Synergistic Signaling of Tumor Cell Invasiveness by Hepatocyte Growth Factor and Hypoxia*

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Background: Hypoxia and growth factors synergistically enhance tumor cell invasiveness through poorly defined mechanisms.

Results: Molecular signaling pathways mediating tumor cell invasion driven by hypoxia and growth factors were identified.

Conclusions: The integration of three major signaling cascades controls invasive synergy.

Significance: This knowledge informs strategies to predict and disrupt tumor invasiveness.

ABSTRACT

Hepatocyte growth factor (HGF) signaling promotes tumor invasiveness in renal cell carcinoma (RCC) and other cancers. In clear cell RCC, VHL loss generates pseudo-hypoxia that exacerbates HGF-driven invasion through β-catenin deregulation. Hypoxia also enhances HGF-driven invasiveness by papillary RCC cells, but in the absence of VHL loss signaling integration involves three parallel routes: (1) hypoxia-induced ROS production and decreased DUSP2 expression leading to enhanced mitogen-activated protein kinase (MAPK) cascade activation; (2) ROS-induced diacylglycerol production by phospholipase Cγ leading to protein kinase C activation and increased protein phosphatase-2A activity, thereby suppressing HGF-induced Akt activation; and (3) a profound shift from HGF-enhanced, proliferation-oriented metabolism to autophagy-dependent invasion and suppression of proliferation. This tripartite signaling integration was not unique to RCC or HGF: in RCC cells, invasive synergy induced by the combination of hypoxia and epidermal growth factor occurred through the same mechanism, and in estrogen receptor positive breast cancer cells, this mechanism was suppressed in the absence of estrogen. These results define the molecular basis of growth factor and hypoxia invasive synergy in VHL-competent papillary RCC cells, illustrate the plasticity of invasive and proliferative tumor cell states and provide signaling profiles by which they may be predicted.

Collectively, kidney cancers are the seventh most common cancer in men and the ninth most common in women (1). Although patients that present with localized disease have a 95% five-year survival rate, it is only 26% for those with advanced disease (1); the development of novel therapeutic approaches needed to improve the latter survival rate will benefit from understanding the molecular basis of kidney tumor invasiveness and metastasis. Clear cell renal cell carcinoma (ccRCC), the most prevalent type of kidney cancer, is associated with the loss of von Hippel-Lindau (VHL) tumor suppressor gene function (1). The VHL gene product, pVHL, is part of an E3 ubiquitin ligase complex that targets hypoxia inducible factors (HIFs) for proteosomal degradation, implicating a wide spectrum of hypoxia response genes in ccRCC oncogenesis (1). pVHL loss also enables robust ccRCC cell invasiveness and morphogenesis in response to hepatocyte growth factor (HGF;2,3), an important
regulator of kidney development and renal homeostasis (4). Interestingly, this does not occur as a consequence of HIF accumulation, but because pVHL negatively regulates cytoplasmic β-catenin abundance downstream of HGF receptor (Met) activation, and pVHL loss enables beta-catenin and HIF mediated transcriptional initiation of an aggressive invasive program (4,5).

HGF signaling also contributes to disease progression, tumor invasiveness and metastasis in kidney cancers other than the clear cell type; in particular, MET kinase domain mutations in a hereditary form of papillary renal carcinoma (PRC) are primary drivers of that disease, and Met overabundance is common in both hereditary and sporadic forms (1,6). VHL loss of function is rare in PRC, but tumor hypoxia is not; hypoxia strongly enhances HGF-mediated invasiveness and metastasis in a variety of model systems, through largely undefined molecular mechanisms (7-13). HGF and hypoxia signaling integration occurs during nephogenesis (14-16), so this effect may represent the aberrant re-initiation of a developmental program. However, HGF-driven beta-catenin transcriptional activity is suppressed when VHL is functional (17), suggesting that the integration of hypoxia and HGF driven cell invasiveness involves other primary intracellular signaling routes downstream of Met. We report here that multiple pathways, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways, mediate the synergistic invasiveness triggered by HGF and hypoxia, which further entails induction of autophagy and growth suppression.

EXPERIMENTAL PROCEDURES

Reagents - Tissue culture media and supplements were obtained from Invitrogen (Carlsbad, CA). Antibodies against pMet (1234/1235), pErk, tErk, pAkt, tAkt, pPKCα/β, tPKCα, LC3B were obtained from Cell Signaling Technology (Danvers, MA). Anti-Met (C-28) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PP2A was obtained from DSHB (Iowa City, Iowa). U0126 and LY294002 PI3K were obtained from Selleckchem (Houston, TX). Purified recombinant human HGF was obtained from ProSpec (Israel). CoCl2 was obtained from Sigma-Aldrich (St. Louis, MO) and DMOG from Cayman Chemical (Ann Arbor, MI). Hypoxia chambers were obtained from Billups-Rothenberg (Del Mar, California).

Cell Culture - ACHN, A549, and MCF7 cells were obtained from ATCC (Manassas, VA). UOK112 cells were generated in the Urologic Oncology Branch, NCI. Cells were cultured RPMI 1640 medium without sodium pyruvate unless noted, containing 10% FBS and antibiotic-antimycotic. Cells were grown in 5% CO2 at 37°C and hypoxic conditions were generated according to manufacturer’s instruction (Billups-Rothenberg). The oxygen level in hypoxic condition was measured as 1.5% +/- 0.1% using an oxygen gas detector (BW Technologies, Calgary, Alberta Canada). Throughout the paper, the term “hypoxia” refers to this oxygen level.

SDS-PAGE, Immunoblot Analysis and Two-Site Immunoassays - Cells were washed with cold PBS, extracted in Laemmli buffer, sonicated, heated for 5 min at 95°C prior to SDS-PAGE and electrophoretic transfer to nitrocellulose membrane. Membranes were blocked with 5% milk in TBST (Tris Buffered Saline, 0.1% Tween 20) for 1h at 25°C and incubated 16h at 4°C with primary antibody in TBST/0.5% milk. Membranes were washed three times with TBST, incubated with horseradish peroxidase-labeled secondary antibody for 1h at 25°C, washed for 3h with TBS prior to ECL detection (Pierce Biotechnology). Phospho- and total Met content in Triton X-100 cell extracts were determined by electrochemiluminescent immunoassay read using a SectorImager 2400 (Meso Scale Discovery, Gaithersburg, MD) as described previously (18).

Phosphatase Assays - PP2A phosphatase activity in immunoprecipitates was determined using para-nitrophenyl phosphate (pNPP) as substrate. For immunoprecipitation, cells were extracted with buffer containing 1% Triton X-100, cleared by centrifugation and aliquots (250 ug cell protein) were incubated with 2 ug PP2A antibody and 20uL protein G sepharose beads for 16h at 4°C. Immunoprecipitates were washed 3 times with cold lysis buffer and 3 times with phosphatase buffer (50mM HEPES, 10mM MnCl2, 1mM DTT and protease inhibitors). The pellet was reconstituted in phosphatase assay buffer and pNPP was added to a final concentration of 20 mM. The reaction mixture was...
incubated in 30°C for 1h and absorbance read at 405nm.

