Introduction

The PI3K–AKT signaling pathway plays a key role in linking the extracellular growth factor stimulation to various cellular processes, including cell growth, proliferation, survival, and angiogenesis (1, 2). Activation of phosphoinositide 3-kinase (PI3K) by growth factor binding to receptor tyrosine kinases (RTK) results in phosphorylation of plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP2) to form PIP3. Consequently, PIP3 recruits other downstream effector proteins to the plasma membrane, prominent among which is the serine/threonine kinase AKT. At the plasma membrane, AKT is further activated through phosphorylation of Thr308 and Ser473 by PKA and mTOR complex 2 (mTORC2). Once fully activated, AKT phosphorylates other target proteins, including the TSC1–TSC2 complex, and FoxOs (3). The TSC1–TSC2 complex inhibits mTOR complex 1 (mTORC1), which functions to promote protein synthesis and cell growth. AKT-mediated phosphorylation of TSC2 inhibits the TSC1–TSC2 complex function, and thus activates mTORC1 signaling (4). FoxO transcription factors mainly function to promote cell-cycle arrest and apoptosis via regulation of diverse arrays of transcription targets. AKT phosphorylation of FoxOs leads to FoxO sequestration in the cytoplasm and inactivates their transcription activities (5–8). Aberrant activation of this signaling network has been observed in virtually all human cancers (9–12). In particular relevance to this study, the recent data from The Cancer Genome Atlas project revealed that the PI3K–AKT pathway is altered in around 30% of renal cell carcinomas (RCC; ref. 13).

RCC accounts for 3% of all malignancies in adults, and ranks among the top ten cancers in the United States (14, 15). Traditional chemotherapy, hormonal therapy, or radiation is not effective in the treatment of advanced RCC. mTOR is a validated therapeutic target in RCC, as mTORC1 hyperactivation has been observed in most human RCC samples (16–18), and several clinical trials established the clinical benefit of mTORC1 inhibitors (19–21). However, the clinical response of
RCC to mTOR inhibition has been modest, and most patients with RCC receiving mTOR inhibitor treatment eventually developed resistance (19–21), which may be associated with mTORC1-mediated feedback regulation of the upstream PI3K–AKT pathway. A number of studies have shown that, although activated PI3K–AKT signaling promotes mTORC1 activation, mTORC1 hyperactivation also leads to feedback shutoff of the PI3K–AKT signaling (22–27). These observations suggest that mTOR inhibitor treatment alone, while inhibiting mTORC1-mediated protein synthesis and cell growth, also stimulates PI3K–AKT-dependent survival and cell-cycle entry, which might explain the relatively moderate impact of mTOR inhibitor in treating RCC (28).

These observations prompted ideas for inhibiting the upstream PI3K–AKT pathway in the treatment of RCC as well as other cancers (29). Indeed, a recent study (30) showed that NVP-BEZ235, a PI3K and mTOR dual inhibitor (31), induced more potent growth arrest and apoptotic responses in RCC cells than did rapamycin, an mTOR inhibitor. Currently, NVP-BEZ235, or other drugs to inhibit the PI3K–AKT pathway, including including FoxO-mediated upregulation of RTK expression, compensatory activation of other oncogenic pathways, mTORC1-mediated feedback regulation of the upstream PI3K kinase (22–27). These observations suggest that mTOR inhibitor treatment alone, while inhibiting mTORC1-mediated protein synthesis and cell growth, also stimulates PI3K–AKT-dependent survival and cell-cycle entry, which might explain the relatively moderate impact of mTOR inhibitor in treating RCC (28).

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In this study, utilizing mouse embryonic fibroblasts (MEF) and various renal cancer cells as model system, we investigated the underlying mechanisms of FoxO-mediated feedback regulation and the role of FoxOs in renal tumor development in response to PI3K–AKT inhibition. We identify a novel feedback mechanism that FoxOs promote PI3K–AKT inhibition-induced AKT Ser473 phosphorylation likely via transcriptional upregulation of Rictor. Inactivation of FoxOs results in more potent cell or tumor growth inhibition in response to PI3K–AKT inhibition. These findings have important implications on the treatment strategies of RCC as well as other cancers.

