Phosphatidylinositol 3-Kinase-AKT-Mammalian Target of Rapamycin Pathway Is Essential for Neuroendocrine Differentiation of Prostate Cancer*

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Hormonal therapy of prostate cancer, by inhibiting androgen production and/or androgen function, is the treatment of choice for advanced prostate cancer. Although most patients respond initially, the effect is only temporary, and the tumor cells will resume proliferation in an androgen-deprived environment. The mechanism for androgen-independent proliferation of cancer cells is unclear. Hormonal therapy induces neuroendocrine differentiation of prostate cancer cells, which is hypothesized to contribute to tumor recurrence by a paracrine mechanism. We studied signal transduction pathways of neuroendocrine differentiation in LNCaP cells after androgen withdrawal, and we showed that both the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathway and ERK are activated, but only the former is required for neuroendocrine differentiation. A constitutively active AKT promotes neuroendocrine differentiation and a dominant negative AKT inhibits it. Activation of AKT by IGF-1 leads to neuroendocrine differentiation, and neuroendocrine differentiation induced by epinephrine requires AKT activation. We also show that the AKT pathway is likely responsible for neuroendocrine differentiation in DU145, an androgen-independent prostate cancer cell line. Therefore, our study demonstrated a novel function of the AKT pathway in prostate cancer progression and identified potential targets that may be explored for the treatment of androgen-independent cancer.

Prostate cancer (PC) 2 is the most common malignancy among men in western countries (1). There are multiple treatment options for PC in early stages. For advanced and metastatic PC, hormonal therapy, consisting of androgen ablation and/or inhibition of androgen action by anti-androgen, is the treatment of choice (2). Although an initial response is seen in most patients receiving hormonal therapy, the effect is temporary, and the tumor eventually recurs and enters the androgen-independent (AI) stage in which the tumor cells proliferate in an androgen-deprived environment. There are no effective therapies for AI PC (3).

The mechanism of AI proliferation of PC is poorly understood, and many hypotheses have been proposed, such as androgen receptor (AR) amplification (4), AR mutation (5), aberrant activation of AR (6), or increased AR sensitivity to low levels of androgen in the prostate (6, 7). In addition, many studies have shown that neuroendocrine (NE) differentiation (NE differentiation) may contribute to AI growth of PC (8–10).

The epithelial compartment of benign prostate consists of luminal secretory cells, basal cells, and a minor component of NE cells that have neuron-like morphology and secret biogenic amines and neuropeptides (11). NE cells are also present in PC as scattered individual cells or small nests among the more abundant secretory type cancer cells. The number of NE cells increases in high grade and high stage tumors and particularly in hormonally treated and AI tumors (9). It is hypothesized that hormonal therapy induces NED and the NE cells contribute to AI growth of PC in the androgen-deprived environment by secreting their products to act on the adjacent non-NE tumor cells in a paracrine fashion (8–10).

Androgen withdrawal of the culture media leads to NED of LNCaP cells, a PC cell line (13), mimicking in vivo observation in PC patients treated hormonally. This finding supports the trans-differentiation model and suggests that NE cells may be derived from the non-NE secretory-type cancer cells (13, 14). However, the signaling pathway involved in the differentiation process is unclear. Here we report our study demonstrating that the PI3K-AKT-mTOR pathway is critically involved in NED.