**MTT Assays** - Cells were plated at a density of 7.5 x 10^3 cells/well in a 96 well plates. Cells were treated and after 24-48h, 20 µL 5mg/ml of MTT was added to each well and plates were incubated at 37°C for 4h. The solution was removed and DMSO was added to dissolve formazan crystals; plates were shaken for 15 min and absorbance at 590nm was read with a reference filter of 620nm.

**RNA Isolation and RT-PCR** - Total RNA was obtained by using the RNasy kit (QIAGEN, Valencia, CA). RNA concentrations were determined spectroscopically at 260nm (ND-1000 spectrophotometer; NanoDrop, Wilmington, DE). RNA (1.0 µg) was subjected to RT with GeneAmp RNA polymerase chain reaction (PCR) kits according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Real time PCR was performed according to the manufacturer’s protocol using 7500 Real-Time PCR System (Applied Biosystems).

**Cell Migration, Invasion and Proliferation Assays** - Cell monolayers grown in 35mm culture dishes in complete growth media were wounded by manual scratching with a pipet tip, washed with PBS and photographed by phase contrast microscopy at time zero. Matched wound region pairs were photographed again after 24h, and percent cell migration was quantified using Image J software V1.47 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012). Invasion assays were performed using 8um pore size BD Bioscience BioCoat Matrigel Invasion chambers or Fluoroblok-BioCoat BD Matrigel Invasion chambers (San Jose, CA) according to the manufacturer’s instructions. Images were captured by light or fluorescence microscopy, and image analysis and quantitation were performed using Image J. For proliferation assays, cells were plated in triplicate at a density of 5 x 10^4 cells/35-mm dish in defined medium. HGF and/or CoCl₂ were added on days 1, 2, and 4. Cells were detached and counted using a hemocytometer on days 3 or 6.

**Cell Cycle Analysis** - ACHN cells were grown without sodium pyruvate and treated with CoCl₂ and/or HGF for 24h. Cells were then harvested, spun, washed, counted and resuspended in PBS and fixed with methanol prior to storage at -20°C for 24h. Thawed cells were spun and resuspended in cold PBS and propidium iodide (50 µg/mL) was added prior to analysis using a BD Biosciences LSRII flow cytometer and data processing using ModFit LT software.

**Statistical Analysis** - Significant differences between two groups were determined by Student's t test using GraphPad Prism 5 software, where p <0.05 was considered statistically significant.

**RESULTS**

**Hypoxia enhances HGF-induced cell invasion but inhibits proliferation** - We examined the effects of hypoxia on HGF/Met-induced invasion by the human renal papillary carcinoma-derived cell lines ACHN and UOK112, the lung adenocarcinoma cell line A549 and the breast cancer derived cell line MCF7. Invasion, measured as migration across matrigel-coated Boyden chambers, was significantly increased by HGF in all cell lines (Fig. 1A). Hypoxia alone (1.5% O₂) also induced cell invasion, generally to a lesser degree than HGF, whereas the combined effects of hypoxia and HGF were synergistic, i.e. 8 - 20-fold greater in ACHN, UOK112 and A549 cells, and at least additively greater in MCF7 (Fig. 1A). Similar effects were observed using the hypoxia mimic agent CoCl₂, which stabilizes HIF1α by competing with iron for binding to prolyl hydroxylase, thereby preventing HIF1α prolyl hydroxylation, pVHL recognition and proteosomal degradation. HGF and CoCl₂ induced cell migration (data not shown) and invasion (Fig. 1B), and the combination significantly enhanced migration and invasion in all cells tested, again less dramatically in MCF7 cells.

The combination of hypoxia and HGF also affected cell proliferation. ACHN cells were plated (day 0) and left untreated or treated with HGF on days 1, 4, 5, 8 and 11; for the latter 3 days one group was treated with CoCl₂ and another received combined HGF/CoCl₂ treatment. A significant increase in proliferation rate associated with HGF treatment relative to all other groups was evident by day 12 (Fig. 1C). CoCl₂ alone had no significant effect; whereas cells treated with
both HGF and CoCl₂ proliferated significantly more slowly relative to those treated with HGF alone, indicating that hypoxia inhibits HGF-induced cell growth (Fig. 1C). Immediately after counting, samples of suspended cells were analyzed for invasiveness and, consistent with prior results, CoCl₂ significantly enhanced the HGF-induced cell invasion (Fig. 1D). As anticipated, all of these HGF-driven effects were associated with immediate and significant Met kinase activation (Fig. 1E); Met activation and Met content (ACHN, 118 ng Met/mg total protein; A549, 66 ng/mg; MCF7, 0.6 ng/mg) were unaffected CoCl₂ treatment (data not shown).

Hypoxia-enhanced MAPK pathway activity augments HGF-driven invasion - Activation of the PI3K and MAPK pathways downstream of Met mediates HGF-driven cell survival, migration, invasion and proliferation. ACHN, A549, UOK112 and MCF7 cells cultured in complete (serum containing) growth media under hypoxic conditions for 24h were immunoblotted for HIF1α, phospho-Akt (pAkt), total Akt (tAkt), phospho-Erk (pErk), and total Erk (tErk) to determine the effects of hypoxia on these pathways. Increased HIF1α protein abundance and pErk/tErk ratio were uniformly observed; the pAkt/tAkt ratio was markedly diminished with hypoxia in ACHN, A549 and UOK112 cell lines, but not in MCF7 (Fig 2A, left panel). The combined effects of HGF-induced Akt and Erk activation and hypoxia were analyzed in ACHN and A549 cells grown in serum-reduced media (Fig. 2A, right panel). HGF treatment increased the pAkt/tAkt and pErk/tErk ratios under normoxic conditions in both cell lines relative to untreated controls (Fig 2A, right panel). HGF and hypoxia had additive effects on the pErk/tErk ratio, whereas hypoxia abrogated HGF effects on the pAkt/tAkt ratio (Fig 2A, right panel). Consistent with these results, time-courses of CoCl₂- or DMOG-induced pseudo-hypoxia were associated with trends of increasing HIF1α abundance and Erk activation, but decreasing Akt activation, over 24h (Fig. 2B). At steady state after 24h of CoCl₂ or DMOG treatment followed by 20 min HGF stimulation, pErk enhancement and suppression of HGF-induced pAkt in ACHN, A549 and UOK112 cells as observed by immunoblotting (Fig. 2C, left panels) were determined to be statistically significant by densitometry (Fig. 2C, right panels).