Materials and Methods

Cell culture studies

786-O, UOK101, and RCC4 are human RCC cells, and are described in our previous publication (18). These renal cancer cells and human embryonic kidney cell line, HEK293T, were obtained from American Type Culture Collection, and passed for less than 6 months after receipt. They are cultured in Dulbecco’s Modified Eagle Medium (DMEM) + 10% FBS. Lentiviruses or retroviruses were produced in HEK293T cells with packing mix (ViraPower Lentiviral Expression System, Invitrogen) and used to infect target cells as per manufacturer’s instruction. FoxO1i/3i/4i L/L, RosaCreERT2 MEFs (18) were isolated from E14.5 embryos by standard methods. Primary FoxO1i/3i/4i L/L, RosaCreERT2 MEFs were treated with 200 nmol/L 4-hydroxytamoxifen (4-OHT) or vehicle for 4 days and then shifted to normal medium (DMEM + 10% FBS) after 4-OHT treatment, resulting in FoxO wild-type (WT) and knockout (KO) MEFs. To measure apoptosis, the cells were stained by Annexin V kit as per manufacturer’s instruction (BD Biosciences) and then subjected to fluorescence-activated cell sorting analysis. Cell growth assay was conducted as described in our previous publication (18).

Reagents

Lentiviral short hairpin RNA (shRNA) vectors targeting human FoxO1 and FoxO3 were described in the previous publication (18). Rictor retroviral vector and Lentiviral shRNA vector targeting human Rictor were described in the previous publication (38). NVP-BEZ235 was purchased from LC Laboratories. BKM120 was ordered from ChemieTek. MK2206 was ordered from Selleck. 4-OHT was purchased from Sigma. The following antibodies were used in this study: Vinculin, FoxO1 (C29H4), FoxO3 (75D8), phospho-FOXO1(Thr24)/FOXO3(Thr32), S6, Ser240/244 phospho-S6, Rictor, Akt, Ser473 phospho-AKT, Thr308 phospho-AKT, GSK3, phospho-GSK3, cleaved caspase-3, Ki-67, and ERBB3 (all from Cell Signaling Technology).

Immunofluorescence

Cells were cultured in chamber slides overnight and fixed with 3.7% formaldehyde in PBS for 10 minutes, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 minutes. Cells were then blocked for nonspecific binding with 10% goat serum in PBS and 0.1% Tween-20 (PBST), and incubated with the antibody against FoxO1 (1:300; Cell Signaling Technology) or FoxO3 (1:300; Cell Signaling Technology) for 1 hour at room temperature, followed by incubation with Alexa Fluor 594 goat anti-rabbit immunoglobulin G (1:1,000, Invitrogen, A11012) for 30 minutes at room temperature. Coverslips were mounted on slides using antifade mounting medium with 4,6-diamidino-2-phenylindole (DAPI). Immunofluorescence images were acquired on a Zeiss Axio Observer Z1 fluorescence microscope. For each channel, all images were acquired with the same settings.

Western blot analysis and fractionation

Tissues were lysed with radioimmunoprecipitation assay buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS) containing complete mini protease inhibitors (Roche) and phosphatase inhibitor cocktail (Calbiochem). Cultured cells were lysed with NP40 buffer (150 mmol/L sodium chloride, 1.0% NP-40, 50 mmol/L Tris, pH 8.0) containing complete mini protease inhibitors (Roche) and phosphatase inhibitor cocktail (Calbiochem). Western blot analyses were obtained utilizing 20 to 40 µg of lysate protein. Fractionation of nuclear and cytoplasmic proteins was done by using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo) according to the manufacturer’s protocol. After fractionation, 30 µg of protein was used for Western blot analysis of FoxOs in the cytoplasm and nucleus. α-Tubulin and lamin-A were used as markers of cytoplasm and the nucleus, respectively.

www.aacrjournals.org Cancer Res; 74(6) March 15, 2014 1683

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Quantitative real-time PCR and ChIP analysis

Total RNA was extracted from cells using RNeasy (Qiagen) and first-strand cDNA was prepared with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI). Real-time PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen) or TaqMan Universal PCR Master Mix (ABI), and was run on Stratagene MX3000P. Chromatin immunoprecipitation (ChIP) experiment was performed using EZ-ChIP Kit (Chemicon) as per the manufacturer’s instructions. The primer sequences used in this study are described in Supplementary Experimental Procedures.

