1 type I receptor kinase), completely inhibited TGF- β 1-induced induction of α -SMA. H89 also prevented TGF- β 1-induced expression of α -SMA, suggesting that PKA contributes to TGF- β 1-induced EMT. We also investigated whether PKA is critical for TGF- β 1-mediated cell migration. As shown in Fig 4D, H89 treatment prevented TGF- β 1-mediated cell migration. To determine whether PKA activation enhances TGF- β 1-mediated induction of EMT, we co-treated A549 cells with a PKA agonist FSK and TGF- β 1, and measured E-cadherin levels. As shown in Fig 4E, FSK did not augment TGF- β 1-mediated downregulation of E-cadherin. Previously, we reported that chronic hypoxia increases TGF- β 1 gene expression through HIF [6]. To determine whether hypoxia induces PKACA via TGF- β 1, we pre-treated A549 cells with SB-431542 and exposed them to normoxia or hypoxia for 2 days, followed by the measurement of PKA activity. As shown in Fig 4F, SB-431542 did not prevent hypoxia-mediated PKA activity, suggesting that hypoxia induces PKA activity independent of TGF- β 1.

3.4. Knockdown of PKACA reduces lung cancer cell EMT, migration, and invasion but does not alter cell proliferation rate

To further validate previous results, we established stable cell lines of A549 with knockdown of PKACA using a lentivirus containing shRNA against human PKACA (A549-shPKACA) and selected a few clones (clone 1, 2, 3, and 5). A549 cells transduced with a control lentivirus (A549-shCTL) were used as control. As shown in Fig 5A, PKACA was successfully suppressed in A549-shPKACA cell lines. Next we chose one cell line (A549-shPKACA1) to determine the effect of deletion of PKACA on the proliferation of A549 cells. As shown in Fig 5B, knockdown of PKACA did not alter the growth rate of A549 cells. To address whether PKACA knockdown induces compensatory effect on other PKA subunits, we measured mRNA levels of PKA subunits in A549, A549-shCTL, and A549-shPKACA1. As expected, A549-shPKACA1 cells express reduced PKACA mRNA levels; however expression of other PKA subunits in A549-shPKACA1 remained unchanged (Fig 5C), suggesting that PKACA knockdown is specific and without compensation of the expression of other PKA subunits.

Since we showed that chemical inhibition of PKA prevented hypoxia- and TGF- β 1-induced EMT (Fig 3 and Fig 4), we validated these results by exposing A549-shCTL and A549-shPKACA1 to normoxia or hypoxia, or treating these cells with TGF- β 1. We determined the expression levels of E-cadherin by Western blot analysis and we found that knockdown of PKACA prevented both hypoxia- and TGF- β 1-induced downregulation of E-cadherin (Fig 5D), suggesting that knockdown of PKACA prevents hypoxia and TGF- β 1-induced EMT. In Fig 2, we also showed that hypoxia induced PKACA via HIF-2 α , thus we investigated whether suppression of HIF-2 α prevents EMT. We exposed A549-shdHIF and A549-shHIF-2 α to normoxia or hypoxia or treated these cells with TGF- β 1 and determined expression levels of E-cadherin. As shown in Fig 5E, knockdown of HIF-2 α also prevented hypoxia- and TGF- β 1-induced EMT. These results confirm that PKACA is necessary for hypoxia- and TGF- β 1-induced EMT. Our results also suggest that activation of PKA by FSK and 8-Br-cAMP is not sufficient to induce EMT in normal conditions (Fig 3F). To validate this observation, we transfected a mouse PKACA (mPKACA) into rat alveolar epithelial cells (RLE-6TN), and exposed these cells to

normoxia or hypoxia or treated with TGF- β 1. As shown in Fig 5F upper panel, transfection of mPKACA resulted in the overexpression of PKACA in RLE-6TN cells. Furthermore, overexpression of PKACA did not alter the basal levels of α -SMA, nor enhanced hypoxia- or TGF- β 1-induced expression of α -SMA, suggesting that overexpression of PKACA is neither sufficient to induce EMT in normal conditions nor to enhance hypoxia- or TGF- β 1-induced EMT. Thus, we conclude that PKACA is necessary but not sufficient to induce EMT.