EXPERIMENTAL PROCEDURES

Materials—LNCaP and DU145 cells were obtained from the American Type Culture Collection (Manassas, VA); FBS, RPMI medium 1640, sodium pyruvate, penicillin, and streptomycin were purchased from Invitrogen; charcoal/dextran-treated FBS was purchased from Hyclone (Logan, UT); Geneljuice® transfection reagent was from Novagen; RNasy® mini kit was from Qiagen (Valencia, CA); transcription reverse transcriptase and homogeneously protein A were from Roche Applied Science; random hexamers was from Promega (Madison, WI); iQ™ SYBR® Green Supermix and Bio-Rad Protein assay kit were from Bio-Rad; monoclonal anti-NSE antibody was from DAKO (Carpinteria,
CA); polyclonal anti-IGF-1 receptor β antibody and monoclonal anti-GAPDH antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-AKT, anti-phospho-AKT, anti-ERK, anti-phospho-ERK, anti-S6K, anti-phospho-S6K antibodies, and monoclonal anti-Tyr(P)-100 antibody were from Cell Signaling (Dancers, MA); RIPA lysis buffer was from Upstate Cell Signaling Solutions (Lake Placid, NY); protease inhibitor mixture and epinephrine (used at 5 μM) were from Sigma; IGF-1 (used at 100 ng/ml) was from R&D Systems (Minneapolis, MN); U0126 (used at 10 μM) and AKT inhibitor IV (used at 20 μM) were from Calbiochem. LY294002 (used at 20 μM) was from Cayman Chemical (Ann Arbor, MI), and rapamycin (used at 10 nM) was from Biomol (Plymouth Meeting, PA).

Cell Culture, Plasmid DNA, and Transfection—LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% FBS. For androgen deprivation, cells were cultured in RPMI 1640 medium with 10% charcoal/dextran-treated FBS. When chemical inhibitors or stimulating agents (IGF-1, epinephrine) were used, the culture media were changed daily with fresh inhibitors.

pCDNA3-cAkt (a constitutively active Akt with a deletion at amino acids 4–129 replaced with a consensus myristoylation domain) (15) and pCDNA-dnAKT (kinase-deficient mutant, K179A) were kindly provided by Dr. Freeman of the University of Rochester. The liposomed mediated plasmid transfection was performed using GeneJuice™ transfection reagent (Novagen). The cells were plated and maintained to 70–80% density in 50-mm plates and then transfected with the plasmid DNAs according to the protocol suggested by the manufacturer. For transient transfection, the cells were harvested 48 h after transfection. For stable transfection, the transfected cells were diluted at 1:5–1:10 and selected with 300 μg/ml G418.

FIGURE 1. Androgen withdrawal of cultured LNCaP cells leads to NED and activation of ERK and AKT pathways. A, in comparison to LNCaP cell cultured in normal FBS, which show epithelial morphology (left panel), LNCaP cells cultured in charcoal-treated (androgen-deprived) FBS (C-FBS) for 6 days (d) show elongated cellular processes typical of NE cells. B, LNCaP cells were cultured in FBS or C-FBS for 3 and 6 days. A real time PCR assay was performed to study the expression of chromogranin A (an NE marker) mRNA. C, LNCaP cells were cultured in FBS or C-FBS for 3 and 6 days. Equal amounts of proteins were resolved by SDS-PAGE and immunoblotted with anti-NSE (an NE marker) and anti-GAPDH antibodies (loading control). Androgen withdrawal led to NED of LNCaP cells as characterized by increased expression of NSE. D–F, Western blots show increased phosphorylation of ERK, AKT, and S6K after androgen withdrawal. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.
Real Time Reverse Transcription-PCR—Total RNA was isolated from cells with the RNeasy kit. RNA was reverse-transcribed by transcriptor reverse transcriptase with random hexamers. The following specific forward and reverse primers were used for NSE, 5'-AGCTGCCCCTGCCTTAC-3' and 5'-GAGACAAACAGC-GTTACTTAG-3', and for chromogranin A, 5'-GCGGTGGAGCCATCAT-3' and 5'-TCTGTGGCTTCACCACACTTTT-TCTC-3'.

Western Blotting—Cells were washed twice with cold phosphate-buffered saline and lysed in RIPA lysis buffer for 30 min on ice. The cells were sheared twice through a

Real time PCR was performed with iQ™ SYBR® Green Supermix in an iCycler iQ System (Bio-Rad) using the SYBR Green Detection protocol. Total reaction volume was 20 μl, and a cycle consists of 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, for a total of 45 cycles followed by 72 °C for 5 min.