Xenograft model

786-O cells infected with either control shRNA or FoxO1/FoxO3 shRNA were injected subcutaneously into nude mice. Once the tumor xenografts reached around 100 mm², mice were randomized into different groups (n = 5/group) and treated once daily by gavage with vehicle, NVP-BEZ235, (Sigma) overnight, and washed once with 1 strost-staining intensity and the percentage of positively stained cells. The immunoreactive signal, corresponding to the target expression level, was calculated on the basis of the average quantification intensity measured as previously described (18). The protein signal was quantified by Image-pro plus 6.0 software (Media Cybernetics). The immunoreactive signal, corresponding to the target expression level, was calculated on the basis of the average staining intensity and the percentage of positively stained cells.

Histology and immunohistochemistry

Tumor samples were fixed in 10% neutral-buffered formalin (Sigma) overnight, and washed once with 1× PBS and then transferred into 70% ethanol and stored at 4°C. Tissues were processed by ethanol dehydration and embedded in paraffin by Histology Core Laboratory (MD Anderson Cancer Center, Houston, TX) according to standard protocols. Sections (5 μm) were prepared for antibody detection and hematoxylin and eosin staining. Immunohistochemical analysis was performed as previously described (18). The protein signal was quantified by Image-pro plus 6.0 software (Media Cybernetics). The immunoreactive signal, corresponding to the target expression level, was calculated on the basis of the average staining intensity and the percentage of positively stained cells.

Results

NVP-BEZ235 treatment-induced AKT Ser473 phosphorylation is significantly compromised in FoxO-deficient cells

Our previous study showed that reactivation of FoxO1 or FoxO3 in renal cancer cells induced potent cell-cycle arrest and apoptosis (18). Paradoxically, in the same cell lines, we observed that FoxO reactivation resulted in significantly increased phosphorylation of AKT (data not shown). Because PI3K inhibition generally leads to FoxO activation, we reasoned that FoxOs may also play a role in PI3K inhibition-induced AKT reactivation. We first tested this hypothesis in the matched FoxO WT and KO MEFs generated by transient 4OHT or vehicle treatment of FoxO1/3/4/L, Rosa26-CreERT2 MEFs. Consistent with findings described in other cancer cell lines (33, 35), we observed that treatment with 50 nmol/L NVP-BEZ235, a PI3K/mTOR dual inhibitor, in WT MEFs led to transient decrease of AKT Ser473 phosphorylation followed by resurgence of AKT Ser473 phosphorylation at later time points (Fig. 1A). NVP-BEZ235 treatment also significantly decreased the phosphorylation levels of AKT substrates such as FoxO and GSK3 (Fig. 1B), and promoted FoxO translocation from cytoplasm to nucleus (Supplementary Fig. S1), confirming the inhibition of the PI3K–AKT pathway by NVP-BEZ235. Mirroring the resurgence of AKT Ser473 phosphorylation, the phosphorylation levels of FoxO and GSK3 also resurged at later time points upon NVP-BEZ235 treatment (Fig. 1B).

Notably, although FoxO deletion in MEFs did not significantly affect the basal level of AKT Ser473 phosphorylation, NVP-BEZ235–induced resuscitation of AKT Ser473 phosphorylation as well as GSK3 phosphorylation at later time points was significantly reduced in FoxO KO MEFs (Fig. 1A and B and Supplementary Fig. S2A). A dose-dependent experiment at 24 hours after NVP-BEZ235 treatment revealed that NVP-BEZ235 treatment increased AKT Ser473 phosphorylation at relatively low dosages (less than 100 nmol/L) in WT MEFs, and the increased AKT Ser473 phosphorylation was significantly compromised in FoxO KO MEFs (Fig. 1C). In line with these data from murine system, further experiments in human renal cancer cells (Fig. 1D and E), including UOK101 (with high expression of FoxO3 and low FoxO1 expression) and 786-O cells (with high expression of both FoxO1 and FoxO3), confirmed that knockdown of FoxOs similarly alleviated NVP-BEZ235–induced AKT Ser473 phosphorylation.