To address whether knockdown of PKACA affects cell migration and invasion, we cultured these cells and determined the migration and invasion under normal and hypoxic conditions. As shown in Fig 6A and 6B, knockdown of PKACA prevented hypoxia-induced cell migration and invasion. Furthermore, we investigated whether activation of other PKA isoforms rescues the hypoxia-mediated migration and invasion in A549-shPKACA1 cells. We pretreated A549-shCTL and A549-shPKACA1 cells with FSK to activate PKA and then exposed them to normoxia or hypoxia for 24 hours or 48 hours to measure migration and invasion, respectively. As shown in Fig 6C, FSK was not sufficient to rescue hypoxia-mediated migration and invasion in A549-shPKACA1, suggesting an isoform specific role of PKA in these processes.

3.5. Overexpression of mouse PKACA rescues hypoxia-mediated migration and invasion in PKACA deficient cells

To further validate that PKACA is truly a key player in hypoxia-mediated migration and invasion, we transfected a shRNA resistant mouse PKACA (mPKACA) into A549-shPKACA1 cells. As shown in Fig 7A and 7B, transfection of mPKACA overexpressed PKACA and increased PKA activity in A549-shPKACA1 cells. We cultured these cells and determined the migration and invasion under normal and hypoxic conditions. Although transiently transfected A549-shPKACA1 cells migrated slower and invaded less than untransfected cells (Fig 6 and 7), overexpression of mPKACA rescued hypoxia-induced cell migration and invasion in A549-

shPKACA1 cells (Fig 7C and 7D), suggesting that PKACA is indeed a key player in hypoxia-mediated lung cancer progression.

4. Discussion

It has long been known that hypoxia induces cancer cell migration, invasion, and metastasis [3, 4]; however, the molecular mechanisms underlying these processes are largely elusive despite much progress. Recently, hypoxia-induced EMT has been implicated in cancer progression [16, 17], hence we investigated whether PKA participates in lung cancer progression via promoting EMT. Our results suggest that hypoxia induces PKA-I and increases PKA activity, which is critical for hypoxia-induced lung cancer cell EMT, migration, and invasion; knockdown of PKACA prevents hypoxia-mediated cancer cell migration and invasion. Taken together, these results suggest that during hypoxia, PKA is a key player in EMT and cancer cell malignancy.

Our results suggest that PKA activity increases under hypoxic conditions only after a prolonged period and that PKACA, PKAR1A, PKAR1B subunits are upregulated (Fig 1), indicating that hypoxia itself does not activate PKA; rather hypoxia induces PKA activity by increasing gene expression of PKA-I. Thus we reason that in our experimental system, hypoxia may not alter cAMP production. Indeed, in primary rat alveolar epithelial cells, hypoxia does not change basal cAMP production or alter adenylate cyclase activity [18].

It is known that PKA-I expression is elevated in fast proliferating cells and many cancer cells express increased levels of PKA-I; whereas PKA-II is mostly expressed in quiescent and differentiated cells, suggesting that PKA isozymes play unique roles in cancer cell proliferation [9, 19]. Furthermore, a switch in PKA isozymes has been reported to regulate cancer cell proliferation potential [9, 19]. We found that hypoxia specifically upregulates PKA-I but not PKA-II in lung cancer cells (Fig 1), suggesting that hypoxia-induced cancer cell malignancy also

depends on PKA-I activity. More importantly, we found that both PKA catalytic and regulatory subunits are upregulated during hypoxia (Fig 1). Previous reports suggest that elevated levels of regulatory subunits increases cAMP sensitivity, lowering the threshold for PKA downstream effects [11]. Thus hypoxia may increase PKA activity via two mechanisms: one is to induce PKACA expression to provide higher PKA activation capacity; the second is to elevate expression levels of regulatory subunits to increase cAMP sensitivity, lowering the threshold for PKA activation.

HIF is a master transcription factor in cellular adaptation to hypoxia. We provide evidence that upregulation of PKA-I is also controlled by HIF. Although both HIF-1 and HIF-2 are necessary for the hypoxia-induced PKACA, knockdown of HIF-1 α or HIF-2 α has little effect on the hypoxia-induced PKAR1A and PKAR1B (Fig 2), suggesting that the PKA catalytic and regulatory subunits are regulated by different mechanisms during hypoxia. Furthermore, knockdown of either HIF-1 or HIF-2 diminishes PKA activity during hypoxia (Fig 2). The basal PKACA expression and PKA activity is unchanged in cells with suppression of HIF-1 α or HIF-2 α (Fig 2). Generation of ROS during hypoxia has been reported to be essential for the stabilization of HIF-1 α and we found that a ROS scavenger prevented hypoxia-induced PKA activity, suggesting that ROS/HIF is critical for the upregulation of PKA during hypoxia.