Western Blotting—Cells were washed twice with cold phosphate-buffered saline and lysed in RIPA lysis buffer for 30 min on ice. The cells were sheared twice through a

FIGURE 2. NED induced by androgen withdrawal is not suppressed by inhibition of ERK. A, LNCaP cells were cultured in FBS or C-FBS for 6 days in the absence or presence of the MEK inhibitor U0126. Equal amounts of cellular proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that U0126 did not inhibit NED. B and C, Western blots show that U0126 inhibited phosphorylation of ERK but increased the phosphorylation of AKT. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.
20-gauge needle and centrifuged at 14,000 rpm for 15 min at 4 °C. The protein concentration in the supernatant was determined with the Bio-Rad protein assay kit. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane with Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked with TBS containing 5% w/v nonfat dry milk, hybridized with primary antibody in 2% w/v nonfat dry milk, followed by incubation with secondary antibody and detected with an ECL kit (Bio-Rad).

**Statistical Analysis**—Statistical significance was determined by t test. The results are expressed as mean ± S.D. from three separate (replicate) experiments.

**RESULTS**

Androgen Deprivation of LNCaP Cells Induces NED and the Activation of ERK and the PI3K-AKT-mTOR Signaling Pathways—As has been shown previously, androgen withdrawal in the culture media of LNCaP cells induced NED, characterized by changes in cell morphology (elongated cellular
processes; Fig. 1A) and increased expression of NE cell markers chromogranin A (Fig. 1B) and NSE (13) (Fig. 1C). Androgen withdrawal also induced phosphorylation of ERK (Fig. 1D) and the serine/threonine kinase AKT (Fig. 1E). Because AKT is a key player in the PI3K-AKT-mTOR signaling pathway and its activity is increased by phosphorylation, our observation suggests that the PI3K-AKT-mTOR pathway may be activated during NED of LNCaP cells. Consistent with this hypothesis, androgen deprivation of LNCaP cells significantly increased the levels of phosphorylation of S6 kinase (S6K) (Fig. 1F), an important downstream effector of mTOR whose level of phosphorylation directly correlates with the activity of the PI3K-AKT-mTOR pathway in PC (16).

**FIGURE 4.** NED induced by androgen withdrawal is suppressed by inhibition of mTOR or AKT. A–C, LNCaP cells were cultured in FBS or C-FBS for 6 days in the absence or presence of the mTOR inhibitor rapamycin. Equal amounts of cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-NSE, anti-GAPDH, anti-S6K, anti-phospho-S6K, anti-ERK, and anti-phospho-ERK antibodies. Rapamycin inhibited NED and the phosphorylation of S6K, but not that of ERK. D, LNCaP cells were cultured in FBS, C-FBS, or C-FBS plus AKT inhibitor IV for 12 h, and a real time PCR assay was performed to measure the mRNA levels of NSE in the cells. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.
withdrawal. Addition of U0126, an inhibitor of MEK that inhibits the activation of ERK, had no effect on the increased NSE level in androgen-deprived LNCaP cells (Fig. 2A). The relative specificity of this compound was demonstrated by showing that U0126 blocked the phosphorylation of ERK (Fig. 2B) but not that of AKT (Fig. 2C), suggesting that activation of ERK is not required for NED of PC.