We also examined the phosphorylation status of several other key signaling molecules in the same experimental conditions. FoxO deletion or knockdown did not significantly affect the levels of S6 phosphorylation under NVP-BEZ235 treatment, suggesting that FoxOs did not affect NVP-BEZ235 inhibition of mTORC1 (Fig. 1A–E). We found that, in MEFs, 50 nmol/L NVP-BEZ235 treatment did not lead to the initial suppression of AKT Ser308 phosphorylation (Fig. 1A and Supplementary Fig. S1B), whereas in UOK101 cells, the kinetics of AKT Thr308 phosphorylation in response to NVP-BEZ235 treatment is similar to that of AKT Ser473 phosphorylation with initial suppression followed by resurgence at later time points (Fig. 1D). Regardless of the differential response of AKT Ser308 phosphorylation in different cell lines, we found that FoxO deficiency did not affect AKT Ser308 phosphorylation under NVP-BEZ235 treatment (Fig. 1A and D). Finally, consistent with previous reports in other cell lines (33), NVP-BEZ235 treatment increased extracellular signal-regulated kinase (ERK) phosphorylation, and FoxO deficiency moderately increased ERK phosphorylation under NVP-BEZ235 treatment (Fig. 1A and C), which may relate to the documented feedback regulation between the AKT and ERK pathways. Together, our data reveal that FoxOs specifically promote AKT Ser473 phosphorylation under NVP-BEZ235 treatment, and NVP-BEZ235 treatment-induced AKT Ser473 phosphorylation is significantly compromised in FoxO-deficient cells.

FoxO deficiency promotes cell proliferation suppression and cell death induction under NVP-BEZ235 treatment

AKT activation plays a major role in promoting cell proliferation and survival (3). We thus examined the impact of FoxO
**Figure 1.** NVP-BEZ235 treatment-induced AKT Ser473 phosphorylation is significantly compromised in FoxO-deficient cells. A and B, FoxO WT and KO MEFs were treated with 50 nmol/L NVP-BEZ235 for different hours as indicated. Cell lysates were then analyzed by Western blotting. C, FoxO WT and KO MEFs were treated with NVP-BEZ235 for 24 hours with different concentrations as indicated. Cell lysates were then analyzed by Western blotting. D, UOK 101 cells infected with either control shRNA or FoxO1/FoxO3 shRNA were treated with 10 nmol/L NVP-BEZ235 for different hours as indicated and then subjected to Western blot analysis. E, 786-O cells infected with either control shRNA or FoxO1/FoxO3 shRNA were treated with 50 nmol/L NVP-BEZ235 for different hours as indicated and then subjected to Western blot analysis.
deficiency on cell proliferation and survival under NVP-BEZ235 treatment. As shown in Fig. 2A, FoxO deletion rendered MEFs more sensitive to NVP-BEZ235–induced cell proliferation suppression. Although NVP-BEZ235 treatment at 50 nmol/L concentration did not induce obvious apoptosis in WT MEFs, it significantly increased apoptosis in FoxO KO MEFs (Fig. 2B). It is important to note that, when applied at very high dosage (more than 250 nmol/L), NVP-BEZ235 very potently suppressed cell proliferation, and FoxO deficiency did not affect high-dose NVP-BEZ235 treatment–induced cell proliferation arrest (Supplementary Fig. S3). In line with these data from MEFs, further experiments confirmed that knockdown of FoxOs in various human renal cancer cells, although modestly increasing cell proliferation, led to more potent cell proliferation suppression and apoptosis induction compared with control shRNA-infected cells under NVP-BEZ235 treatment (Fig. 2C–F). Together, our data strongly suggest that FoxO deficiency promotes cell proliferation suppression and apoptosis induction in response to NVP-BEZ235 treatment, thus rendering renal cancer cells more susceptible to NVP-BEZ235 treatment.

**FoxO deficiency potentiates NVP-BEZ235–induced renal tumor suppression in vivo**

The data presented above in cell line studies prompted us to further examine the roles of FoxOs in renal tumor growth in response to NVP-BEZ235 treatment in vivo using the xenograft model. To this end, we injected the same amount of 786-O cells infected with either FoxO1/3 shRNA or control shRNA into nude mice. Once the tumor xenografts reached 100 mm³, mice were treated once daily with vehicle or NVP-BEZ235 (30 mg/kg/d) for additional 20 days, and the tumor samples were collected for various analyses at the endpoint. In line with the data from in vitro analyses (Fig. 2F), our in vivo experiments revealed that, although FoxO knockdown moderately increased tumor size and weight in vivo, more dramatic decline in tumor size and weight were observed in FoxO knockout tumor group compared with control group with NVP-BEZ235 treatment (Fig. 3A–C).