The functional impact of the differential effects of hypoxia on HGF-induced Erk and PI3K pathway activation was investigated using the pharmacological inhibitors of these pathways U0126 and LY294002, respectively. Migration by ACHN (Fig. 3A) and UOK112 cells (Fig. 3B) incubated for 16h in CoCl₂ +/- HGF was unaffected by LY294002 but significantly inhibited by U0126, suggesting that the independent and combined effects of hypoxia and HGF were at least in part Erk dependent. Reinforcing these observations, we found that ACHN cells ectopically expressing a constitutively active form of Mek displayed significantly greater 24h invasiveness than GFP control transfectants, that this effect was further increased by concurrent CoCl₂ treatment, and that ACHN cells expressing a dominant negative form of Mek were non-invasive (Fig. 3C).

ROS mediates hypoxia enhanced Erk activation and HGF-driven invasion - Intracellular reactive oxygen species (ROS), including free radicals such as superoxide, hydroxyl radical and H₂O₂, are often generated at high levels under hypoxic conditions. ROS production in ACHN cells treated with CoCl₂ or DMOG for 4 to 16h increased significantly over time (Fig. 4A); combined treatment with HGF did not further enhance ROS levels (data not shown). In the added presence of the ROS-scavenger sodium pyruvate (NP; 10 mM), CoCl₂ or DMOG induced ROS accumulation was curtailed more than 50% (Fig 4B). CoCl₂ or DMOG-induced ACHN cell invasion, and pseudo-hypoxia enhancement of HGF-driven invasion, was significantly diminished by NP treatment, indicating partial dependence of this effect on ROS accumulation (Fig. 4C). ROS accumulation over 24h was also functionally linked to hypoxia-induced Erk activation and hypoxic suppression of HGF-stimulated Akt activation in ACHN, because both events were blocked by the ROS scavengers NP and diphenyleneiodonium (DPI; Fig. 4D). HGF-induced Erk activation was independent of ROS (Fig. 4D). Similar results were seen when ACHN cells were treated with CoCl₂ or DMOG in the absence or presence of NP (Fig. 4E and F) or DPI (data not shown).
Erk requires both threonine 202 and tyrosine 204 phosphorylation for full activity; members of the subgroup of dual specificity phosphatases (DUSPs) known as MAPK phosphatases (MKPs) are uniquely capable of comprehensively regulating Erk signaling. There are 11 well characterized MKP family members; those frequently associated with Erk inactivation include MKP3, MKP4, and phosphatase of activated cells 1 (PAC1 or DUSP2). Real time PCR analysis of mRNA abundance for various DUSP family members under hypoxic or normoxic conditions with or without HGF treatment for 24h showed that under hypoxia, DUSP2 transcript abundance was significantly reduced in ACHN and A549 (Fig. 5A) and moderately reduced in UOK112 and MCF7 (Fig. 5B). In contrast to the other cell lines tested, MCF7 cells showed significantly increased DUSP1 expression with the combination of HGF and hypoxia, potentially attenuating their invasive response to these conditions. DPI treatment of hypoxic ACHN and A549 cells blocked hypoxia-associated DUSP2 suppression, indicating ROS dependence of this effect (Fig. 5 C). DUSP2 protein content was also significantly decreased by hypoxia and similarly ROS dependent in ACHN and A549 (Fig. 5D).

ROS mediates PKCα and PP2A activation leading to hypoxic Akt suppression - Hypoxia suppressed HGF-induced pAkt in all cell lines except MCF7. Investigating the biochemical link between hypoxia and pAkt suppression, lysates from ACHN and A549 cells treated with CoCl$_2$ for 24h were immunoblotted for total PP2A, pPTEN, or pPDK1 -known regulators of Akt phosphorylation - but no changes in the levels of these proteins were observed (data not shown). A prior study (19) showed that cell lines (including A549) treated with the protein kinase C (PKC) activator 12-O-tetradecanoylphorbol-13-acetate (TPA) displayed decreased pAkt. Consistent with our observations, they also noted that some cell lines (including MCF7) did not display TPA-mediated pAkt suppression. Oxidative stress has been reported to trigger PKC translocation from the cytosol to the plasma membrane and PKC activation. To test whether hypoxia induced ROS was activating PKC, ACHN and A549 cells were maintained in normoxic or hypoxic conditions in the absence or presence of DPI for 24, then treated with HGF for 20 min prior to immunoblot analysis of phospho- and total PKCα (pPKCα and tPKCα, respectively). HGF treatment induced PKCα activation in normoxic cells regardless of DPI (Fig. 6A, B) or NP treatment (data not shown). Hypoxia also induced PKCα activation in these cell lines, and this reversed by DPI treatment, suggesting that activation resulted from ROS accumulation (Fig. 6A, B).

Evidence that ROS-induced PKC activation was linked to hypoxia-associated suppression of HGF-driven Akt activation was obtained using the cell permeable PKC inhibitor bisindolylmaleimide I (BIM). ACHN, A549 and MCF7 cells were pretreated with BIM, incubated in normoxic or hypoxic conditions and then treated briefly with HGF. As expected, BIM treatment suppressed all HGF- and hypoxia-associated increases in pPKC, and it also reversed the hypoxia-associated suppression of HGF-driven Akt activation observed in ACHN and A549 (Fig. 6C, top and middle panels), suggesting that indeed, ROS-induced PKC activation mediated this effect. Consistent with the results presented in Figures 1 and 2, hypoxia did not suppress Akt activation in MCF7 cells, and BIM treatment had no effect on Akt activation (Fig. 6C, bottom panel). BIM treatment did not inhibit Erk activation by HGF, hypoxia, or the combination (Fig. 6C), nor did it inhibit invasion by ACHN or A549 cells (data not shown), indicating that these effects were PKC independent.

We also investigated the potential role of PP2A in mediating Akt dephosphorylation downstream of PKCα. As noted above, we did not observe any change in the level of PP2A in cells treated with CoCl$_2$ for 24h, however, prior reports have shown that PKCα is a primary mediator of PP2A activation (20) and that PP2A negatively regulates Akt by direct dephosphorylation in other cell types under different conditions (21). To determine whether PP2A is activated by PKC in hypoxia, cells were pretreated with BIM or DPI and incubated in normoxic or hypoxic conditions for 24h followed by brief HGF treatment. PP2A was immunoprecipitated from cell lysates and
phosphatase assays were performed using para-nitrophenyl phosphate (pNPP) as substrate. ACHN and A549 cells showed increased PP2A phosphatase activity under hypoxic conditions (Fig. 7A) or (for A549 only) with CoCl₂ treatment (Fig. 7B). Pretreatment with BIM or DPI returned hypoxia-stimulated PP2A activity levels to control, indicating PKC pathway dependence. Neither ACHN nor A549 showed any increase in PTEN activity after 24h of CoCl₂ treatment (data not shown).

