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Suppression of Glycogen Synthase Kinase 3 Activity Reduces Tumor Growth of Prostate Cancer In vivo

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BACKGROUND. Glycogen synthase kinase 3 (GSK-3) has been regarded as a potential therapeutic target for multiple human cancers. We previously reported that suppression of GSK-3 activity with lithium chloride (LiCl) or small chemical inhibitors impaired cellular DNA synthesis and reduced cell proliferation in prostate cancer cells. Therefore, in this study, we extended this in vitro findings to in vivo settings in order to establish a proof of concept that inhibition of GSK-3 activity is feasible in suppressing tumor growth of prostate cancer in vivo.
METHODS. In this study, we used three GSK-3 inhibitors, LiCl, TDZD-8, and L803-mts, which are structurally unrelated and non-ATP competitive. Human prostate cancer cell lines PC-3 and C4-2 were used for nude mouse xenograft models. The autochthonous transgenic prostate cancer TRAMP mice were used for testing GSK-3 inhibitor's effect on tumor development. Anti-Ki-67 and BrdU immunohistochemistry was used to determine cell proliferation. The pE2F-TA-LUC (E2F-LUC) luciferase reporter assay and gene specific small interferencing RNA technique were used to examine C/EBP involvement in GSK-3 inhibitor-induced E2F-1 suppression.
RESULTS. Using mouse xenograft models, we demonstrated that LiCl and TDZD-8 significantly suppressed tumor development and growth of subcutaneous xenografts derived from human prostate cancer cells. Similarly, in the TRAMP mice, TDZD-8 and L803-mts reduced the incidence and tumor burden in the prostate lobes. Consistent with our previous in vitro findings, GSK-3 inhibitors significantly reduced BrdU incorporation and Ki67-positive cells in xenograft tumors and mouse cancerous prostates compared to the control. Further analysis revealed that following GSK-3 inhibition, C/EBPα, a negative cell cycle regulator, was remarkably accumulated in xenograft tumors or in cultured prostate cancer cells. Meanwhile, knocking down C/EBPα expression abolished GSK-3 inhibition-induced suppression of E2F1 transactivation, suggesting that C/EBPα accumulation is involved in GSK-3 inhibition-induced anti-tumor effect.
CONCLUSION. Taken together, these results suggest that GSK-3 inhibition has the potential as a therapeutic strategy for prostate cancer intervention, although further pre-clinical and clinical testing are desirable. *Prostate 71: 835–845, 2011.* © 2010 Wiley-Liss, Inc.

KEY WORDS: GSK-3; prostate cancer; C/EBPα; tumor growth; xenograft; TRAMP

INTRODUCTION

Prostate cancer is one of the most common malignancies around the world [1]. The mainstays of treatment for advanced prostate cancers remains the removal of androgens, known as androgen ablation [2]. Unfortunately, for reasons not completely understood, essentially all patients become hormone refractory, a condition known as castration resistant prostate cancer, with no means to cure [3]. This condition ultimately leads to death usually at a median of 2 years after its development [4]. Thus, protein kinase-targeted therapy that have the ability to slow down disease progression at the end-stage of castration-resistant clinical diseases would provide additional hope in controlling advanced prostate cancers [5,6].

Glycogen synthase kinase 3 (GSK-3) is a family of serine/threonine kinases expressed ubiquitously, and consists of two isoforms in humans, namely GSK-3α and GSK-3β [7]. They have 97% sequence homology within their kinase domains, but GSK-3α has an extended N-terminal glycine-rich tail. Unlike other protein kinases, GSK-3 is constitutively active and its phosphorylation upon the substrates usually results in inactivation and proteolytic degradation, such as β-catenin and snail [8]. It has been shown that GSK-3 regulates a wide range of cellular functions, including glycogen metabolism, transcription, translation, cytoskeletal regulation, intracellular vesicular transport, cell cycle progression, and apoptosis [9].