Since we have previously shown that hypoxia induces EMT in lung cancer cells [6], we set out to investigate whether PKA plays a role in EMT during hypoxia. Our results show that PKA inhibitors H89 and KT5720 but not MEK inhibitor U0126 prevent hypoxia-mediated EMT (Fig 3). Since EMT promotes cell migration and invasion, we have tested whether PKA affects cell migration and invasion and have shown that H89 prevents A549 cell migration and invasion during hypoxia (Fig 3), further confirming our observation. Accordingly, Tkachenko and colleagues suggest that PKA activity in the leading edge is required for the rapid cell protrusion in migrating cells [20]. However, activation of PKA alone is not sufficient to induce EMT (Fig 3F), which is consistent with our previous results that HIF alone is not sufficient to induce EMT [6].

TGF- β 1 is a potent cytokine known to induce EMT in many tissues [21] and we found that TGF- β 1 induces gene expression of PKACA (Fig 4). Furthermore, inhibition of PKA activity by H89 prevents TGF- β 1-induced EMT and migration, whereas activation of PKA does not augment TGF- β 1-induced EMT, suggesting that PKA triggers one of TGF- β 1 pathways to induce EMT. In other cells, such as colon cancer cells, TGF- β 1 can activate PKA in a cAMP-independent mechanism [22, 23]. In that pathway, TGF- β 1 treatment phosphorylates Smad4, which interacts with PKA regulatory subunits and releases catalytic subunits, leading to activation of PKA [22, 23]. In another article, Yang and colleagues reported that TGF- β 1 induces EMT in AML-12 murine hepatocytes via activation of PKA as evidenced by the increased phosphorylation of CREB and in vitro kinase assay with kemptide as substrate [24]. Thus TGF- β 1 may adopt two distinct mechanisms to induce PKA activity in a tissue-specific manner: in an acute mechanism, TGF- β 1 phosphorylates Smad to interact with PKA holoenzyme to release PKA catalytic subunit; in another prolonged mechanism, TGF- β 1 can induce gene expression of PKA to increase PKA activity. Previously we have shown that HIF can induce TGF- β 1 [6] so we investigated whether hypoxia-induced PKA depends on TGF- β 1 signaling. Our results suggest that TGF- β 1 pathway is not required for PKA upregulation during hypoxia (Fig 4), suggesting that hypoxia induces PKA and TGF- β 1 independently.

To further confirm that PKA plays a role in lung cancer cell progression, we have established a cancer cell line with genetically knockdown of the PKACA subunit (Fig 5). We found that knockdown of PKACA did not alter cell proliferation, but prevented hypoxia- and TGF- β 1-mediated EMT (Fig 5). Furthermore, knockdown of PKACA reduced migration and invasion of the lung cancer cells under hypoxic conditions, suggesting that PKA is critical for cancer progression (Fig 6). This observation is in line with previous reports which suggest that low PKA activity is associated with loss of tumorigenesis [11]. Interestingly, activation of other PKA isoforms is not sufficient to rescue the reduced migration and invasion of A549-shPKACA1 during hypoxia (Fig 6C) and there is no compensation mechanism in PKACA knockout cells (Fig

5C), suggesting an isoform specific role of PKA in lung cancer progression. Furthermore, introduction of a shRNA resistant mouse PKACA in PKACA deficient human lung cancer cells restore hypoxia-mediated migration and invasion (Fig 7), suggesting that PKACA is indeed critical for hypoxia-induced cancer progression.

5. Concluding remarks

Taken together, we have provided a first line of evidence that hypoxia-induced PKA contributes to EMT and subsequent migration and invasion of lung cancer cells. Other studies have shown that PKA can interact with RAS oncogene pathway to enhance tumorigenesis and tumor progression [11]. Recent reports also suggest that PKA can directly interact with EGFR receptor, a main therapeutic target for the treatment of lung cancer [25, 26]. Thus, PKA may participate in multiple signal transduction pathways in the progression of lung cancer, positioning it as a central therapeutic target in the treatment of lung cancer; however, further studies are warranted.

Acknowledgments

The work was supported in part by UIC Faculty Scholarship Support Program (GZ), the University of Illinois Cancer Center Pilot Subsidy Program (GZ), the Kabbes Scholarship for undergraduate research (DS).

