AMPK Reverses the Mesenchymal Phenotype of Cancer Cells by Targeting the Akt–MDM2–Foxo3a Signaling Axis

Chih-Chien Chou1, Kuen-Haur Lee1,2, I-Lu Lai1, Dasheng Wang1, Xiaokui Mo3, Samuel K. Kulp1, Charles L. Shapiro4, and Ching-Shih Chen1,5

Abstract

In cancer cells, the epithelial–mesenchymal transition (EMT) confers the ability to invade basement membranes and metastasize to distant sites, establishing it as an appealing target for therapeutic intervention. Here, we report a novel function of the master metabolic kinase AMPK in suppressing EMT by modulating the Akt–MDM2–Foxo3 signaling axis. This mechanistic link was supported by the effects of siRNA-mediated knockdown and pharmacologic activation of AMPK on epithelial and mesenchymal markers in established breast and prostate cancer cells. Exposure of cells to OSU-53, a novel allosteric AMPK activator, as well as metformin and AICAR, was sufficient to reverse their mesenchymal phenotype. These effects were abrogated by AMPK silencing. Phenotypic changes were mediated by Foxo3a activation, insofar as silencing or overexpressing Foxo3a mimicked the effects of AMPK silencing or OSU-53 treatment on EMT, respectively. Mechanistically, Foxo3a activation led to the transactivation of the E-cadherin gene and repression of genes encoding EMT-inducing transcription factors. OSU-53 activated Foxo3a through two Akt-dependent pathways, one at the level of nuclear localization by blocking Akt- and IKKβ-mediated phosphorylation, and a second at the level of protein stabilization via cytoplasmic sequestration of MDM2, an E3 ligase responsible for Foxo3a degradation. The suppressive effects of OSU-53 on EMT had therapeutic implications illustrated by its ability to block invasive phenotypes in vitro and metastatic properties in vivo. Overall, our work illuminates a mechanism of EMT regulation in cancer cells mediated by AMPK, along with preclinical evidence supporting a tractable therapeutic strategy to reverse mesenchymal phenotypes associated with invasion and metastasis. Cancer Res; 74(17): 1–13. © 2014 AACR.

Introduction

Epithelial–mesenchymal transition (EMT) is a critical process in malignant progression that confers to epithelial cancer cells the ability to breach basement membranes and metastasize to distant sites (1–3). Recent studies have also implicated EMT in the development of chemoresistance (4, 5) and acquisition of cancer stem cell–like properties (6, 7). In light of such findings, EMT regulators have been proposed as predictive markers of metastatic propensity and response to cancer treatment. At the molecular level, EMT is characterized by loss of the epithelial cell adhesion molecule E-cadherin, which enables cells to increase motility and invasiveness through the disruption of intercellular contacts (8). This loss of E-cadherin is accompanied by concomitant increases in the expression of mesenchymal-associated genes, including those encoding vimentin, N-cadherin, fibronectin, integrins, and α-smooth muscle actin, which bestow a motile phenotype on cancer cells through changes in cellular architecture and cell–matrix interactions (9, 10). From a mechanistic perspective, many signaling pathways, such as those mediated by TGFβ (11), Ras (12), IGFIR (13), and GSK3β (14), have been linked to EMT induction under different cellular contexts through increased expression of Snail and/or other transcriptional repressors of E-cadherin, including Zeb-1, Twist, and Slug. More recently, the oncogenic transcription/translation factor Y-box–binding protein-1 (YB-1; ref. 15) and the tumor suppressor Foxo3a (16, 17) have also been identified as players in the regulation of EMT. Although YB-1 promotes EMT by activating translation of mRNA encoding Snail and other EMT-inducing transcription factors in noninvasive breast epithelial cells (15), Foxo3a was shown to suppress cell motility by regulating YB-1 and E-cadherin expression through negative Twist regulation in urothelial cancer cells (17).
In the past decade, the role of adenosine monophosphate–activated protein kinase (AMPK) as a metabolic tumor suppressor has received much attention, in part, due to the reported effect of metformin, a pharmacologic activator of AMPK, in reducing risk and/or mortality in certain types of cancers, especially those of the breast (18), pancreas (19), and prostate (20), among patients with type II diabetes (18, 21–23). These chemopreventive effects are consistent with the ability of metformin to suppress tumorigenesis and xenograft tumor growth in various animal models of cancer (reviewed in ref. 24). Together, these findings have led to multiple clinical trials of metformin in the treatment of different cancer types (www.clinicaltrials.gov).