To study whether PI3K-AKT-mTOR pathway is required for NED of LNCaP cells, inhibitors that target key molecules of this pathway were added to LNCaP cells cultured in androgen-deprived LNCaP cells were transiently transfected with pcDNA3-cAKT (expressing a constitutively active AKT) or pcDNA3-dnAKT (expressing a dominant negative AKT) or cultured in c-FBS (positive control of NED). The cells were harvested 48 h after transfection, and a real time PCR assay was performed to study the expression of NSE mRNA and showed that cAKT, not dnAKT, induced NED. B, equal amounts of protein from control LNCaP (transfected with empty vector), LNCaP-cAKT, and LNCaP-dnAKT cells were immunoblotted with anti-AKT antibody and anti-GAPDH antibodies. C and D, LNCaP, LNCaP-cAKT, and LNCaP-dnAKT cells were cultured in FBS or in C-FBS for 6 days. Equal amounts of proteins were immunoblotted with anti-NSE, anti-GAPDH, anti-S6K, and anti-phospho-S6K antibodies to show that cAKT induced NED and dnAKT inhibited androgen withdrawal-induced NED. E, LNCaP or LNCaP-cAKT cells were cultured in FBS or C-FBS for 6 days in the absence or presence of rapamycin. Equal amounts of proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that cAKT-induced NED was inhibited by rapamycin. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.
LY294002, a PI3K inhibitor, and rapamycin, an inhibitor of mTOR, significantly inhibited the expression of NSE (Fig. 3A and Fig. 4A). The specificity of LY294002 was demonstrated by its ability to inhibit the phosphorylation of AKT (Fig. 3B) but not ERK phosphorylation (Fig. 3C). Similarly, we demonstrated that rapamycin inhibited the phosphorylation of S6K, a downstream molecule of mTOR (Fig. 4B), but not ERK (Fig. 4C). Additionally, we studied whether inhibition of AKT by a chemical inhibitor (AKT inhibitor IV) may inhibit NED. Because treatment of LNCaP cells with this compound for longer than 12 h causes significant cell apoptosis, we performed our study for 12 h (complete NED occurs in 6 days).

LNCaP cells cultured in charcoal-treated FBS for 12 h showed increased NSE mRNA but not its protein (data not shown). We showed that AKT inhibitor IV abolished the increased expression of NSE mRNA after androgen withdrawal (Fig. 4D). Taken together, these results suggest that the PI3K-AKT-mTOR pathway, but not the Ras-MEK-ERK pathway, is required for androgen withdrawal-induced NED of PC.

Activated AKT Leads to NED of LNCaP Cells—Results from the above experiments suggest that the PI3K-AKT-mTOR pathway is activated in LNCaP cells after androgen withdrawal and is required for NED. We next studied whether activation of AKT by other means also promoted NED. We transiently transfected LNCaP cells with pcDNA3-cAkt, which expresses a constitutively active AKT with a deletion of amino acids 4–129 replaced with a consensus myristoylation domain, or pcDNA3-dnAKT, which expresses a dominant negative (kinase-deficient mutant) AKT (K179A). A real time PCR assay 48 h after transfection showed that expression of c-AKT, not dnAKT, induced the expression of NSE mRNA in LNCaP cells (Fig. 5A), suggesting that activated AKT induces NED, and the kinase activity of AKT is required for this function.

To confirm the results obtained with transient transfection experiments, we established stable cell lines of LNCaP cells. The LNCaP cells were transfected with pcDNA3-cAKT and pcDNA3-dnAKT, respectively and selected with G-418. Cells that survived the selection were pooled and designated as LNCaP-cAKT and LNCaP-dnAKT cells, respectively. Western blot showed significant overexpression of AKT in these cells in comparison to the parental LNCaP cells (Fig. 5B)