MCF7 breast cancer-derived cells stood apart from the other lines analyzed here in that the combined effects HGF and hypoxia (or hypoxia mimetics) on migration and invasion were significant but less pronounced (Figures 1A, B) and HGF-stimulated Akt activation was not suppressed by hypoxia (Fig 2A, left) nor associated with increased PP2A activity (Fig. 7A, lower left), despite ROS-mediated PKCα activation (Fig. 6A, B). Prior studies have shown that PP2A activation is estrogen dependent in MCF7 cells (22). Indeed, addition of β-estradiol also increased PP2A activity in our studies, and under these conditions it was further enhanced by hypoxia (Fig. 7A, lower right), an effect that was linked to ROS-mediated PKCα activation by its reversal with DPI or BIM treatment. MCF7 cells treated with or without β-estradiol were then examined for hypoxia-associated Akt suppression by immunoblotting for phospho- and total Akt. Control MCF7 cells again displayed HGF-stimulated Akt activation regardless of oxygen level, but hypoxic suppression of Akt activation was observed in the presence of β-estradiol (Fig. 7C); β-estradiol had no effect on pAkt levels in ACHN or A549 (data not shown). Thus the hypoxia signaling pathway extending to Akt suppression was intact in MCF7 cells, but subject to further regulation by estrogen signaling.

The connection between increased PP2A activity and suppression of HGF-driven Akt activation was further interrogated in ACHN and A594 cells using the PP2A inhibitor okadaic acid. Hypoxia-associated suppression of HGF induced Akt activation in both cell lines was reversed by okadaic acid treatment to the level observed in normoxic cells (Fig. 7D), strongly implicating PP2A in hypoxic regulation of HGF-activated Akt signaling.

**Hypoxic suppression of HGF-driven cell proliferation and metabolism** - The hypoxia mimetic CoCl₂ suppressed ACHN cell growth stimulated by HGF (Fig. 1C). This effect was reproduced in UOK112 and A549 cells, but not in MCF7 (Fig. 8A), consistent with the distinct requirement for concomitant estrogen signaling as described above. Similar to these findings, results of MTT assays indicative of mitochondrial metabolic activity performed on ACHN, UOK112 and A549 cells grown in hypoxic or normoxic conditions showed hypoxic suppression of HGF-stimulated metabolism, an effect that was not seen in MCF7 (Fig. 8B, left). MTT analysis of CoCl₂ treated ACHN and UOK112 cells followed the same trend seen in cells subjected to hypoxia (Fig. 8B, right). Cell cycle analysis of ACHN cells by flow cytometry revealed that HGF treatment alone increased the S phase cell population, whereas CoCl₂ treatment resulted in G1 arrest (Fig. 8C). MTT analysis of ACHN, UOK112 (Fig. 8D), A549 and MCF7 cells (Fig. 8E) grown in normoxic or hypoxic conditions, and ACHN grown in CoCl₂ (data not shown), treated with U0126 or LY294002 revealed a strong dependence of HGF-induced metabolic increase on PI3K signaling; neither inhibitor affected hypoxic suppression of HGF-stimulated cell metabolism.

ROS and PKCα activation mediated the observed hypoxic suppression of HGF-stimulated metabolism in ACHN cells, as this was reversed by either BIM or DPI treatment (Fig. 9A). HGF-stimulated mitochondrial metabolism in MCF7 cells was not suppressed by hypoxia (Fig. 9B, top), unless cells were treated concomitantly with β-estradiol to restore PP2A activation (Fig. 9B, bottom); under these conditions, either BIM or DPI treatment reversed the observed hypoxic suppression. Similarly, HGF-mediated cell growth in MCF7 cells was not suppressed by hypoxia (Fig. 9C, left), unless cells were treated with β-estradiol to restore PP2A activation (Fig. 9C, right).

**Autophagy induction critically supports invasiveness driven by HGF with hypoxia** - Hypoxia has been shown to induce autophagy in multiple cell lines and tissues. When autophagy is initiated, cytoplasmic microtubule-associated protein 1 light chain 3 (LC3-I), is cleaved, lipidated and inserted as LC3-II into autophagosome membranes (23), where it
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becomes associated with the ubiquitin binding protein sequestosome 1 (SQSTM1, p62). Lysosomal degradation of autophagosomes leads to decreased SQSTM1 abundance during autophagy. A549 and UOK112 cells grown in hypoxia displayed decreased SQSTM1 and increased LC3-II accumulation (Fig. 10A); LC3-II was also increased in ACHN cells treated with CoCl₂ or DMOG (Fig. 10B). Increased recruitment of LC3 to autophagosomes can be visualized microscopically as an increased number of LC3 puncta (24,25). Fluorescence immunostaining for LC3 in ACHN and UOK112 revealed accumulation of LC-3 puncta with 24h CoCl₂ treatment, whereas untreated and HGF treated cells did not (Fig. 10C).

Prior studies have shown that enhanced tumorigenesis by constitutively active Akt is linked to inhibition of autophagy (26) and that inhibition of Akt promotes autophagy (27). To determine whether hypoxia-associated Akt inhibition initiated autophagy, ACHN cells harboring lentiviral expression constructs encoding constitutively active (ca) or dominant negative (dn) forms of Mek or Akt were incubated under normoxic or hypoxic conditions for 24h incubation in serum-reduced media (Fig. 10D). Hypoxic GFP infected control cells displayed increased pErk, unaltered pAkt and increased LC3-II formation, indicating autophagy induction (Fig. 10D, left). Ectopic expression of ca- or dn Mek exerted the anticipated effects on Erk activation but did not alter hypoxia-induced LC3-II formation (Fig. 10D, middle), suggesting that autophagy initiated by hypoxia is MAPK cascade independent. In contrast, cells expressing ca- or dnAkt displayed the expected effects on pAkt and, consistent with the prior studies mentioned, constitutive Akt activation blocked hypoxia-induced LC3-II formation while constitutive Akt suppression led to increased LC3-II formation even in normoxia (Fig. 10D, right). These results indicate that hypoxia-associated Akt suppression initiates autophagy.

In ACHN cells, the autophagosome inhibitor chloroquine had little effect on invasion driven by either HGF or CoCl₂ alone, but significantly suppressed invasion driven by combined HGF and CoCl₂ treatment (Fig. 11A). In follow-up, ACHN and A549 cells were transfected with expression constructs for EGFP (control), or EGFP-tagged wild type LC3 (wt-LC3) or mutant LC3 (LC3DG120A), which blocks autophagosome formation by preventing the cleavage of pro-LC3 required for the formation of LC3-I and LC3-II. MTT assays performed on these transfectants over a two-day period confirmed that hypoxia suppressed metabolism and that disruption of autophagosome formation by the LC3 mutant form significantly reversed this effect (Fig. 11B). Cell invasion by these transfectants across Matrigel-coated Boyden chambers in the presence or absence of HGF and/or hypoxia followed the expected pattern of control<hypoxia<HGF<HGF + hypoxia, but the combined effect of HGF and hypoxia was reduced 66% in the mutant LC3 transfectants relative to wild type transfectants (Fig. 11C). Thus, autophagosome formation provided critical support for invasiveness driven by the combination of HGF and hypoxia.