From a mechanistic perspective, AMPK inhibits tumorigenesis by targeting tumor metabolism and mTOR-associated oncogenic signaling pathways (25–29). However, it warrants attention that the role of AMPK as a metabolic sensor might vary in a cell type–or context-specific manner as emerging evidence suggests that tumor cells might also use AMPK activation as a survival strategy to undergo metabolic adaptation in the face of environmental stresses (30, 31), such as hypoxia (21), acidosis (22), and nutrient deprivation (21, 23). These dichotomous effects underscore differences in signaling networks downstream of AMPK in response to different stress signals. Nonetheless, metabolic stress, such as serum starvation, can also activate AMPK-independent signaling pathways, including those mediated by Akt and ERKs, in aggressive cancer cell lines as part of the metabolic adaptation to hostile environments (32).

Conventional AMPK activators, such as metformin and 2-deoxyglucose, facilitate AMPK activation by generating an intracellular energy deficit, as manifested by increased AMP:ATP ratios (24). This energy deficit also represents a form of metabolic stress, which might induce AMPK-independent cellular responses. To circumvent this problem, we developed an allelosteric AMPK activator, OSU-53 (structure, Fig. 1D), via the pharmacologic exploitation of the off-target effect of the peroxisome proliferator-activated receptor (PPAR)γ agonist ciglitazone on AMPK activation (33, 34). OSU-53 directly activates the kinase activity of recombinant AMPK α1β1γ2 (IC50, 0.3 μmol/L vis-à-vis 8 μmol/L for AMP) by binding to the autoinhibitory domain (33, 34), and this mode of action is supported by the ability of OSU-53 to activate AMPK in MDA-MB-231 cells that are deficient in its upstream regulator liver kinase B1 (LKB1; ref. 34). By using OSU-53 as a pharmacologic probe, we report a novel functional activity of OSU-53 in inhibiting EMT by targeting the Akt–MDM2 signaling. This unique mode of activation contrasts with a previous report that AMPK increased the transcriptional activity of Foxo3a through direct phosphorylation without affecting its subcellular localization in nutrient-deprived HEK 293T cells (35).

Pursuant to our previous finding that AMPK inhibits cancer cell proliferation by perturbing both metabolic and oncogenic signaling pathways (33, 34), the present study identifies a novel mechanism by which AMPK activation suppresses the metastatic potential of cancer cells, which might foster new therapeutic strategies for metastatic cancer.

Materials and Methods

Cell lines and antibodies

Cancer cell lines were purchased from the American Type Culture Collection, and nonmalignant human epithelial cells of the mammary gland (MEC) and prostate (PrEC) were obtained from Lonza Biologics, Inc. All cells were used in fewer than 6 months of continuous passage. Media used for the maintenance of these cells are as follows: MCF-7, DMEM/F-12 (Life Technologies), MDA-MB-468 and MDA-MB-231, DMEM (Life Technologies), MECs, mammary epithelial growth medium (Lonza Biologics), 4T1, RPMI-1640 (Life Technologies); LNCaP and PC-3, RPMI-1640 (Life Technologies), DU-145, MEM (Life Technologies), PrECs, prostate epithelial growth medium (Lonza Biologics), all of which were supplemented with 10% FBS. Cells were incubated at 37°C in a humidified incubator containing 5% CO2. Antibodies against various proteins were obtained from the following sources: mouse monoclonal antibodies: Snail, and ubiquitin, Cell Signaling Technology; E-cadherin, BD Biosciences; GFP, Santa Cruz Biotechnology; β-TrCP, Invitrogen; β-actin, MP Biomedicals. Rabbit antibodies: Thr(P)742–AMPK, AMPK, Ser(P)253–Foxo3α, Foxo3α, vimentin, YB-1, Ser(P)2448–mTOR, mTOR, Thr(P)389/p70S6K, p70S6K, Ser(P)173–Akt, Thr(P)170–Akt, Akt, Ser(P)202/204/183/181–IKKβ, Cell Signaling Technology; Twist, Santa Cruz Biotechnology. Alexa Fluor dye–conjugated phalloidin (Alexa Fluor 488) was purchased from Invitrogen. Goat antibody against Histone H3 was from Santa Cruz Biotechnology.

Cell viability assays

Cell viability was assayed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in six replicates. Cells were seeded and incubated in 96-well plates in the respective medium with 10% FBS for 24 hours, and then exposed to various concentrations of test agents dissolved in DMSO in 5% FBS-supplemented medium. The medium was removed and replaced by 200 μL of 0.5 mg/mL MTT in 10% FBS-containing medium, and cells were incubated at 37°C for 2 hours. Supernatants were removed, and the MTT dye was solubilized in 120 μL/well of DMSO. Absorbance at 570 nm was determined on a plate reader.