Previous studies have shown a link of GSK-3 overexpression or deregulation with human cancer development and progression [10]. In human cancer tissues, high levels of GSK-3α mRNA/protein were found in thymus and reproductive organs including prostate compared to other organs [11–14]. Compared to other cell lines, human prostate cancer PC-3 cells posses a higher GSK-3α kinase activity in parallel with enhanced tyrosine phosphorylation [15]. Meanwhile, cytoplasmic accumulation of GSK-3β protein in prostate cancers was found to correlate with disease progression [16]. In agreement with previous reports, we also found aberrant GSK-3β activation (Y216 phosphorylation) in highly aggressive prostate cancer cells [17]. Consistently, suppressing GSK-3 activity reduced prostate cancer cell proliferation in vitro [18–20].

Due to the high therapeutic potential of targeting GSK-3 in many different human diseases, so far more than 30 GSK-3 inhibitors have been identified or synthesized [21]. There are two groups of GSK-3 inhibitors, ATP competitive and non-ATP competitive. As a clinical drug for mental disease, lithium ion is a non-ATP competitive GSK-3 inhibitor [22,23]. Interestingly, lithium uptake significantly reduced cancer incidence compared to the controls both in clinical observation and animal studies [24–26], indicating a possible value of lithium in human cancer intervention. Consistently, we found that lithium could inhibit prostate cancer cell growth in vitro by attenuating DNA replication [18].

Because of the close relationship between GSK-3 and cyclin-dependent kinases (CDKs), any given ATP competitive GSK-3 inhibitors often interfere with CDK activity. By contrast, non-ATP competitive GSK-3 inhibitors, such as lithium ion, TDZD-8, and short peptide L803-mts, have no effect on CDKs or other protein kinases [21,27,28]. TDZD-8 is a non-ATP competitive synthetic small molecule [27]. Peptide inhibitor L803-mts was designed as a pseudo-substrate to compete with endogenous proteins that can be phosphorylated by GSK-3 [28]. It has been shown that L803-mts selectively suppresses GSK-3 activity in vitro and in vivo [28,29]. This unique feature is more clinically relevant, because this type of inhibitors will be more specific to GSK-3 substrates and hence the potential side effect due to a broader GSK-3/CDKs inhibition will be significantly limited once implied in vivo systemically [21]. In agreement with this notion, no side effect (psychological and physiological parameters) was observed when L803-mts was delivered chronically in mice [29].

Since we previously found that GSK-3 inhibition attenuated cell proliferation of prostate cancer in vitro [18], in this study, we sought to extend these in vitro findings to an in vivo setting to explore the anti-tumor potential of GSK-3β inhibitors in prostate cancer. Using mouse xenograft model and a spontaneous mouse prostate cancer model, we demonstrated that GSK-3 inhibitors significantly suppressed tumor development and growth. We also documented that CCAAT/enhancer binding protein alpha (C/EBPα) is accumulated in GSK-3 inhibitor-treated prostate cancer cells, which might represent a mechanism involved in GSK-3 inhibitor-induced anti-tumor effect.

MATERIALS AND METHODS

Cell Culture, Antibodies, and Reagents

PC-3 and C4-2 cells were maintained in a humidified atmosphere of 5% CO_2, RPMI 1640 supplemented with 10% fetal bovine serum (FBS) plus antibiotics (Invitrogen, Carlsbad, CA). TDZD-8 was obtained from Calbiochem (San Diego, CA). L803-mts (*N*-Myristol-GKEAPPAPPQSpP) was synthesized by GeneMed Synthesis as previously described [28]. Antibodies for C/EBPα (clone G-10), C/EBPβ (clone c-19), Ki-67 (clone H-300), and Actin were purchased from Santa Cruz Biotech (Santa Cruz, CA). Lithium chloride (LiCl) and

other chemicals were purchased from Sigma (St. Louis, MO). Where indicated the inhibitor was added to the cell culture media from a 1,000-fold concentrated stock. Control cultures received similar amounts of the solvent only. Final concentrations of the solvent did not exceed 0.1%.