REFERENCES

- [1] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. CA: a cancer journal for clinicians 2008;58(2):71-96.
- [2] Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, Beer DG, Powell CA, Riely GJ, Van Schil PE, Garg K, Austin JHM, Asamura H, Rusch VW, Hirsch FR, Scagliotti G, Mitsudomi T, Huber RM, Ishikawa Y, Jett J, Sanchez-Cespedes M, Sculier J-P, Takahashi T, Tsuboi M, Vansteenkiste J, Wistuba I, Yang P-C, Aberle D, Brambilla C, Flieder D, Franklin W, Gazdar A, Gould M, Hasleton P, Henderson D, Johnson B, Johnson D, Kerr K, Kuriyama K, Lee JS, Miller VA, Petersen I, Roggli V, Rosell R, Saijo N, Thunnissen E, Tsao M, Yankelewitz D. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer;6(2):244-285.
- [3] Finger EC, Giaccia AJ. Cancer metastasis reviews;29(2):285-293.
- [4] Semenza GL. Nat Rev Cancer 2003;3(10):721-732.
- [5] Kalluri R, Weinberg RA. The Journal of clinical investigation 2009;119(6):1420-1428.
- [6] Zhou G, Dada LA, Wu M, Kelly A, Trejo H, Zhou Q, Varga J, Sznajder JI. American Journal of Physiology - Lung Cellular & Molecular Physiology 2009;297(6):L1120-1130.
- [7] Pearce LR, Komander D, Alessi DR. Nature reviews Molecular cell biology;11(1):9-22.
- [8] Taylor SS, Kim C, Cheng CY, Brown SHJ, Wu J, Kannan N. Biochimica et biophysica acta 2008;1784(1):16-26.
- [9] Neary CL, Nesterova M, Cho YS, Cheadle C, Becker KG, Cho-Chung YS. Oncogene 2004;23(54):8847-8856.
- [10] Naviglio S, Caraglia M, Abbruzzese A, Chiosi E, Di Gesto D, Marra M, Romano M, Sorrentino A, Sorvillo L, Spina A, Illiano G. Expert opinion on therapeutic targets 2009;13(1):83-92.

- [11] Chiaradonna F, Balestrieri C, Gaglio D, Vanoni M. *Frontiers in bioscience : a journal and virtual library* 2008;13:5257-5278.
- [12] Zhou G, Dada LA, Chandel NS, Iwai K, Lecuona E, Ciechanover A, Sznajder JI. *FASEB J* 2008;22(5):1335-1342.
- [13] Murray AJ. *Science signaling* 2008;1(22):re4.
- [14] Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. *J Biol Chem* 2000;275(33):25130-25138.
- [15] Davies SP, Reddy H, Caivano M, Cohen P. *The Biochemical journal* 2000;351(Pt 1):95-105.
- [16] Hill RP, Marie-Egyptienne DT, Hedley DW. *Seminars in radiation oncology* 2009;19(2):106-111.
- [17] Jiang J, Tang Y-l, Liang X-h. *Cancer biology & therapy*;11(8):714-723.
- [18] Baloglu E, Ke A, Abu-Taha IH, Bartsch P, Mairbaur H. *American journal of physiology Lung cellular and molecular physiology* 2009;296(3):L500-509.
- [19] Cho-Chung YS, Nesterova MV. *Annals of the New York Academy of Sciences* 2005;1058:76-86.
- [20] Tkachenko E, Sabouri-Ghomi M, Pertz O, Kim C, Gutierrez E, Machacek M, Groisman A, Danuser G, Ginsberg MH. *Nature cell biology*;13(6):660-667.
- [21] Willis BC, Borok Z. *Am J Physiol Lung Cell Mol Physiol* 2007;293(3):L525-534.
- [22] Zhang L, Duan CJ, Binkley C, Li G, Uhler MD, Logsdon CD, Simeone DM. *Molecular and cellular biology* 2004;24(5):2169-2180.
- [23] Chowdhury S, Howell GM, Rajput A, Teggart CA, Brattain LE, Weber HR, Chowdhury A, Brattain MG. *PloS one*;6(5):e19335.
- [24] Yang Y, Pan X, Lei W, Wang J, Shi J, Li F, Song J. *Cancer research* 2006;66(17):8617-8624.
- [25] Pao W, Chmielecki J. *Nature reviews Cancer*;10(11):760-774.