Further analyses of the tumor samples by Western blotting (Fig. 3D) and immunohistochemical experiments (Fig. 3E and F) confirmed that NVP-BEZ235 significantly induced AKT Ser473 phosphorylation in renal tumor
samples obtained at endpoint (20 days after NVP-BEZ235 treatment), and that FoxO knockdown alleviated the increase of AKT Ser473 phosphorylation induced by NVP-BEZ235 treatment in tumors. FoxO knockdown also potentiated NVP-NEZ235 treatment-induced apoptosis and cell-cycle arrest in renal tumors, as evidenced by cleaved caspase-3 and Ki-67 immunohistochemical analyses (Fig. 3E and F). It should be pointed out that FoxO knockdown did not affect NVP-BEZ235-induced mTORC1 inactivation as evidenced by S6 phosphorylation (Fig. 1A and C). Collectively, our data strongly suggest that, under NVP-BEZ235 treatment, FoxOs regulate renal tumor growth likely through its upregulation of AKT Ser473 phosphorylation, and FoxO deficiency potentiates PI3K inhibition-induced renal tumor suppression in vivo.

**FoxOs upregulate Rictor expression under NVP-BEZ235 treatment**

Both in vitro and in vivo studies presented above prompted us to further dissect the underlying mechanisms by which FoxOs regulate AKT Ser473 phosphorylation under NVP-BEZ235 treatment. Previous studies showed that FoxOs mediate AKT or PI3K inhibition-induced upregulation of certain RTKs, particularly ERBB3, in breast cancer cells (36), which likely relates to FoxO-mediated AKT reactivation as shown in our studies. However, we found that there was no appreciable difference of ERBB3 expression levels between FoxO WT and FoxO KO MEFs (Fig. 4A).

Because our data showed that FoxOs regulate AKT Ser473 phosphorylation, but not AKT Ser308 phosphorylation or S6 phosphorylation, in response to NVP-BEZ235 treatment (Fig. 1A–E), we reason that FoxOs may specifically regulate...
mTORC2 function under NVP-BEZ235 treatment, as mTORC2 is responsible for AKT Ser473 phosphorylation (38). To this end, we examined the expression levels of mTORC2 components under NVP-BEZ235 treatment. These analyses revealed that NVP-BEZ235 treatment significantly induced the expression of Rictor, an essential component of mTORC2, in WT MEFs, and that FoxO deletion in MEFs abolished NVP-BEZ235–induced Rictor expression (Fig. 4A and B). In line with this, FoxO knockdown attenuated NVP-BEZ235–induced Rictor expression in both renal cancer cells in vitro (Fig. 4C and D) and xenograft renal tumor samples in vivo (Fig. 4E). FoxO knockdown did not affect ERBB3 expression in response to NVP-BEZ235 treatment in renal cancer cells (Fig. 4C and D). Finally, ChIP analysis documented that endogenous FoxO1 and FoxO3 can directly bind to the FoxO-binding element identified in Rictor promoter region, and NVP-BEZ235 treatment significantly increased FoxO1/3 binding to FoxO-binding element in Rictor promoter (Fig. 4F and G). Taken together, our data suggest that, in response to NVP-BEZ235 treatment, FoxOs directly bind to Rictor promoter and upregulate Rictor expression, which likely is responsible for the resurgence of AKT Ser473 phosphorylation at later time points under NVP-BEZ235 treatment. Our data are also consistent with a previous report showing that overexpression of FoxO is sufficient to induce Rictor expression (39).
Rictor mediates FoxO regulation of AKT Ser473 phosphorylation, cell proliferation, and cell survival in response to NVP-BEZ235 treatment

Next we studied whether Rictor plays any causal role in FoxO function in PI3K inhibition-mediated feedback regulation. To this end, we examined whether reexpression of Rictor in FoxO-deficient cells would rescue the decrease of AKT Ser473 phosphorylation, cell proliferation and survival under NVP-BEZ235 treatment. As shown in Fig. 5A, reexpression of Rictor in FoxO KO cells restored AKT Ser473 phosphorylation to WT level under NVP-BEZ235 treatment. As expected, reexpression of Rictor did not affect AKT Ser308 phosphorylation (Fig. 5A). Accordingly, although reexpression of Rictor in FoxO KO cells did not affect cell proliferation or apoptosis under vehicle treatment condition, Rictor reexpression significantly rescued NVP-BEZ235–induced cell proliferation suppression and apoptosis induction phenotypes observed in FoxO KO cells (Fig. 5B and C). In addition, we showed that knockdown of Rictor in both UOK101 and 786-O renal cancer cells potentiated cell proliferation suppression and cell death induction under NVP-BEZ235 treatment (Supplementary Fig. S4). Together, our data strongly suggest that Rictor is at least one key downstream target of FoxOs to mediate NVP-BEZ235–induced feedback regulation.