Animal Experiments and Immunohistochemistry

All animal studies were conducted under an approved Institutional Animal Care and Use Committee protocol. For xenograft experiments in nude mice, PC-3 or C4-2 cell suspension (2×10^6 cells in 0.1 ml of RPMI 1640 media) were inoculated subcutaneously (s.c.) into the rear flanks (2 sites/mouse) of 6-weeks-old male nude mice (Charles River, Wilmington, MA).

For the protocol A of LiCl treatment, animals were randomly divided into two groups (n = 5) on next day after PC-3 cell inoculation. One group received intra-peritoneal (i.p.) LiCl injection at a daily dose of 2.0 mg/kg bodyweight. LiCl was dissolved in PBS. Control animals received PBS alone in the same volume. Xenograft development and growth were recorded by measuring tumor dimensions twice a week with a caliper. Tumor volume was calculated by the formula of $L \times W \times H \times 0.5236$ [28]. Treatment lasted for 8 weeks. At the end of treatment, tumors were extirpated, and the final tumor size and weight, as well as animal weight were measured and recorded.

For the protocol B, treatment began once xenograft tumors became palpable or 30 mm^3 in size, LiCl or PBS injection was conducted as above for a 2-week course. Tumor size and wet-weight were measured at necropsy.

For TDZD-8 treatment, when PC-3 or C4-2 xenografts were in size of 30 mm^3, animals were randomized into two groups (n = 10). TDZD-8 was dissolved in a slow-releasing formula containing 50% *N,N*-dimethylacetamide (DMA), 45% polyethylene glycol 400 (PEG-400), and 5% Tween-80. Drugs or the solvent in 100 µl volume was injected intraperitoneally (i.p.) three times a week. TDZD-8 was used at a dose of 4.0 mg/kg bodyweight per injection. Tumor growth and measurement were monitored as above.

For the TRAMP model system, C57BL/6-TRAMP mice were obtained from NCI-Frederick Mouse Models of Human Cancer Consortium Repository and maintained as instructed. Male transgenic mice at age of 10 weeks were used in the experiments. TDZD-8 (4.0 mg/kg bodyweight), L803-mts (1.0 µM/injection), or the solvent in 100 µl volume was injected i.p. three times a week for a period of 4 weeks. L803-mts were dissolved in the same slow-releasing solvent as TDZD-8. At the end of experiments, necropsy was performed on all animals under anesthesia with ketamine/xylazine mixture. All prostate lobes together with seminal vesicles were harvested. The wet-weight of prostate lobes plus seminal vesicles was recorded after dissecting from the surrounding tissues like the urinary bladder and urethra tract. Prostate wet-weight was normalized against animal body weight individually before statistic analysis. All other major organs were inspected for frank toxicity or evidence of metastasis. Harvested tissues were fixed in 4% buffered para-formaldehyde overnight and then subjected to paraffin embedding. H&E staining was performed for histological analysis.

BrdU Labeling Assay, Immunohistochemistry, and Western Blot Assay

For BrdU in vivo labeling, the animals were injected i.p. with 0.5 ml of a 10-mM BrdU solution 2 hr before necropsy/sacrifice. To detect BrdU labeled cells in tissues, paraffin-embedded tissue sections were immunostained for incorporated BrdU using ZYMED® BrdU Staining Kit (Invitrogen). To detect apoptotic cell death in xenograft tumors, terminal deocynucleotide transferase dUTP nick end labeling (TUNEL) assay was conducted using Apo-BrdU-IHC™ In Situ DNA Fragmentation Assay Kit (BioVision, Mountain View, CA). Both assays were carried out as described in our previous publication [30].

Immunostaining for anti-Ki-67 and anti-C/EBPα was conducted as described in our previous publication [30]. Briefly, paraffin-embedded tissue sections were de-paraffinized and re-hydrated. Antigen recovery was achieved by boiling the sections in citrate acid buffer (10 mM, pH 6.0). The sections were incubated with the primary antibodies overnight at 4°C and visualized using a Zymed™ LAB-SA Detection System (Invitrogen).