Transient transfection

Cells were transfected using an Amaxa Nucleofection system (Amaxa Biosystems) according to the manufacturer's
instructions. The plasmids encoding constitutively active (CA)-Akt, pcDNA-MDM2, and HA-ubiquitin were obtained from Addgene, and Foxo3a shRNA from Origene. Foxo3a-GFP plasmid was kindly provided by Dr. Mickey C.T. Hu (Stanford University School of Medicine, Stanford, CA). Treatments were initiated 24 hours after completion of 48-hour transfection. Expression of various plasmids was confirmed by immunoblotting analysis.

**RT-PCR analysis**

Total RNA was isolated using TRIzol (Invitrogen) and then reverse-transcribed to cDNA using the Omniscript RT Kit (Qiagen). The PCR products were resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. The sequences of the PCR primers used are listed in Supplementary Table S1. The cycle numbers for the RT-PCR of each target gene were as follows for the human and mouse cell lines, respectively: Foxo3a, 31 and 35; E-cadherin, 32 and 26; vimentin, 28 and 28; YB-1, 30 and 30; Snail, 32 and 35; claudin-1, 30 and 38; β-actin, 26 and 26.

**In vitro Foxo3a ubiquitination analysis**

Cells were transfected with the expression vector for HA-tagged ubiquitin (HA-Ub) in combination with either the plasmid for MDM2 or the empty vector (pcDNA3). Cells then were cultured in 6-well plates for 24 hours and then treated with 5 μmol/L OSU-53 for 12 hours, followed by cotreatment with the proteasome inhibitor MG132 (10 μmol/L) for an additional 12 hours. Cells were harvested into M-PER buffer containing 1% protease inhibitor cocktail and centrifuged at 13,000 × g for 20 minutes. The supernatants were collected, and one-tenth volume of each supernatant was stored at 4°C for use as the input sample for immunoblotting. The remainder was incubated with anti-Foxo3a antibody overnight at 4°C and incubated with protein A/G-agarose for 30 minutes. The immunoprecipitates were centrifuged, collected, washed,
suspended in 2× SDS sample buffer, and subjected to Western blot analysis with antibodies against HA-tag, ubiquitin, or Foxo3a.

**Immunoblotting**

Growing cells were harvested by scraping and lysed in the presence of SDS lysis buffer/protease inhibitor cocktail. An equal amount of protein from each sample was loaded per lane, separated by SDS–PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane, and then probed with specific antibodies. Secondary antibodies conjugated to horseradish peroxidase and Western Lighting Chemiluminescence Reagent Plus (PerkinElmer) were used to develop images.

**Chromatin immunoprecipitation assay**

After crosslinking with 1% formaldehyde for 10 minutes at room temperature, cells were washed with ice-cold PBS three times and whole-cell extracts were prepared with lysis buffer. Cellular DNA fragments of approximately 500 bp were generated by sonication. For immunoprecipitation, cell lysates were incubated with 2 μg of anti-Foxo3a antibody at 4°C for 16 hours with rotation. After incubation with 30 μL of protein A/G agarose beads at 4°C for additional 2 hours, beads were washed three times with high-salt buffer (20 mmol/L Tris–HCl, 500 mmol/L NaCl, 2 mmol/L EDTA, and 0.5% NP-40), followed by another three washes with low-salt buffer (10 mmol/L Tris–HCl, 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Nonidet P-40, and 0.01% SDS). Proteins were eluted from the beads with 500 μL of Tris–EDTA buffer containing 1% SDS, and crosslinking was reversed by exposure to 65°C for 16 hours. After digestion by 0.5 mg/mL proteinase K at 50°C for 2 hours, DNA was extracted by phenol/chloroform and precipitated by absolute alcohol. The purified DNA was analyzed by PCR using primers for five Foxo3-binding elements (FBE) in the E-cadherin promoter. Primer sequences are listed in Supplementary Table S1.

**Immunofluorescent imaging of F-actin cytoskeletal structure**

Immunofluorescent imaging was performed according to a reported procedure (34). In brief, treated cells were washed with cold PBS, fixed with 4% formaldehyde for 10 minutes at 37°C, permeabilized with 0.5% Triton X-100 for 5 minutes at room temperature, and then blocked with 3% BSA overnight at 4°C. After washing with PBS, the cells were incubated with Alexa Fluor 488–conjugated phalloidin in the presence of 1% BSA for 1 hour at room temperature (for F-actin). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) contained in the VECTASHIELD mounting medium (Vector Laboratories). Confocal images were obtained with an Olympus FV1000 confocal microscope (Olympus Corp.) using the 40× oil immersion lens.