LNCaP-cAKT cells had a significantly increased level of NSE in comparison with that in the parental LNCaP cells when cultured in normal FBS (Fig. 5C), suggesting that activation of the AKT pathway promotes NED of LNCaP cells. mTOR mediates the function of AKT in inducing NED because a downstream effector of mTOR, S6K, was constitutively activated in the LNCaP-cAKT cells cultured in normal FBS (Fig. 5D). Addition-
FIGURE 6. IGF-1, an activator of AKT, induces NED of LNCaP cells. A, LNCaP cells were cultured in FBS in the absence or presence of IGF-1 for 6 days. Equal amounts of proteins were immunoprecipitated (IP) with an anti-IGF-1-Rβ antibody and immunoblotted (IB) with an anti-phosphotyrosine antibody. B, LNCaP cells were cultured in FBS in the absence or presence of IGF-1 or in C-FBS (positive control of NED) for 6 days. Equal amounts of protein were immunoblotted with anti-AKT and anti-phospho-AKT antibodies. C, LNCaP cells were cultured in the absence (left panel) or presence (right panel) of IGF-1 for 6 days to show that IGF-1 induced NE-like morphology. D, LNCaP cells were cultured in FBS, C-FBS, or in FBS with IGF-1 for 6 days. A real time PCR assay was performed to study the expression of chromogranin A mRNA. E, equal amounts of proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that IGF-1 induces NED. F, LNCaP cells were cultured in FBS, C-FBS, or FBS plus IGF-1 in the absence or presence of rapamycin for 6 days. Equal amounts of proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that rapamycin inhibited IGF-1-induced NED. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.
FIGURE 7. NED induced by epinephrine (Epi) requires AKT signaling. A, LNCaP cells were cultured in the absence (left panel) or presence (right panel) of epinephrine for 6 days to show that epinephrine induced NE-like morphology. B, LNCaP cells were cultured in FBS, C-FBS, or FBS with epinephrine for 6 days. A real time PCR assay was performed to study the expression of chromogranin A mRNA. C and D, Western blots show that epinephrine induced NED and also induced phosphorylation of AKT. E, LNCaP cells were treated with epinephrine for 6 days in the absence or presence of rapamycin. Untreated LNCaP cells cultured in FBS or C-FBS were used as negative and positive controls, respectively. Equal amounts of proteins were immunoblotted with an anti-NSE and an anti-GAPDH antibody to show that rapamycin inhibits NED induced by epinephrine. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.
ally, rapamycin, an mTOR inhibitor, inhibited NED of the LNCaP-cAKT cells (Fig. 5E). Expression of the dominant negative AKT significantly inhibited androgen withdrawal-induced NED (Fig. 5C) as well as phosphorylation of S6K (Fig. 5D), confirming that the PI3K-AKT-mTOR pathway is required for NED of PC.

**IGF-1 Activates AKT and Induces NED of LNCaP Cells**—We next studied whether activation of endogenous AKT by a stimulus other than androgen withdrawal may induce NED. We chose to study the effects of IGF-1, which activates AKT and has been implicated in the progression of PC in many studies (17). IGF-1 induced tyrosine phosphorylation of IGF-1 receptor β and the phosphorylation of AKT as expected (Fig. 6, A and B). Interestingly, addition of IGF-1 to LNCaP cells cultured in normal media induced NE morphologic changes similar to what is observed in cells cultured in androgen-deprived media (Fig. 6C) and also significantly increased the expression of chromogranin A and NSE (Fig. 6, D and E), suggesting that it induces NED. Importantly, NED induced by IGF-1 was inhibited by rapamycin (Fig. 6F), suggesting that IGF-1-induced NED is also mediated by the PI3K-AKT-mTOR pathway.

**Epinephrine Activates AKT and Induces NED**—Results from the previous experiments support the hypothesis that AKT is essential for the induction of NED of PC by androgen withdrawal. However, other agents, such as β-adrenergic receptor agonist epinephrine, can also stimulate NED of LNCaP cells (14, 18), but it was unclear whether AKT is involved under such conditions. Thus, we tested if NED of LNCaP cells induced by epinephrine also requires activation of AKT. Addition of epinephrine to LNCaP cells induced morphologic changes of NED and significant increases in the expression of chromogranin A and NSE as reported previously (Fig. 7, A–C) (14). Additionally, it also induced phosphorylation of AKT (Fig. 7D). Epinephrine-induced NED was significantly inhibited by the mTOR inhibitor rapamycin (Fig. 7E), suggesting that AKT also plays an essential role in this process.