DISCUSSION

Hypoxia plays a key role in many aspects of cancer biology including angiogenesis, epithelial-mesenchymal transition, tumor invasion and metastasis (28-33). In patients with invasive breast cancer, loss of vascular pericytes from hypoxia was associated with Met activation, epithelial to mesenchymal transition and increased metastasis (31). Similar hypoxia-related shifts
from rapid tumor growth to increased metastasis have been reported for uveal melanoma (7), cervical cancer (8) and oral squamous carcinoma cells (12). This is also frequently seen in patients who have undergone radiation treatment for various cancers, where treated areas show increased hypoxia, decreased tumor density but increased metastasis (28,32). As shown here and in prior studies, the combination of hypoxia and HGF/Met signaling strongly promotes tumor cell invasiveness and metastasis (10,11,13), motivating us to make a thorough and cohesive investigation of the integration of these signaling pathways.

ROS production can induce and/or activate several members of MAPK pathway (34-37). In some of these models, ROS production was associated with hypoxia, and inhibition of MAPK activation with antioxidants functionally implicated ROS in this process, but cell motility or invasion was not interrogated (35,38). HIF1α-mediated ROS accumulation has been causally linked to increased breast cancer cell invasion, but MAPK activation was not assessed (39). We show here that hypoxia, as well as CoCl2- and DMOG-induced pseudo-hypoxia, led to increased ROS, Erk1/2 activation and invasion in four tumor cell lines. ROS accumulation, Erk activation and invasion were suppressed by ROS scavengers, and by pharmacologic or dominant negative Mek inhibition, whereas constitutive Mek activation enhanced invasion. The portion of this hypoxia pathway above Erk was independent of Met abundance or activation in all four cell lines, and thus additive to HGF-induced Erk activation, which is critical for HGF-driven invasion as shown here and supported by prior work (40-43).

The observed hypoxic suppression of DUSP2 transcript and protein content is likely to provide, wholly or in part, hypoxic Erk activation and invasiveness. A prior report showed that DUSP2 expression was reduced or absent in many human cancers, inversely correlated with that of HIF1α and with cancer malignancy, and causally linked to increased Erk activation and chemoresistance in tumor cell lines (6). Although invasion was not analyzed in that study, other reports have shown that overexpression of family members DUSP1 or DUSP6 reduced tumor cell invasion (44,45). We assert that the coordinated changes in HIF1α, ROS, phospho-Erk and DUSP2 shown here define the primary signaling pathway by which hypoxia promotes invasion in papillary RCC cells, and that Erk is the critical node for synergistic invasiveness induced by HGF and hypoxia (Fig. 14).

Akt is a master regulator of cell metabolism and HGF-driven cell proliferation; the integration of this pathway with hypoxia signaling pathway is complex. In normoxic conditions, HGF-induced Akt activation is essential for cell proliferation but independent of invasion. In hypoxia, substantial suppression of HGF-induced Akt activation resulted in cell cycle arrest and decreased proliferation. This occurred through ROS-mediated PLCγ activation, DAG production and consequent PKC activation and, in turn, increased PP2A activity, Akt dephosphorylation and inactivation. Hypoxic Akt suppression was associated with induction of autophagy, a protective mechanism that enhances cell viability in adverse conditions (51-53). Disruption of autophagosome formation by chloroquine, or by ectopic expression of an uncleavable LC3 mutant, significantly reduced hypoxia-enhanced, HGF-induced cell invasion, indicating that autophagosomes provide critical support for invasive synergy in a hypoxia-attenuated metabolic state. Moreover, constitutive Akt activation blocked LC3 proteolytic conversion, implying that Akt suppression is also important. Thus, Akt activation state is not completely unrelated to the invasive synergy mediated by the integration of HGF and hypoxia signaling: its role is passive in that suppression is necessary.

Interestingly, our results show that adaptation to hypoxia is estrogen receptor (ER) regulated in breast-cancer derived MCF-7 cells. Hypoxia did not induce autophagy in MCF7 (Fig. 9B, left) and failed to suppress HGF-stimulated Akt activation (Fig. 3A). ER activation enabled hypoxia to induce suppression of HGF-stimulated pAkt (Fig. 8C), autophagy induction manifested as dramatically reduced metabolism (Fig. 10B), and growth suppression (Fig. 10C). Thus, the failure of MCF7 to adapt to hypoxia through induction of autophagy is not due to an absence of the signaling pathway, but to its suppression in the absence of ER activation. ER-enabled metabolic suppression by hypoxia was dependent on ROS production and PKC activation (Fig. 10B), but ER-driven PP2A activation was more complex: the majority of this increase was independent of ROS and PKC, but a lesser though statistically significant portion was
BIM and DPI sensitive (Fig. 8A). PP2A activation by ER under normoxic conditions has been reported and was coincident with ER-PP2A physical association (54). The observation that ER-mediated PP2A activation only leads to decreased pAkt in the presence of hypoxia (Fig. 8D) suggests that either this minor additional increase in PP2A activity is functionally relevant, or that another hypoxia-related event is needed for PP2A to dephosphorylate Akt beyond increased catalytic activity; both possibilities are illustrated schematically in Figure 14. A recent study showed that primary cells derived from ER negative breast tumors grew significantly faster that those from ER positive tumors, and together with other attributes, authentically reflected rates of disease progression and outcome (55). Our analysis of MCF7 suggests that failure to activate the ER pathway, mimicking ER negative breast tumors, would lead to increased Akt activation, cell survival and proliferation even in a hypoxic microenvironment. Overall, our results illustrate the plasticity of invasive and proliferative tumor states, cohesively outline the convergence of hypoxia and growth factor signaling pathways that strongly promotes tumor cell invasiveness, and provide signaling profiles that may help distinguish rapidly growing tumors from those with greater likelihood for metastasis.
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FIGURE LEGENDS

FIGURE 1. Hypoxia enhances HGF-driven invasion and suppresses proliferation. A. Cell types indicated were incubated in normoxia (N, left) or hypoxia (H, right) and otherwise left untreated (unfilled bars) or treated with HGF (0.5 nM; light gray bars), hypoxia (dark gray bars), or HGF + hypoxia (black bars) for 24h in matrigel-coated Boyden chambers and cell invasion across the membranes was quantitated photomicrographically. This gray scale scheme is used in bar graphs throughout the paper; bars represent the mean of triplicate samples +/- SD; some error bars are too small to be visible. Data are representative of three independent experiments (*p<0.001 vs untreated, **p<0.05 HGF vs HGF + hypoxia). B. Cell types indicated were incubated in normoxia (N, left) or hypoxia (H, right) and otherwise left untreated (unfilled) or treated with HGF (light gray), CoCl₂ (100 µM; dark gray), or HGF + CoCl₂ (black) for 24h in matrigel-coated Boyden chambers and cell invasion across the membranes was quantitated photomicrographically. Bars represent the mean of triplicate samples +/- SD. Data are representative of three independent experiments (*p<0.001 vs untreated, **p<0.05 HGF vs HGF + hypoxia). C. Proliferation of ACHN cells left untreated (circles), or treated with HGF (squares), 100 µM CoCl₂ (triangles), or HGF + CoCl₂ (inverted triangles). HGF treatments were on days 1 and 4; HGF and/or CoCl₂ treatments were on days 5, 8 and 11. Cells were counted on days 0, 3, 6, 9 and 12. D. Cells from panel C were detached after counting, placed in matrigel coated Boyden chambers for invasion assays (*p<0.05, **p=0.006). Bars represent the mean of triplicate samples +/- SD; some error bars are too small to be visible. E. Cell types indicated below each graph were treated with CoCl₂ (100 µM) for 24h, followed by 20 min exposure to HGF (0.5 nM) before extraction with non-ionic detergent and measurement of phospho-Met (pMet) by electrochemiluminescent immunoassay. Bars represent the mean of triplicate samples +/- SD; some error bars are too small to be visible.