- [26] Tortora G, Damiano V, Bianco C, Baldassarre G, Bianco AR, Lanfranccone L, Pelicci PG, Ciardiello F. *Oncogene* 1997;14(8):923-928.

FIGURE LEGENDS

FIGURE 1. Hypoxia stimulates PKA activity and increases gene expression of PKA-I subunits.

A) A549 cells were exposed to normoxia (0h) or hypoxia for 1, 5, 24, and 48 hours (1h, 5h, 24h, and 48h, respectively) to measure PKA activity as described in the Methods. Three independent experiments were performed in triplicates. The PKA activity was normalized and compared to that of cells exposed to normoxia (0h). Data are expressed as mean+SEM. **, $p < 0.01$. B) A549 cells were exposed to normoxia (N) or hypoxia (H) for 24 hours and the abundance of phospho-CREB and CREB was measured by Western blot analysis. Tubulin was used as equal loading. C) A549 cells were exposed to normoxia (N) or hypoxia for 24 (H24) or 48 hours (H48). Total RNA was extracted, and the relative mRNA amount of catalytic and regulatory subunits of PKA was determined by qRT-PCR. Four independent experiments were performed in triplicates. The results were normalized and compared to that of the normoxia group (N). Data are expressed as mean+SEM. *, $p < 0.05$. D) A549 cells were exposed to normoxia (N) or hypoxia (H) for 24 hours. The abundance of proteins was measured by Western blot analysis. Tubulin was used as equal loading. The ratio of PKA subunits/Tubulin was normalized to that of normoxic cells and was indicated above the gel images.

FIGURE 2. HIF is required for elevated PKA activity during hypoxia. A549, A549-shdHIF, A549-shHIF-1 α , and A549-shHIF-2 α were exposed to normoxia (N) or hypoxia (H) for 2 days, followed with the measurements of the amount of PKA α catalytic subunit (PKACA) (A), PKAR1A (B), and PKAR1B (C) mRNA by qRT-PCR and PKA activity (D). Multiple independent experiments were performed (A, $n=5$; B, $n=4$; C, $n=3$; D, $n=3$) in triplicates. The results were normalized to that of normoxic cells (N). Data are expressed as mean+SEM. * ($p < 0.05$) and ** ($p < 0.01$) represent the significant difference when compared to normoxic A549 cells (N). # ($p < 0.05$), ## ($p < 0.01$), and n.s. (no significance) compare the difference between normoxia and

hypoxia group within the same cell line. In the top panel of D), representative Western blot images of PKACA and Tubulin of these cell lines cultured in normoxia were shown and the ratio of PKACA/Tubulin was normalized to that of A549 cells and was indicated above the gel images. Data were calculated from 4 independent experiments and are expressed as mean+SEM. E) A549 cells were pretreated with ROS scavenger Euk-134 (Euk; 10 μ M and 20 μ M) or the same volume of DMSO for one hour and then exposed to normoxia (N) or hypoxia (H) for 24 hours, followed with the measurement of PKA activity. Three independent experiments were performed in triplicates. The results were normalized to normoxic cells treated with DMSO. ** ($p<0.01$) represents the significant difference when compared to normoxic cells treated with DMSO. # ($p<0.05$) represents the significant difference between hypoxic cells pretreated with DMSO and (Euk 20 μ M). n.s., no significance between hypoxic cells treated with 10 μ M Euk and DMSO.

FIGURE 3. PKA activity is necessary for hypoxia-induced EMT. RLE-6TN cells were pre-treated with 10 μ M PKA inhibitor H89 (A), 20 μ M PKA inhibitor KT5720 (B), or MEK1/2 inhibitor U0126 (C) for 2 hours and then exposed to normoxia (N) or hypoxia (H) for 4 days. Mock treated cells were added with the same volume of DMSO. The expression of α -SMA protein was determined with Western blot analysis. The grey density of the band was determined by ImageJ and the amount of α -SMA was normalized to that of Tubulin. The ratio of α -SMA/Tubulin of the normoxic cells (in panel B) or DMSO treated normoxic cells (in panel A and C) was set as 100. The bar graphs represent at least three independent experiments (A, n=3; B, n=3; C, n=4). **, $p<0.01$. D) A549 cells were cultured on 35 mm dishes overnight to reach confluence, following pretreatment with H89 or DMSO and then exposed to normoxia (N) or hypoxia (H) with wounds created by a 250 μ l tip. The width of the wounds was measured as described above and the difference between the width before and after migration was presented as the migration