**In vitro migration and invasion assays**

Assays were performed using Falcon Cell Culture Inserts (8-mum pore size) in a 24-well format (BD Biosciences) according to the vendor’s instructions. In the migration assay, cells (10⁴ cells/well) in 0.5 mL of serum-free medium containing OSU-53 at the indicated concentration were seeded onto the membranes of the upper chambers, which had been inserted into the wells of 24-well plates containing 10% FBS-supplemented medium. After 18 hours, the cells were fixed with 100% methanol and stained with 5% Giemsa (Merck). Unmigrated cells remaining in the upper chambers were removed by wiping with a damp cotton swab leaving those that had migrated to the underside of the membranes. The membranes were mounted on glass slides, and the numbers of cells in three randomly chosen high-power fields were counted. For the invasion assay, cells (10⁵ cells/well) in 0.5 mL of serum-free medium containing OSU-53 at the indicated concentration were seeded onto Matrigel-coated membranes of the upper chambers. The lower chambers contained the same amount of OSU-53 in 10% FBS-supplemented medium. After 24 hours, noninvasive cells remaining on the upper surface of the membranes were removed with a cotton swab. Cells on the lower surface of the membrane were fixed in 100% methanol and stained with 0.1% crystal violet for 10 minutes. The membranes were mounted on glass slides, and the numbers of cells in three randomly chosen high-power fields were counted. All experiments were performed three times.

**In vivo metastasis study**

Orthotopic xenograft tumors were established in female BALB/c mice (BALB/cAnNCr, 5–7 weeks of age; NCI, Frederick, MD) by injecting 4T1 cells (2.5 × 10⁶ cells/mouse) into the right inguinal mammary fat pad in a total volume of 0.1 mL of PBS. Mice were randomized to three groups (n = 6), which received the following treatments 24 hours after implantation: (i) OSU-53 at 50 mg/kg; (ii) OSU-53 at 100 mg/kg; and (iii) vehicle (0.5% methylcellulose/0.1% Tween 80 in water). Treatments were administered once daily by oral gavage. Primary tumor volumes were calculated from weekly caliper measurements (volume = width² × length × 0.52). Body weights were measured weekly. At terminal sacrifice, tumors were harvested, snap-frozen in liquid nitrogen, and stored at −80°C until used for biomarker assessment by Western blotting. Lungs were collected and...
fixed in 10% neutral buffered formalin for assessment of metastatic burden. Using a dissection microscope, the numbers of tumors visible on the surfaces of all lung lobes from each mouse were counted and their diameters were measured. All experimental procedures using live animals were conducted in accordance with protocols approved by The Ohio State University Institutional Animal Care and Use Committee.

Statistical analysis

Differences among group means in the viability, migration, and invasion assays for each cell line and in the in vivo experiment were analyzed using one-way ANOVA followed by the Dunnett multiple comparisons test. The analyses were performed using SPSS software (SPSS Inc.). Differences in the rates of concentration-dependent changes in viability, migration, and invasion in the MDA-MB-231 cell line were analyzed by trend analysis using SAS 9.3 software (SAS, Inc.). Differences were considered significant at \( P < 0.05 \).

Results

Evidence that AMPK regulates EMT in breast and prostate cancer cells

Consistent with the premise that the role of AMPK as a metabolic sensor varies in a cell type/context-specific manner, we observed a differential cellular response among a series of breast and prostate cancer cell lines to metabolic stress in eliciting AMPK activation. Although cell lines with a nonaggressive, epithelial phenotype (MCF-7 and LNCaP) were highly sensitive to serum deprivation–induced AMPK activation, as manifested by phosphorylation/expression levels of AMPK and its downstream target acetyl-CoA carboxylase (ACC; Fig. 1A), cell lines with mesenchymal characteristics were resistant to this metabolic stress as no significant change (MDA-MB-468, MDA-MB-231, and 4T1) or even reduction (DU-145, 8 \( \mu \)mol/L; PC-3, 5 \( \mu \)mol/L; Fig. 1D). In contrast, MECs and PrECs were resistant to OSU-53, indicating a discriminative antiproliferative effect between malignant and nonmalignant cells. In addition, OSU-53 dose-dependently increased the phosphorylation of AMPK and ACC, accompanied by parallel changes in the expression of various EMT effectors, including Foxo3a, E-cadherin, claudin-1, vimentin, YB-1, and Snail, in four cell lines displaying mesenchymal traits, MDA-MB-231, 4T1, PC-3, and MDA-MB-468, in a manner consistent with the reversal from a mesenchymal to an epithelial phenotype (Fig. 2A). As MDA-MB-231 cells lack endogenous E-cadherin expression due to promoter hypermethylation (37), decreased Snail, a transcriptional repressor of E-cadherin, was not accompanied by E-cadherin upregulation. Therefore, we used claudin-1 as an additional epithelial marker to demonstrate the gain of epithelial character in response to OSU-53. It is noteworthy that OSU-53 had no appreciable effect on any of the aforementioned biomarkers in MECs and PrECs, reflective of the lack of response of these normal epithelial cells to the drug’s antiproliferative activity. This drug effect on EMT was also noted with AICAR and metformin in 4T1 and PC-3 cells (Fig. 2B), indicating that this was not an OSU-53–specific response. RT-PCR analysis indicates that, with the exception of Foxo3a, these changes in the expression of EMT effectors were mediated at both mRNA and protein levels (Fig. 2C). Moreover, siRNA-mediated knockdown of AMPK diminished the effects of OSU-53 on the expression of these EMT effectors in both cell lines, supporting the central role of AMPK in mediating this phenotypic change (Fig. 2D). On the basis of these findings, we hypothesized that the effect of OSU-53 on EMT was associated with its ability to increase Foxo3a expression at the posttranslational level, which, in turn, modulated the gene transcription of various EMT-associated effectors.