FIGURE 2. Hypoxia activates the Erk pathway while suppressing Akt. A. Left: cells cultured in complete (serum containing) medium incubated under normoxic (-) or hypoxic (+) conditions for 24h were immunoblotted for HIF1α, pAkt, Akt, pErk, or Erk as indicated. Right: cells cultured in serum-reduced medium were incubated under normoxic (-) or hypoxic (+) conditions for 24h followed by 20 min exposure to HGF (0.5 nM), then extracted with non-ionic detergent and immunoblotted HIF1α, pAkt, Akt, pErk, or Erk as indicated. B. ACHN cells were treated with CoCl₂ (100 µM; left) or DMOG (500 µM; right) at the indicated times (h) and lysates were immunoblotted for HIF1α, phospho-Akt (pAkt), total Akt (tAkt), phospho-Erk (pErk), or total Erk (tErk). C. Cells were treated with CoCl₂ or DMOG for 24h followed by 20 min treatment with HGF as indicated. Left, cell lysates immunoblotted for pAkt, Akt, pErk, or Erk. Right, densitometric quantitation of the immunoblot for the following treatment groups: untreated (white), HGF (light gray), CoCl₂ (dark gray), DMOG (stripe), HGF + CoCl₂ (black), HGF + DMOG (gray stripe) (*p<0.05).

FIGURE 3. Migration and invasion driven by HGF and enhanced by hypoxia is Erk activation dependent. A. ACHN cell migration by untreated (control) cells or following treatment with LY294002 (10 µM) or U0126 (1 µM). After 30 min, HGF (0.5 nM; light gray bars), CoCl₂ (100 µM; dark gray bars) or both (black bars) were added as indicated for 24h. Values represent the mean of triplicate samples +/- SD. Data are representative of three independent experiments (*p<0.05 vs untreated, **p<0.001). B. Migration by UOK112 cells as described for Panel A. C. Invasion by ACHN cells infected with vectors encoding GFP, constitutively active (ca) Mek, or dominant negative (dn) Mek for 48h in the absence (unfilled) or presence of CoCl₂ (dark gray) for 24h (*p<0.001). Lysates prepared from infected cells were also used for dot blots (inset): immunoblotting for pErk and Ponceau S (PS) stain as loading control.
FIGURE 4. Hypoxia induced ROS production leads to enhanced Erk activation and increased invasiveness. A. ROS level, expressed as mean fold increase over untreated cells +/- SD, in cells treated with CoCl2 (100 µM; gray) or DMOG (500 µM; stripe) at the indicated times, or H2O2 (100 µM) for 1h (unfilled). B. ROS level, expressed as mean fold increase over untreated cells +/- SD, in cells treated with CoCl2 or DMOG for 24h in the additional absence or presence of sodium pyruvate (1 mM; NP) for 24h prior (*p<0.005). C. Cell invasion was measured in matrigel-coated Boyden chambers among the following treatment groups: untreated (white), HGF (0.5 nM; light gray), CoCl2 (dark gray), DMOG (stripe), HGF + CoCl2 (black), HGF + DMOG (gray stripe). Bars represent the mean of triplicate samples +/- SD. Data are representative of three independent experiments (*p<0.05). D. Cells were incubated in the absence or presence of NP or DPI (5 µM) for 24h followed by an additional 24h under normoxic or hypoxic conditions; cells were then treated with HGF for 20 min as indicated prior to immunoblot analysis for pAkt, Akt, pErk, or Erk. E. ACHN cells were cultured in either the absence or presence of NP prior to CoCl2 or DMOG treatment for 24h, followed by 20 min of HGF treatment prior to immunoblot analysis for pErk or Erk. F. Densitometric quantitation of the immunoblot shown in panel E: untreated (white), HGF (light gray), CoCl2 (dark gray), DMOG (stripe), HGF + CoCl2 (black), HGF + DMOG (gray stripe) (*p<0.001, **p<0.01).

FIGURE 5. Hypoxia-induced ROS accumulation decreases DUSP2 expression. A. Cells were incubated in HGF (0.5 nM; light gray), hypoxia (dark gray) or hypoxia with HGF (black) for 24h prior to analysis of DUSP1 or DUSP2 mRNA abundance, expressed as mean fold change vs untreated normoxic cells (n = 3; *p<0.01). B. UOK112 and MCF7 cells were incubated in normoxia (light gray), hypoxia (dark gray) or hypoxia with HGF (black) for 24h prior to analysis of DUSP1 or DUSP2 mRNA abundance, expressed as mean fold change relative to untreated normoxic cells (n=3; *p<0.01). C. Cells were incubated in hypoxia (unfilled), normoxia with DPI treatment (5 µM; gray), or hypoxia with DPI treatment (black) for 24h prior to analysis of DUSP2 mRNA abundance, expressed as mean fold change untreated normoxic cells (n = 3; *p<0.01). D. Cells were incubated in the absence or presence of DPI for 24h followed by an additional 24h under normoxic or hypoxic conditions prior to immunoblotting for DUSP2, pErk or GAPDH.

FIGURE 6. PKCα activation mediates hypoxia-induced Akt suppression. A. Cells were incubated in the absence or presence of DPI (5 µM) for 24h followed by an additional 24h under normoxic or hypoxic conditions, then treated with HGF (0.5 nM) for 20 min as indicated prior to immunoblot analysis for pPKCα or PKCα. B. Densitometric quantitation of immunoblot shown in panel A; bars represent the mean of triplicate measurements +/- SD, *p<0.05 relative to control (immediately above bar) or between groups as indicated by lines. C. Cells were incubated in the absence or presence of BIM (5 nM) for 24h under normoxic or hypoxic conditions, then treated with HGF (0.5 nM) for 20 min as indicated prior to immunoblot analysis for pPKCα, PKCα, pAkt, Akt, pErk, or Erk.