**NED in DU145 PC Cells Requires PI3K-AKT-mTOR Pathway**—To confirm that the function of AKT signaling in NED is not limited to LNCaP cells only, we studied NED in DU145 PC cells. Unlike LNCaP cells that require androgen for proliferation, DU145 is an androgen-independent cell line that proliferates in the absence of androgen (19). Interestingly, unlike LNCaP cells, DU145 cells showed NED even when cultured in normal FBS as there were high levels of NSE and chromogranin A in such cells (Fig. 8, A–C). Similar to what was observed in LNCaP cells, rapamycin inhibited NED in DU145 cells whereas U0126 did not (Fig. 8C), confirming that PI3K-AKT-mTOR pathway, but not ERK, is required for NED. We confirmed the specificity of the inhibitors by showing that U0126 inhibited phosphorylation of ERK and rapamycin inhibited S6K phosphorylation (Fig. 8, D and E).

**DISCUSSION**

Progression of PC to the hormone refractory state is the major obstacle in PC therapy and has been an important focus of PC research. Many studies have shown that NED may play an important role in the progression of PC to AI state. In the transgenic adenocarcinoma of the mouse prostate model, NED is more extensive in poorly differentiated tumors and in recurrent AI tumors after castration (20). In the CWR22 human PC xenograft tumor model, castration induces an increase in tumor NE cells prior to tumor recurrence (21). LNCaP xenografts do not normally grow in castrated mice because they are androgen-dependent. However, they can grow in castrated hosts when NE cells from a mouse NE tumor (NE-10) are transplanted on the opposite flank, confirming that NE tumor cells can promote AI growth of PC (22). In the presence of androgen, the same NE cells can enhance migration and metastasis of PC cells (23).

We have shown that NE cells do not express AR (24). It is thus conceivable that hormonal therapy, which causes apoptosis of the AR-positive secretory cells, will not eliminate NE tumor cells. The NE tumors cells may then establish paracrine networks to induce AI proliferation of PC. Consistent with this hypothesis, we have shown that the NE cells in human PC secrete IL-8, a mitogenic and angiogenic factor for many tumors, including PC, and the surrounding non-NE tumor cells express increased levels of IL-8 receptor CXCR1 (25).

LNCaP is a very useful model because androgen withdrawal induces NED, mimicking *in vivo* situations in PC patients receiving hormonal therapy (13). With this model, we and others have shown that protein-tyrosine phosphatases may play a role in NED (26–28). Additionally, ERK (27, 29) has been reported to be important in NED. In this study, we were able to reproduce the previously published finding that androgen withdrawal in LNCaP cells induces the activation of ERK. However, in contrast to previous reports (27, 29), we found that inhibition of ERK activation did not suppress NED. One explanation is the possible difference in the chemical inhibitors used. The two previously reported studies used MEK inhibitor PD98059, while our study used the newer MEK inhibitor U0126, and there could be differences in the specificities of the two inhibitors. There may also be differences in other experimental conditions. For example, it has been reported that the LNCaP cells of different passage numbers show significantly different properties (30). For that reason, we performed all our experiments with LNCaP cells under passage 40.

AKT is an important signaling molecule in mammalian cells. It is activated by PI3K and inhibited by tumor suppressor gene PTEN (31, 32). Loss of function of *PTEN* is seen in some human PC cases (33). LNCaP harbors a point mutation in *PTEN* (34, 35), which may allow activation of AKT readily in such cells. We, as well as others (30, 36), have shown that the activity of