Foxy3a as a master regulator of OSU-53–mediated suppression of EMT

To interrogate the role of Foxo3a as a downstream effector of AMPK in regulating EMT, we investigated the effect of shRNA-mediated knockdown of Foxo3a on the expression of EMT markers in MCF-7 and LNCaP cells. Foxo3a knockdown promoted EMT in these two epithelial cell lines, which was evidenced by reduced E-cadherin expression in conjunction with concomitant increases in the expression of the mesenchymal markers YB-1 and Snail (Fig. 3A). Conversely, ectopic expression of Foxo3a mimicked the ability of OSU-53 to reverse the mesenchymal characters of MDA-MB-231, 4T1, and PC-3 cells (Fig. 3B). Moreover, RT-PCR analysis indicated that, reminiscent of the effect of OSU-53 (Fig. 2C), Foxo3a-induced changes in the expression of these EMT effectors were mediated at the mRNA level (Fig. 3B).
Pursuant to these findings, we used the shRNA-mediated knockdown of Foxo3a to verify its role in regulating the OSU-53–mediated suppression of EMT in MDA-MB-231 and PC-3 cells. Reminiscent of its effects in MCF-7 and LNCaP cells, silencing of Foxo3a accentuated the mesenchymal character of these cells, as manifested by changes in pertinent EMT-associated markers (Fig. 3C). Although OSU-53 (5 μmol/L) was able to induce AMPK activation in these Foxo3a-silenced MDA-MB-231 and PC-3 cells, its ability to alter the EMT status of these cells was lost as no substantial effect on various EMT markers was observed (Fig. 3C).

Previously, Foxo3a was reported to regulate E-cadherin expression through the negative regulation of Twist in urothelial cancer cells (17). In this study, we obtained evidence that Foxo3a directly activated E-cadherin transcription in OSU-53–treated MDA-MB-468, PC-3, and MDA-MB-231 cells. Analysis of the E-cadherin gene promoter identified five potential Foxo-binding elements (AAACA) in different regions of the promoter (FBE1–5; Fig. 3D, top). Chromatin immunoprecipitation (ChIP) analysis demonstrated that OSU-53 at 5 μmol/L promoted the selective binding of Foxo3a to FBE3 and FBE4 in all three cell lines (bottom). Together with the ability of ectopic Foxo3a to increase E-cadherin gene expression, the data suggest that Foxo3a directly binds to the E-cadherin promoter to activate its transcription following OSU-53 treatment.

OSU-53 stimulates Foxo3a nuclear localization and protein stabilization by suppressing Akt signaling

In light of the ability of OSU-53 to increase Foxo3a expression at the posttranscriptional level (Fig. 2A and C), we investigated the drug effect on its cellular distribution by

![Figure 2. OSU-53 facilitates the reversal of the mesenchymal phenotype of MDA-MB-231, 4T1, PC-3, and MDA-MB-468 cells via an AMPK-dependent mechanism. A and B, Western blot analysis of the concentration-dependent effects of the AMPK activators OSU-53 (A) and AICAR and metformin (B) on the phosphorylation/expression of AMPK and its downstream target ACC, Foxo3a, and various EMT effectors in the indicated cell lines, including MDA-MB-231, 4T1, PC-3, and MDA-MB-468 cells versus MECs and PrECs. C, RT-PCR analysis of the concentration-dependent effects of OSU-53 on the mRNA levels of Foxo3a and other EMT effectors in 4T1 and PC-3 cells. D, Western blot analysis of the effects of siRNA-mediated knockdown of AMPK on OSU-53–induced changes in the phosphorylation/expression of AMPK, Foxo3a, and various EMT effectors in 4T1 and PC-3 cells. Immunoblots and images of PCR products are representative of three independent experiments. All drug treatments were for 48 hours.](https://doi.org/10.1158/0008-5472.CAN-14-0135)
immunocytochemistry. As shown, exposure to OSU-53 (5 μmol/L) led to complete nuclear translocation in all three cell lines examined (MDA-MB-231, 4T1, and PC-3 cells; Fig. 4A). Together, these findings suggest that OSU-53 induces the activation of Foxo3a through increased protein stability and nuclear localization. We rationalized that these drug effects might be associated with the previously reported activity of OSU-53 in facilitating protein phosphatase (PP) 2A–mediated Akt inactivation (34), which is supported by the following findings.