FoxOs modulate the feedback response on AKT Ser473 phosphorylation and renal tumor growth by other PI3K or AKT inhibitors

In our data presented above, we use NVP-BEZ235 as a pharmacologic means to inhibit the PI3K–AKT pathway. To rule out the possibility that the effect is specific to NVP-BEZ235, we also extended our experiments to other PI3K or AKT inhibitors, including BKM120 (a pan-PI3K inhibitor) and MK-2206 (an AKT inhibitor). First, we showed that FoxO KO cells also exhibited significantly reduced AKT Ser473 phosphorylation under the treatment of BKM120 (Fig. 6A) or MK-2206 (Fig. 6B). In addition, our data from different cell lines, including MEFs and renal cancer cells, showed that FoxO deficiency similarly potentiated cell proliferation suppression and cell death induction in response to either BKM120 to MK-2206 treatment (Fig. 6C–J). Finally, we also examined the roles of FoxOs in renal tumor growth in response to BKM120 or MK-2206 treatment in vivo using the similar xenograft model described above. As shown in Fig. 7A–F, our data showed that FoxO knockdown potentiated BKM120 or MK-2206–treatment–induced renal tumor suppression in vivo, which aligns well with NVP-BEZ235 treatment data (Fig. 3A–C). Together, our data strongly suggest that FoxO-mediated feedback regulation described here results from the pharmacologic inhibition of the PI3K–AKT pathway rather than a specific effect of NVP-BEZ235 treatment.

Discussion

The PI3K–AKT–mTOR pathway is hyperactivated in many human cancers, including RCC. The moderate clinical benefit of the mTOR inhibitor observed in several human cancer clinical trials and the mTORC1 inhibition-mediated feedback activation of PI3K–AKT have prompted ideas for inhibiting the upstream PI3K–AKT in cancer treatment (28). However, the relief of feedback regulation and the resulting activation of other oncogenic signaling pathways caused by PI3K–AKT inhibition likely will limit the clinical utilization of such inhibitors in cancer treatment.

Using NVP-BEZ235 as a pharmacologic approach to inhibit PI3K, our study reveals a novel mechanism of PI3K inhibition-induced feedback regulation in RCC cells, which involves FoxO-mediated Rictor upregulation and AKT reactivation via Ser473 phosphorylation. Specifically, our data show that, although short-term treatment of NVP-BEZ235 leads to immediate PI3K–AKT inactivation and FoxO activation, long-term treatment of NVP-BEZ235 leads to FoxO binding to Rictor.

Figure 5. Rictor mediates FoxO regulation of AKT Ser473 phosphorylation, cell proliferation, and cell survival in response to NVP-BEZ235 treatment. A, FoxO WT and KO MEFs with stable expression of either empty vector (EV) or Rictor were treated with 0 or 50 nmol/L NVP-BEZ235 for 24 hours; cell lysates were then analyzed by Western blotting. B, FoxO WT and KO MEFs with stable expression of either empty vector or Rictor were treated with 0 or 50 nmol/L NVP-BEZ235 for different days as indicated and subjected to cell proliferation analysis. C, FoxO WT and KO MEFs with stable expression of either empty vector or Rictor were treated with 0 or 50 nmol/L NVP-BEZ235 for 24 hours. The apoptosis rates of the cells were then detected by Annexin V staining assay.
promoter and upregulation of Rictor expression, resulting in the resurgence of AKT Ser473 phosphorylation likely via activation of mTORC2. Because NVP-BEZ235 is a PI3K/mTOR dual inhibitor, it should also inhibit mTORC2. However, NVP-BEZ235 treatment at low dosage (such as 50 nmol/L) likely only partially inhibits the activity of mTORC2 (as well as PI3K). We propose that NVP-BEZ235–induced Rictor upregulation will offset the partial inhibitory effect of NVP-BEZ235 on mTORC2, leading to resurgence of AKT Ser473 phosphorylation at later time points. When applied at very high dosage (more than 250 nmol/L), we reason that the inhibitory effect of NVP-BEZ235 on mTORC2 and PI3K is so potent that Rictor upregulation will not be enough to offset it. In line with this model, NVP-BEZ235–induced resurgence of AKT Ser473 phosphorylation was only observed at relatively low dosages of NVP-BEZ235 (compare Fig. 2A with Supplementary Fig. S3). Because drug treatment at high dosages is generally toxic to patients with cancer, the observation from our study focusing on low dosages is likely to be of clinical relevance. Finally, we extend our data to other PI3K or AKT inhibitors, suggesting that FoxO–mediated feedback regulation described here likely results from the pharmacologic inhibition of the PI3K–AKT pathway. Whether this mechanism contributes to the tumor recurrence in patients with renal cancer receiving PI3K or AKT inhibitor treatment awaits further investigation in the future clinical trials.