FIGURE 7. Activation of PP2A by PKCα mediates hypoxia-induced Akt suppression. A. ACHN (upper left), A549 (upper right) and MCF7 cells (lower left) were treated without (control) or with BIM (5 nM) or DPI (5 µM) under normoxic (unfilled bars) or hypoxic (dark gray bars) conditions for 24h prior to brief HGF stimulation (under normoxia, light gray bars, under hypoxia, black bars). Another set of MCF7 cells (lower right) were treated with β-estradiol (10 nM) for 24h, then with or without BIM or DPI under hypoxic or normoxic conditions for another 24h prior to brief HGF stimulation. Cell lysates were immunoprecipitated with anti-PP2A antibody and phosphatase activity assessed using pNPP as substrate. B. A549 treated as in panel A, but substituting CoCl2 treatment (100 µM) for hypoxia. C. MCF7 cells were incubated in the absence or presence of β-estradiol (10 nM) for 24h followed by an additional 24h under normoxic (N) or hypoxic (H) conditions, then treated with HGF (0.5 nM) for 20 min as indicated prior to immunoblot analysis for pAkt or Akt. D. ACHN and A549 cells were incubated in the absence or
presence of okadaic acid (1 nM) for 24h under normoxic (N) or hypoxic (H) conditions, then treated with HGF (0.5 nM) for 20 min as indicated prior to immunoblot analysis for pAkt and Akt.

**FIGURE 8.** Hypoxia blocks cell proliferation and the associated increase in metabolic activity driven by HGF. A. Mean cell number over time +/- SD for control (circles) and cells treated with CoCl$_2$ (100 µM; triangles), HGF (0.5 nM; squares) or HGF + CoCl$_2$ (inverted triangles) on days 0, 3 and 6 (*p<0.01 vs control, **p<0.01 HGF vs HGF + CoCl$_2$). B. Left, mitochondrial metabolic activity (mean MTT value relative to control +/- SD, n=3) in ACHN, UOK112, A549 and MCF7 cells left untreated (unfilled) treated with HGF (light gray), hypoxia (dark gray), or HGF + hypoxia (black). Right, mitochondrial activity in ACHN and UOK112 cells treated with HGF (light gray), CoCl$_2$ (dark gray), or HGF + CoCl$_2$ (black) (*p<0.003). C. Cell cycle analysis of ACHN cells, bars represent mean percentage of cells in each phase +/- SD (n=3) for untreated (unfilled), HGF treated (light gray), CoCl$_2$ treated (dark gray) or HGF + CoCl$_2$ treated (black) sample groups. Inset shows detail of changes in S phase distribution (*p<0.0001 vs untreated, **p=0.0005 HGF vs HGF + CoCl$_2$). D. Mitochondrial activity (mean MTT value fold over control +/- SD) in ACHN (top) and UOK112 (bottom) cells left untreated (unfilled) or treated with HGF (light gray), hypoxia (dark gray), or HGF + hypoxia (black) and similar sample groups treated additionally with U0126 (1 µM) or LY294002 (10 µM) as indicated (*p<0.0001). E. As described for panel D except using A549 (left) and MCF7 (right) cells.

**FIGURE 9.** Hypoxia-induced metabolic suppression in PRC cells is mediated by ROS and PKCα, and requires ER pathway co-activation in MCF7 breast cancer cells. A. Mitochondrial activity in ACHN left untreated (unfilled) or treated with HGF (0.5 nM; light gray), hypoxia (dark gray), or HGF + hypoxia (black) and similar sets treated additionally with BIM (5 nM) or DPI (5 µM) as indicated (*p<0.0001 vs control, **p<0.0001 HGF vs HGF/hypoxia, ***p<0.0001 HGF/hypoxia vs BIM/HGF/hypoxia and DPI/HGF/hypoxia). Bars represent the mean of triplicate samples +/- SD (n=3); some error bars are too small to be visible. B. Mitochondrial activity in MCF7 cells incubated in the absence (top) or presence (bottom) of β-estradiol (10 nM) for 24h followed by an additional 24h under normoxic or hypoxic conditions with or without HGF and additionally with BIM or DPI; bar fill scheme follows that of panel A (*p<0.001 vs control, **p<0.001 hypoxia vs BIM/HGF/hypoxia and DPI/HGF/hypoxia). C. Proliferation of MCF7 cells incubated in the absence (left) or presence (right) of β-estradiol, expressed as mean cell number over time +/- SD (n=3) for control (circles) and groups treated with CoCl$_2$ (triangles), HGF (squares) or HGF + CoCl$_2$ (inverted triangles) on days 0, 3 and 6 (*p<0.01 vs control, **p<0.01 HGF vs HGF + CoCl$_2$).

**FIGURE 10.** Hypoxic Akt suppression induces autophagy. A. A549 or UOK112 lysates prepared from cells incubated in normoxic or hypoxic conditions for 24h in the absence or presence of HGF (0.5 nM) were immunoblotted for SQSTM1, LC3B or GAPDH. B. Extracts prepared from ACHN cells left untreated or treated with CoCl$_2$ (100 µM) or DMOG (500 µM) for 24h followed by 20 min of HGF treatment were immunoblotted for LC3B, or GAPDH. C. Immunofluorescence microscopic localization of LC3 in UOK112 and ACHN cells left untreated or treated with HGF, CoCl$_2$ or HGF + CoCl$_2$ for 24h. D. ACHN cells were infected with GFP or constitutively active (ca) Mek, caAkt, dominant negative (dn) Mek, or dnAkt for 48h, then incubated in normoxic or hypoxic conditions for 24h prior to immunoblot analysis for HIF1α, pAkt, Akt, pErk, or Erk, LC3B or GAPDH.

**FIGURE 11.** Hypoxia induced autophagy critically supports HGF-driven cell invasion. A. Invasion by ACHN cells left untreated (unfilled) or treated with HGF (0.5 nM; light gray), CoCl$_2$ (100 µM; dark gray), or HGF + CoCl$_2$ (black), in the absence or presence of chloroquine (50 µM) for 24h (*p<0.05). B. Mitochondrial activity (mean MTT value relative to control +/-SD, n=3) in ACHN (top) and A549 (bottom) cells on day 1 (left) or day 2 (right) after transfection with plasmids for EGFP, LC3wt-GFP, or mutLC3-GFP expression (*p<0.05). C. Mean ACHN (top) or A549 (bottom) cell invasion as measured...
using matrigel-coated Fluoroblok Boyden chambers (+/- SD, n=3) by normoxic untreated cells (unfilled bar at left in each group, too low to be visible), HGF treated (light gray), hypoxic (dark gray) or HGF treated hypoxic cells (black) that had been transfected with EGFP, LC3wt-GFP, or mutLC3-GFP. Invading cells were visualized by fluorescence microscopy (*p<0.005 vs untreated normoxic cells, **p<0.0002, ***p<0.01).