distance. Four independent experiments were carried out in triplicates and the results are expressed as mean+SEM. * ($p<0.05$) and ** ($p<0.01$) indicate the significant difference when compared to DMSO/N group. E) A549 cells were cultured on BD Matrigel invasion chambers, pretreated with H89 or DMSO, and exposed to normoxia (N) or hypoxia (H) for 48 hours. Invaded cells were stained and counted under microscopic fields at x200 magnification in each filter using a calibrated ocular grid. Experiments were carried out in triplicates and repeated four times. The results were compared to that of normoxic cells treated with DMSO and data are expressed as mean+SEM. **, $p<0.01$. F) RLE-6TN cells were pre-treated with various doses of PKA activator forskolin (FSK) or 8-Br-cAMP, or exposed to normoxia (N) or hypoxia (H) for 4 days. In the mock treatment, cells were added with the same volume of DMSO. Expression of α -SMA and vimentin was determined by Western blot analysis. Tubulin was used as the control for equal loading.

FIGURE 4. PKA activity is necessary for the induction of EMT by TGF- β 1. A) A549 cells were treated with TGF- β 1 for 2 days and the expression of PKACA and E-Cadherin were measured by Western blot analysis with tubulin as loading control. B) A549 cells were treated with TGF- β 1 for the indicated time period. Cells were lysed and PKA activity was measured as described previously. Results from three independent experiments (each was carried out in triplicates) were presented as mean+SEM. C) A549 cells were pre-treated with 10 μ M TGF- β 1 receptor tyrosine inhibitor SB431542 (SB) or H89 for 2 hours and then incubated with (+) or without (-) TGF- β 1 (5 ng/ml) for 4 days. The same volume of DMSO were added in the culture media as mock treatment. Protein levels of α -SMA were determined by SDS-PAGE and Western blot analysis and normalized to the amount of tubulin. Results from four independent experiments are presented as mean+SEM. ** ($p<0.01$) represents the significant difference when compared DMSO/TGF- β 1(-) group. ## ($p<0.01$) represents the significant difference between DMSO/TGF- β 1(+) and SB/TGF- β 1(+) or H89/TGF- β 1(+). D) A549 cells were cultured on 35 mm dishes

overnight to reach confluence, following pretreatment with H89 or TGF- β 1 and mock treated cells were added with the same volume of DMSO. The migration of these cells was measured as described above. Results from five independent experiments (each was carried out in triplicates) are presented as mean+SEM and compared to that of DMSO/TGF- β 1(-) group. **, $p < 0.01$. E) A549 cells were pre-treated with 50 μ M FSK or the same volume of DMSO and followed with addition of 5 ng/ml TGF- β 1 and incubated for 4 days. Protein levels of E-Cadherin were determined by Western blot analysis. F) A549 cells were pre-treated with 10 μ M TGF- β 1 receptor tyrosine inhibitor SB431542 or the same volume of DMSO for 2 hours and then exposed to normoxia (N) or hypoxia (H) for 2 days. PKA activity was measured, normalized, and compared to that of DMSO/N group. Data are expressed as mean+SEM. Experiments were performed in triplicates and were repeated four times. * ($p < 0.05$) and ** ($p < 0.01$) indicates the significant difference when compared to the DMSO/N group. # ($p < 0.05$) indicates the significant difference between SB431542 treated cells exposed to N or H.

FIGURE 5. Suppression of PKACA does not alter proliferation of A549 cells. A) A549 cells were infected with PKACA shRNA lentiviral particles (shPKACA) or control shRNA lentiviral particles (shCTL) and were selected by media containing puromycin dihydrochloride. Resulting puromycin-resistant single clones were collected and expanded and the expression of PKACA was analyzed by Western blot analysis. The bar graph represents results from three independent experiments which is compared to the A549 cells. **, $p < 0.01$. B) A549 cells were plated in the density of 200000 cells/60 mm dishes and the cell numbers were counted after 1, 3, 5, and 7 days. The experiments were carried out in triplicates and repeated three times. Data are expressed as the average number of cells +SEM. C) A549, A549-shCTL, and A549-shPKACA1 cells were cultured and total RNA was extracted. The relative mRNA amount of catalytic and regulatory subunits of PKA was determined by qRT-PCR. Four independent RNA preparations from each cell line were obtained for qRT-PCR and one preparation of RNA from