Our data showed that FoxOs function to promote cell proliferation and survival under pharmacologic inhibition of the PI3K–AKT pathway, which is different from the antiproliferative and proapoptotic function FoxOs exert under other stress conditions (5). Thus, FoxOs could play very...
different functions by regulating different sets of transcriptional targets under different cell lineages or stimuli, which is in line with a previous report (40). Consistent with this, we found that FoxO deficiency did not affect the expression levels of FoxO targets mediating apoptosis response, such as Bim and p27, under NVP-BEZ235 treatment (Supplementary Fig. S5).

Our data from both gain-of-function (Rictor reexpression in FoxO-deficient cells) and loss-of-function (Rictor knockdown in FoxO-proficient cells) experiments identified Rictor as an important downstream target of FoxO to mediate PI3K inhibition-induced feedback. It should be pointed out that moderate overexpression of Rictor in FoxO KO cells did not significantly increase AKT Ser473 phosphorylation under vehicle treatment condition (Fig. 5A), suggesting that NVP-BEZ235-induced AKT Ser473 phosphorylation may involve Rictor and other factors (which may be regulated by PI3K inhibition via FoxO-independent mechanisms). Clearly, the response to pharmacologic inhibition of the PI3K–AKT pathway may involve multiple mechanisms. Our study strongly suggests that FoxO-Rictor axis is at least one important mechanism involved in this feedback regulation.

Similar to other targeted therapies, it is likely that only a fraction of patients with cancer will respond positively to PI3K–AKT inhibition therapy. Thus, it will be critical to identify appropriate biomarker(s) that can be used to stratify patients with cancer for PI3K–AKT inhibition treatment. Our study suggests that FoxO may serve as a novel biomarker to stratify patients with renal cancer for PI3K or AKT inhibitor treatment. Specifically, because FoxO-deficient renal tumors are more sensitive to PI3K–AKT inhibition than FoxO-proficient renal tumors, we propose that patients with renal cancer with loss of (or low) FoxO expression or activity will respond better to PI3K–AKT inhibition than those with high FoxO expression or activity. In addition, we propose that inhibition of FoxO (or key FoxO targets involved in feedback regulation, such as Rictor) may...
synergize with PI3K–AKT inhibition in renal cancer treatment. Our study thus challenges the current paradigm of reactivating FoxO in cancer treatment based on FoxO generally being considered as a tumor suppressor. Finally, because the PI3K–AKT pathway is also activated in many other cancers, our data may provide important insights into the treatment of other cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A. Lin, B. Gan
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Lin, H.-L. Piao, L. Zhuang, B. Gan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Lin, B. Gan
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Acknowledgments
The authors thank Zhen Xing, Ye Chen, Kingsley Charles V, Xin Wang, and Li Wang for insightful discussion and technical assistance.

Grant Support
This work was supported by grants from the MD Anderson Cancer Center, NIH, U.S. Department of Defense (TS093049), Cancer Prevention and Research Institute of Texas (RP130020), and the Sidney Kimmel Foundation (B. Gan). B. Gan is a Kimmel scholar, an Ellison Medical Foundation New Scholar, and a member of the MD Anderson Cancer Center (CA016672).

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Received June 18, 2013; revised December 5, 2013; accepted January 11, 2014; published OnlineFirst January 21, 2014.

Published OnlineFirst January 21, 2014; DOI: 10.1158/0008-5472.CAN-13-1729


FoxO Transcription Factors Promote AKT Ser473 Phosphorylation and Renal Tumor Growth in Response to Pharmacologic Inhibition of the PI3K–AKT Pathway

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doi: 10.1158/0008-5472.CAN-13-1729

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