First, Akt promotes nuclear exclusion of Foxo3a through direct phosphorylation at three consensus sites (32Thr, 253Ser, and 315Ser; ref. 38), or via phosphorylation by its downstream effector IKKβ (39, 40). Accordingly, our data show the concurrent downregulation of phosphorylated Akt and IKKβ, which we conclude gave rise to reduced 253Ser-Foxo3a phosphorylation (exemplified in PC-3 cells; Fig. 4B), leading to the cytoplasmic-to-nuclear translocation of Foxo3a in OSU-53–treated cells (in all three cell lines; Fig. 4C).

Second, we hypothesized that decreased 166Ser-MDM2 phosphorylation consequent to Akt inactivation might underlie the ability of OSU-53 to upregulate Foxo3a, as MDM2 is the E3 ligase responsible for Foxo3a ubiquitination and degradation (41, 42), and its nuclear entry is regulated by Akt-mediated phosphorylation (43). As shown, OSU-53 treatment led to a dose-dependent decrease in Foxo3a and MDM2 phosphorylation (Fig. 4B) and the parallel exit of MDM2 from the nucleus into the cytoplasm in MDA-MB-231, 4T1, and PC-3 cells (Fig. 4C). This drug-induced trafficking of Foxo3a and MDM2 into different cellular compartments was verified by coimmunoprecipitation, which revealed reduced physical interactions between Foxo3a and MDM2 in the

Figure 3. Foxo3a plays a role in regulating EMT in breast and prostate cancer cells. A, shRNA-mediated knockdown of Foxo3a facilitated EMT in the epithelial-type MCF-7 and LNCaP cells, as revealed by loss of the epithelial marker E-cadherin and gain of the mesenchymal markers vimentin, YB-1, and Snail. B, Western blot and RT-PCR analyses of the effect of the ectopic expression of Foxo3a on the protein and mRNA expression, respectively, of various EMT markers. Immunoblots and images of PCR products are representative of three independent experiments. C, effect of shRNA-mediated knockdown of Foxo3a on OSU-53–mediated AMPK activation and reversal of mesenchymal character in MDA-MB-231 and PC-3 cells. D, top, depiction of five putative Foxo3a-binding elements (FBE1–5) in the promoter region of the E-cadherin (CDH1) gene. The filled bars indicate the location of exons. Bottom, evidence that Foxo3a directly activates CDH1 gene expression in response to OSU-53. ChIP analysis of the effects of OSU-53 on Foxo3a binding to FBE1–5 of the CDH1 promoter in MDA-MB-468, PC-3, and MDA-MB-231 cells. The sizes of PCR amplicons for each of the five putative FBEs is shown on the left. Cells were treated with OSU-53 for 48 hours.
nuclear extracts of all three cell lines examined after exposure to OSU-53 (Fig. 5A).

Furthermore, the role of MDM2 in OSU-53–induced Foxo3a accumulation was demonstrated by the following experiments. Ectopic expression of MDM2 abrogated the effect of OSU-53 on Foxo3a expression (Fig. 5B). Furthermore, coimmunoprecipitation analysis showed that the ability of ectopic MDM2 to abolish OSU-53–mediated Foxo3a upregulation was attributable to increased Foxo3a ubiquitination relative to the pcDNA control (Fig. 5C). Equally important, ectopic expression of a constitutively active form of Akt, AktT308D/S473D, manifested by increased 253Ser-Foxo3a phosphorylation, abrogated the OSU-53–induced increase in Foxo3a expression (Fig. 5D). Together, these findings confirmed that OSU-53 facilitated Foxo3a activation by targeting the Akt–MDM2 axis (Fig. 6A).

OSU-53 inhibits invasive phenotype

The in vitro efficacy of OSU-53 in suppressing cancer cell invasiveness was illustrated by its dose-dependent inhibition of the migration and invasion of MDA-MB-231 and PC-3 cells after 24 hours of treatment in Boyden chamber assays.