A549 cells was used as control and its value was set as 1. The results were compared to that of A549 cells. *, $p < 0.05$. D) A549-shCTL and A549-shPKACA1 cells were exposed to normoxia (N) or hypoxia (H), or treated with TGF- β 1 (5 ng/ml) for 4 days. Cells were lysed and the expression of E-Cadherin protein was determined by Western blot analysis. The amount of Tubulin was used as loading control. E) A549-shdHIF and A549-shHIF-2 α cells were exposed to normoxia (N) or hypoxia (H), or treated with TGF- β 1 (5 ng/ml) for 4 days. Cells were lysed and the expression of E-Cadherin protein was determined by Western blot analysis. The amount of Tubulin was used as loading control. F) RLE-6TN cells were transfected with mPKACA plasmid (mPKACA) or empty vector (Vec), counted, and replated. Replated cells were exposed to normoxia (N) or hypoxia (H), or treated with TGF- β 1 (5 ng/ml) for 4 days. Cells were lysed and cell lysates were used to detect expression levels of α -SMA by Western blot analysis.

FIGURE 6. Hypoxia-mediated migration and invasion are PKA dependent. A) A549, A549-shCTL, and A549-shPKACA1 cells were plated on 35mm dishes to reach confluence and a wound was created by scratching with microtips. The cells were then exposed to normoxia (N) and hypoxia (H) for 24 hours and migration distance were measured as previously described and was presented as the difference between the original and the final width. The experiments were performed in triplicates and repeated three times. The data are expressed as mean+SEM. * ($p < 0.05$) and ** ($p < 0.01$) represent the significant difference when compared to normoxic A549 cells (N). # ($p < 0.05$) and n.s. (no significance) compare the difference between normoxia and hypoxia within the same cell line. B) A549, A549-shCTL, and A549-shPKACA1 cells were plated on the Matrigel-coated invasion chambers and were incubated in normal and hypoxic condition for 48 hours. The total number of invaded cells was counted under the microscope (x200 magnification). Data are expressed as the average number of cells/field+SEM. Three independent experiments were performed in triplicates. * ($p < 0.05$) represents the significant difference when compared to normoxic A549 cells (N). # ($p < 0.05$) and n.s. (no significance)

compare the difference between normoxia and hypoxia within the same cell line. C) A549-shCTL and A549-shPKACA1 cells were pretreated with FSK (50 μ M) or the same volume of DMSO for 1 hour and then exposed to the normoxia or hypoxia for 24 hours (for migration assay, top panel) or 48 hours (for invasion assay, bottom panel). The migration and invasion of these cells were measured as described. The results from four independent experiments (each was carried out in triplicates) are expressed as mean+SEM. * ($p<0.05$) and ** ($p<0.01$) represent the difference when compared to normoxic A549-shCTL cells without FSK treatment (N/shCTL/FSK(-)).

FIGURE 7. Overexpression of mouse PKACA rescues hypoxia-mediated migration and invasion in PKACA deficient A549 cells (A549-shPKACA1 cells). A549-shPKACA1 cells were transfected with mPKACA plasmid (mPKACA) or empty vector (Vec), counted, and replated. A) Replated cells were lysed and cell lysates were used to detect PKACA expression by Western blot analysis. B) Transfected cells were collected and cleared crude cell lysates were used to measure PKA activity as described in the Methods. Three independent experiments were performed in triplicates. The PKA activity was normalized and compared to that of cells transfected with empty vector (Vec). Data are expressed as mean+SEM. **, $p<0.01$. C) Transfected cells were plated on 35mm dishes to reach confluence and a wound was created by scratching with microtips. The cells were then exposed to normoxia (N) and hypoxia (H) for 24 hours and migration distance were measured as previously described. The experiments were performed in triplicates and repeated three times. The data are expressed as mean+SEM. ** ($p<0.01$) represents the significant difference when compared to normoxic A549-shPKACA1 cells transfected with empty vector (Vec). D) Transfected cells were plated on the Matrigel-coated invasion chambers and were incubated in normal and hypoxic condition for 48 hours. The total number of invaded cells was counted under the microscope (x200 magnification). Data are expressed as the average number of cells/field+SEM. Three independent experiments were

performed in triplicates. ** ($p < 0.01$) represents the significant difference when compared to normoxic A549-shPKACA1 cells transfected with empty vector (Vec).