**FIGURE 12.** Invasive synergy and suppression of proliferation driven by EGF and hypoxia. A. Cells incubated under normoxic (-) or hypoxic (+) conditions for 24h followed by 20 min exposure to EGF (50 ng/mL) were extracted with non-ionic detergent and immunoblotted for HIF1α, pEGFR, EGFR, pPKCα, PKCα, pAkt, Akt, pErk, or Erk as indicated. B. Cell growth (mean cell number over time +/- SD, n=3) for control (circles) and groups treated with CoCl₂ (triangles), EGF (squares) or EGF + CoCl₂ (inverted triangles) as measured on days 0, 3 and 6 (*p<0.05). C. Invasion by ACHN cells incubated in normoxia (unfilled, left, too low to be visible), EGF (light gray), hypoxia (dark gray) or EGF with hypoxia (black) for 24h; bars represent mean +/- SD, n=3, *p<0.01 vs control.

**FIGURE 13.** Schematic depiction of synergistic signaling of tumor cell invasiveness by HGF and hypoxia. Left, normoxic HGF induction of cell invasion and proliferation mediated predominantly by the Erk and Akt pathways, respectively. Right, integration of HGF and hypoxia signaling pathways leading to synergistic invasion, induction of autophagy and suppression of proliferation. Red molecules are active, red arrows/arrowheads indicate activated pathways; red rotated “T” indicates active inhibition; green molecules indicate decreased expression or activity; gray arrows or rotated “T” indicate silenced pathways.
Figure 2

A

HIF1α
pAkt
Akt
pErk
Erk
hypoxxia
ACHN A549 UOK MCF7

B

HIF1α
pAkt
Akt
pErk
Erk
hr 0 2 4 8 16 24
CoCl2 DMOG

C

ACHN
pAkt
Akt
pErk
Erk

A549
pAkt
Akt
pErk
Erk

UOK112
pAkt
Akt
pErk
Erk

HGF
CoCl2 DMOG

densitometry (fold change)

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Figure 3

A

![Bar chart showing migration (SI x 10^4) for ACHN cells under different conditions: control, LY294002, and U0126.](image)

B

![Bar chart showing migration (SI x 10^4) for UOK112 cells under different conditions: control, LY294002, and U0126.](image)

C

![Bar chart showing invasion (SI x 10^-1) for different conditions: GFP, caMEK, and dnMEK.](image)
Figure 4

A

ROS level (fold vs control)

hr 4 8 16 CoCl₂ DMOG H₂O₂

control

NP

NP

B

ROS level (fold vs control)

control NP NP

0 10 20

C

invasion area (SI x 10⁻⁵)

control NP

0 0.4 0.8 4 5

D

pAkt tAkt pErk tErk

HGF hypoxia NP DPI

E

pErk tErk

HGF CoCl₂ DMOG H₂O₂

control NP

F

pErk/tErk (fold change)

untreated NP

0 2 4 6 **
Figure 5

(A) mRNA expression (fold change vs control)

DUSP1  DUSP2

ACHN

A549

(B) mRNA expression (fold change vs control)

DUSP1  DUSP2

UOK112

MCF7

(C) DUSP2 mRNA (fold change vs control)

ACHN  A549

(D) Western blot analysis

ACHN  A549

DUSP2  pErk  GAPDH  Hypoxia  DPI
Figure 6

A

|       | ACHN     | A549     | MCF7     |
|-------|----------|----------|----------|
| pPKCα | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| tPKCα | ![Image](image4) | ![Image](image5) | ![Image](image6) |

B

|       | ACHN     | A549     | MCF7     |
|-------|----------|----------|----------|
|       | ![Image](image7) | ![Image](image8) | ![Image](image9) |

C

|       | ACHN     | A549     | MCF7     |
|-------|----------|----------|----------|
| pPKCα | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| tPKCα | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| pAkt  | ![Image](image16) | ![Image](image17) | ![Image](image18) |
| tAkt  | ![Image](image19) | ![Image](image20) | ![Image](image21) |
| pErk  | ![Image](image22) | ![Image](image23) | ![Image](image24) |
| tErk  | ![Image](image25) | ![Image](image26) | ![Image](image27) |

Densitometry (fold change)

|       | ACHN     | A549     | MCF7     |
|-------|----------|----------|----------|
|       | ![Image](image28) | ![Image](image29) | ![Image](image30) |
Figure 7

A

PP2A activity (fold change)

ACHN

MCF7

MCF7 + β-estradiol

Control BIM DPI

B

ACHN

A549

Control BIM DPI

C

pAKT tAKT

HGF β-estradiol

D

pAkt tAkt

HGF OkA

ACHN A549
Figure 8

A. Cell number (x 10^5) over days for ACHN, UOK112, A549, and MCF7.

B. MTT (fold vs control) for ACHN, UOK112, A549, and MCF7.

C. Percentage of cells in cycle phase for ACHN, UOK112, A549, and MCF7.

D. MTT (fold vs control) for ACHN, UOK112, A549, and MCF7.

E. MTT (fold vs control) for A549 and MCF7.
Figure 9

A

B

C

MCF7

MCF7 + β-estradiol

MTT (fold vs control)

MTT (fold vs control)

cell number (x 10^5)

days

control BIM DPI

control BIM DPI

control BIM DPI

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Figure 10

A

SQSTM1
LC3 IB
LC3 IIB
GAPDH
HGF
hypoxia
A549
UOK112

B

LC3 IB
LC3 IIB
GAPDH
HGF
hypoxia
N
CoCl₂
DMOG

C

untreated
HGF
CoCl₂
HGF+CoCl₂

UOK112

ACHN

D

HIF1α
pAkt
tAkt
pErk
tErk
LC3 IB
LC3 IIB
GAPDH
hypoxia
GFP
caMek
dnMek
caAkt
dnAkt
Figure 12

A

| HIF1α | ACHN | A549 |
|-------|------|------|
| pEGFR |      |      |
| EGFR  |      |      |
| pPKCα |      |      |
| tPKCα |      |      |
| pAkt  |      |      |
| tAkt  |      |      |
| pErk  |      |      |
| tErk  |      |      |

EGF
hypoxia

- -
- +
- +
- +
- +
- +
- +
- +

B

| cell number (x 10^6) |
|----------------------|
| days 0 3 6 |

ACHN

- * |
A549

- * |

C

| invasion area (SI x 10^-5) |
|-----------------------------|
| EGF hypoxia |

ACHN

- * |

A549

- * |
Figure 13

- External stimuli
- Signaling events
- Biological effects

**Normoxia**
- HGF
- Erk
- DUSP2
- Akt
- PP2A
- Metabolic shift
- Invasion
- Proliferation

**Hypoxia**
- Estrogen
- ROS
- PLCγ
- DAG
- PKC
- ER
- Autophagy
- Metabolic shift

**Cell cycle**
- Akt
- Invasion
- Proliferation
Synergistic Signaling of Tumor Cell Invasiveness by Hepatocyte Growth Factor and Hypoxia
Young H. Lee, Bethanie L. Morrison and Donald P. Bottaro

J. Biol. Chem. published online June 9, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.580597

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