Figure 4. OSU-53 facilitates Foxo3a accumulation by trafficking Foxo3a and MDM2 into different cellular compartments. A, immunocytochemical analysis of the effect of OSU-53 on the nuclear localization of ectopically expressed GFP-tagged Foxo3a in MDA-MB-231, 4T1, and PC-3 cells after 24 hours of treatment. B, Western blot analysis of the concentration-dependent effects of OSU-53 on the phosphorylation and expression levels of Akt, IKKβ, Foxo3a, and MDM2 in PC-3 cells after 48 hours of treatment. C, Western blot analysis of the concentration-dependent effects of OSU-53 on the phosphorylation and/or expression levels of Foxo3a and MDM2 in the cytoplasmic versus nuclear fractions of MDA-MB-231, 4T1, and PC-3 cells. Histone H3 and β-actin were used as internal markers for the nucleus and cytoplasm, respectively. Immunoblots and immunocytochemistry images are representative of three independent experiments. Cells were treated with OSU-53 for 48 hours.
Although exposure of either cell line to OSU-53 within the same dose range caused a dose-dependent reduction in cell viability within 24 hours of treatment (all $P$ values $< 0.005$; left), this drug-mediated inhibition of cell motility and invasion was not attributable to cell death as the rates of the concentration-dependent decreases in invasion and migration were significantly greater than that in viability (all $P$ values $< 0.001$; right).

In addition, the effect of OSU-53 on the metastatic potential of these two cell lines was interrogated by the three-dimensional colony formation assay, which is frequently used to assess cancer cells’ metastatic capacity (44). As shown, OSU-53 dose-dependently inhibited the ability of MDA-MB-231 and PC-3 cells to form invasive colonies, as indicated by reduced colony size and loss of stellate morphology (Fig. 6C). In addition, staining of MDA-MB-231 cells with FITC-conjugated phalloidin revealed significant decreases in F-actin stress fibers in response to OSU-53 treatment (Fig. 6D). Together, these findings suggest the ability of OSU-53 to inhibit the metastatic phenotype of mesenchymal-type cancer cells.

**In vivo suppressive effect of OSU-53 on tumor metastasis**

Pursuant to the above *in vitro* findings, we assessed the efficacy of OSU-53 in blocking tumor metastasis *in vivo* in the 4T1 syngeneic tumor model. 4T1 cells ($2.5 \times 10^4$) were injected into the mammary fat pads of female Balb/c mice, and OSU-53, at 50 or 100 mg/kg per day, was given by oral gavage once daily, beginning at 24 hours after tumor cell implantation for 3 weeks. OSU-53 was well tolerated by tumor-bearing mice as no change in body weight was noted in either drug-treated group throughout the course of the treatment (Fig. 7A, inset). However, in contrast with the tumor-suppressive effect of OSU-53 noted in the MDA-MB-231 xenograft tumor model (34), 4T1 tumors were resistant to OSU-53 as tumor growth was only modestly inhibited relative to the control ($P > 0.05$; Fig. 7A), indicative of the aggressive nature of these murine breast cancer cells. Nevertheless, Western blot analysis of tumor lysates indicated that OSU-53 dose-dependently increased AMPK and ACC phosphorylation (Fig. 7B). Reminiscent of our *in vitro* findings, this AMPK activation was associated with reduced Akt phosphorylation (all $P$ values $< 0.001$; Fig. 6B, center two panels).
phosphorylation and increased Foxo3a expression, accompanied by the gain of the epithelial marker E-cadherin and loss of mesenchymal markers vimentin, YB-1, Snail, and Twist (Fig. 7B). Equally important, this phenotypic switch in OSU-53–treated mice was associated with reductions in the number and size of metastatic nodules on the surface of the lung as compared with the controls (Fig. 7C).

Discussion

Beyond its role in regulating energy homeostasis (45), AMPK is increasingly recognized as a metabolic tumor suppressor in light of its ability to suppress lipogenesis and mTOR signaling (27, 28). Our data suggest that AMPK is differentially regulated in cancer cell lines expressing an epithelial versus mesenchymal phenotype. Relative to epithelial-type cancer cell lines, such as MCF-7 and LNCaP, cancer cell lines with mesenchymal characteristics were resistant to AMPK activation in response to serum deprivation (Fig. 1A). In light of the role of AMPK in regulating EMT, this finding raises a possibility that these aggressive cancer cell lines might relinquish metabolic stress-induced AMPK activation as a strategy to maintain their mesenchymal characteristics under nutrient-deprived conditions. For example, MDA-MB-231 cells lack the expression of LKB1 (46), thereby incapacitating the function of the LKB1–AMPK pathway as a cellular energy-sensing checkpoint to regulate cell proliferation. Nevertheless, these mesenchymal-type cancer cells still responded to OSU-53 by undergoing AMPK activation, leading to a switch to an epithelial-like phenotype.