**FIGURE 8. Basal NED in DU145 cells requires PI3K-AKT-mTOR pathway.** A–C, DU145 cells were cultured in FBS, FBS + rapamycin, or FBS + U0126, respectively. Real time PCR assays and Western blot show that rapamycin, not U0126, inhibited basal NE differentiation. D and E, the relative specificity of the inhibitors is confirmed by showing inhibition of ERK phosphorylation by U0126 and inhibition of S6K phosphorylation by rapamycin. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.
AKT in LNCaP cells is significantly increased after androgen withdrawal. Moreover, we have shown that activation of AKT after androgen withdrawal is inhibited by a PI3K inhibitor, suggesting that androgen withdrawal may activate a signaling molecule upstream of PI3K, such as a protein-tyrosine kinase or a G protein, leading to phosphorylation and activation of AKT. AKT participates in a variety of cellular processes, including proliferation, apoptosis, and survival, and is considered a key player in many tumors, including PC (37). Up-regulation/activation of AKT has been reported in a number of tumors, including PC (38–41). However, the mechanism of action of AKT in various cancers is not clear and is likely cell type—and organ-dependent. Transgenic mice expressing activated AKT in the prostate develop prostate intraepithelial neoplasia, a precursor lesion of PC, which is antagonized by the rapamycin analog RAD001, an inhibitor of mTOR (42). Similarly, in most other studies, AKT activation appears to be generally associated with malignant transformation and cell proliferation (33, 43, 44). Therefore, our observation that activation of AKT induces NED of LNCaP cells appears surprising at first because NE cells, having a differentiated phenotype, are generally nonproliferative (24). In support of our conclusion that AKT may have functions more diverse than originally anticipated, it has been shown recently that AKT actually blocks motility and invasion of breast cancer cell lines (45, 46).

It has been reported previously that AR may actively repress the NE phenotype of PC cells and inhibition of AR signaling leads to NED (47). It is therefore possible that activation of AKT may inhibit AR signaling, leading to NED. Consistent with this hypothesis, it has been shown that WNT signaling suppresses AR protein level (48) and induces NED in LNCaP cells (49). Similarly, HB-EGF, a ligand of the EGF receptor, can inhibit AR signaling (50) as well as inducing NED in LNCaP cells (29). It is possible that both Wnt signaling and activation of EGF receptor by HB-EGF may stimulate NED of LNCaP cells through inhibition of AR signaling, similar to the effect of androgen deprivation of the culture media. Interestingly, the AR inhibitory function of HB-EGF also requires the PI3K-AKT-mTOR pathway but not the activation of ERK (51), supporting our conclusion that PI3K-AKT-mTOR pathway, not ERK, is critical in NED of PC.

Although our data indicate that the PI3-AKT-mTOR pathway, not ERK, is critical for NE differentiation, the true intracellular process may be more complex, and there may be cross-talk between the two pathways. For example, we have consistently observed increased phosphorylation of AKT after U0126 treatment in androgen-deprived LNCaP cells. Similarly, Zhuang et al. (52) reported that blockade of ERK1/2 by U0126 resulted in an increase in Akt phosphorylation in renal proximal tubular cells treated with H2O2. The potential cross-talk of the two pathways needs more investigation but that is beyond the scope of this study.

Our study shows that activation of the PI3K-AKT-mTOR pathway is necessary and sufficient for NED of PC. Activation of this pathway is required for NED of LNCaP cells induced by either androgen deprivation or epinephrine; and activation of the pathway by either overexpression of a constitutively active AKT or treatment with IGF-1 leads to NED. Inhibition of the pathway at the levels of PI3K, AKT, or mTOR all leads to inhibition of NED. In general, our data showed that inhibition of the pathway did not completely inhibit the increased level of NSE. This may be due to incomplete inhibition of the PI3-AKT-mTOR pathway as phosphorylation of the downstream molecule S6K was not completely inhibited in these experiments. Nonetheless, our study does not exclude the possibility that other pathways may also participate in the NED process.

Currently, multiple clinical trials are in progress targeting PI3K-AKT-mTOR signaling pathway in PC patients based on the observation that this pathway may be a key player in malignant transformation and cell proliferation (33). We show here for the first time that AKT is critically involved in NED of PC after androgen deprivation. This novel finding has important implications in interpreting the results of the clinical trials and should also help investigators in designing future treatment strategies for AI PC. It is reasonable to hypothesize that the combination of hormonal therapy, which induces NED through the PI3K-AKT-mTOR pathway, and an agent targeting the PI3K-AKT-mTOR pathway may suppress the proliferation of PC while inhibiting NED, thus possibly delaying/preventing the emergence of AI PC.

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