By using OSU-53, we demonstrated the role of AMPK in blocking EMT in aggressive cancer cells in vitro and in vivo by activating Foxo3a signaling. Although AMPK has been reported as an upstream regulator of Foxo3a, the underlying mechanism remains undefined (47). In this study, we obtained several lines of evidence that OSU-53 activates Foxo3a through two distinct Akt-dependent pathways (Fig. 6A). First, OSU-53 facilitated the nuclear localization of Foxo3a by...
downregulating Akt- and IKKβ-mediated phosphorylation (Fig. 4B). Second, OSU-53 increased the protein stability of Foxo3a by blocking MDM2-mediated degradation (Fig. 5). Mechanistically, OSU-53 had no effect on MDM2 expression, but suppressed Akt-mediated MDM2 phosphorylation. This dephosphorylation promoted MDM2 nuclear exit, thereby leading to the physical separation of MDM2 from Foxo3a in different cellular compartments (Fig. 4C).

This mode of regulation differs from that described in a recent report in which AMPK activation in nutrient-deprived HEK 293T cells led to increased transcriptional activity of Foxo3a, however, without affecting its cytoplasmic localization through direct phosphorylation at multiple sites distinct from those targeted by Akt (35). This discrepancy might reflect differences in cellular context, i.e., nutrient deprivation–induced versus allosteric activation of AMPK, and in cell types, i.e., nonmalignant 293T versus aggressive cancer cells, which underscores the intricate relationship between AMPK and Foxo3a (47).

Our data suggest that Foxo3a acted as a master regulator of OSU-53–mediated inhibition of EMT by regulating the expression of E-cadherin, YB-1, Snail, and Twist (Fig. 6A). The ability of Foxo3a to activate E-cadherin gene transcription is especially noteworthy, as other mechanisms have been implicated in Foxo3a-mediated E-cadherin expression, including those mediated by estrogen receptor (ER)α (16) and Twist (17), which underlies the complexity of the role of Foxo3a in regulating EMT in different cell systems. Considering the role of YB-1 in promoting EMT through the upregulation of Snail and other transcriptional repressors of E-cadherin (15), there exists an intricate interplay between Foxo3a and YB-1 in mediating the effect of OSU-53 on the mesenchymal phenotype of cancer cells. In addition, Foxo3a has also been reported to play a key role in the abilities of epigallocatechin-3-gallate and berberine to reverse the invasive phenotype of ERα–positive breast cancer and melanoma cells through distinct mechanisms, i.e., induction of ERα expression (16) and suppression of ERK activity and COX-2 expression (48), respectively. Together, these findings suggest the multifaceted mechanisms of Foxo3a signaling under different cellular contexts.

In summary, we obtained evidence that AMPK plays a role in regulating EMT in cancer cells by upregulating Foxo3a signaling through an Akt-dependent mechanism. Moreover, the effectiveness of oral OSU-53 to suppress metastasis in vivo provides a proof-of-concept that allosteric activation of AMPK by small-molecule agents represents a therapeutically relevant...
strategy to reduce the invasiveness and metastatic capacity of aggressive cancer cells. Lead optimization of OSU-53 to generate more potent derivatives for preclinical development is currently under way.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.-C. Chou, K.-H. Lee, S.K. Kulp, C.L. Shapiro, C.-S. Chen

Development of methodology: K.-H. Lee, D. Wang, C.-S. Chen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-C. Chou, I.-L. Lai, S.K. Kulp, C.-S. Chen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.-C. Chou, K.-H. Lee, X. Mo, S.K. Kulp, C.L. Shapiro, C.-S. Chen

References


Writing, review, and/or revision of the manuscript: C.-C. Chou, S.K. Kulp, C.-S. Chen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Wang, C.-S. Chen

Study supervision: S.K. Kulp, C.-S. Chen

Grant Support

This work is supported by NIH grants R01 CA112250 and R21 158807, the Stefanie Spielman Fund for Breast Cancer Research, and the Lucius A. Wing Endowed Chair Fund at The Ohio State University Medical Center (C.-S. Chen), and a Pelotonia Postdoctoral Fellowship (The Ohio State University Comprehensive Cancer Center; I.-L. Lai).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 16, 2014; revised June 9, 2014; accepted June 10, 2014; published OnlineFirst July 3, 2014.
Role of AMPK in Regulating EMT


AMPK Reverses the Mesenchymal Phenotype of Cancer Cells by Targeting the Akt–MDM2–Foxo3a Signaling Axis


Cancer Res  Published OnlineFirst July 3, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-0135

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/07/21/0008-5472.CAN-14-0135.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2014/08/16/0008-5472.CAN-14-0135. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.