For Western blot, after treatment, cells were harvested, rinsed with PBS, and lysed on ice in a radio-immunoprecipitation assay (RIPA) buffer (Cell Signal, Inc., MA). Equal amount of proteins from each lysates was loaded onto SDS–PAGE gels and immunoblotted (IB) with antibodies as indicated in the figures.

siRNA Transfection and Luciferase Gene Reporter Assay

The Silencer® Select Validated siRNAs against human C/EBPα and C/EBPβ genes were purchased from Ambion, Inc. (Austin, TX) and used according to the manufacturer's manual. The pE2F-TA-LUC (E2F-LUC) reporter construct and the internal control reporter pCMV-SEAP (CMV-SEAP) were described in our previous publications [17,18]. Cells plated in 6-well tissue culture plates were transfected with the siRNAs for 2 days, followed by another transfection with the

reporter constructs using the Cytofectene™ reagent (Bio-Rad, Hercules, CA). After treatment, culture supernatants and cell lysates were collected for reporter assays. The luciferase reporter activity of each sample was normalized against the corresponding SEAP activity and protein content prior to calculation of the fold induction value relative to the controls.

RNA Extraction and Reverse Transcription-PCR

Total cellular RNAs were prepared using TriZol reagent (Invitrogen). To assess mRNA expression, a reverse transcription-PCR (RT-PCR) method was used as described in our previous publication [18]. RT-PCR was carried out using a RETROscript Kit (Ambion) as per manufacturer's instructions. The primers and PCR conditions were described as follows: for human C/EBPα gene (forward 5′-aaccttgtgccttggaaatg-3′; backward 5′-ccctatgtttccaccccttt-3′; Ref. [4]); human C/EBPβ gene (forward 5′-gacaagcacagcgacgagta-3′; backward 5′-agctgctccaccttcttctg-3′). Human S18 ribozyme RNA (forward 5′-gttcacccactaatagggaac gtg-3′; backward 5′-gattctgacttagaggcgttcagt-3′) was used as the internal control. The primers were synthesized by IDT (Coralville, IA). The amplification profile was as follows: 95°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min running in a total of 25 cycles. After amplification, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

Statistical Analysis

All cell culture-based experiments were repeated three times. Western blotting and immunostaining results are presented from a representative experiment. The mean and SEM for tumor growth, prostate wet-weight, BrdU labeling, Ki-67, and C/EBPα immunostaining index as well as luciferase reporter activities were shown. The significance of differences between groups were analyzed as described in our previous publications [17,18,30] using the SPSS computer software (SPSS, Inc., Chicago, IL).

RESULTS

GSK-3 Inhibition Reduces Tumor Growth of Prostate Cancer Xenografts in Nude Mice

We and others have shown that GSK-3 inhibition reduces androgen receptor (AR)-mediated gene expression, AR protein levels and cell proliferation in androgen-responsive prostate cancer cell lines in vitro [17–20]. In this study, we extended these in vitro findings to an in vivo setting, xenograft models. We utilized two castration-resistant prostate cancer cell lines, the AR-positive C4-2 and the AR-negative PC-3, in our experiments. For LiCl treatment, we used two different strategies (protocols A and B), of which protocol A was to assess the xenograft tumor development/growth, while protocol B was to assess the effect of GSK-3 inhibition on growth rate of existing tumors. Therefore, in protocol A GSK-3 inhibitor LiCl was delivered at the day after PC-3 cell inoculation and in protocol B LiCl treatment was started when the xenografts were established (palpable or 30 mm^3 in size). In both protocols, LiCl was delivered at a daily dose of 2.0 mg/kg of bodyweight. This lithium dose level was based on a previous report where a biological effect was observed in rat models [31], and is more than 100-fold lower that a therapeutic dose for mental diseases in mouse [32] or human [33]. In both protocols, no obvious abnormality of daily activities, such as apathy, asthenia, movement, drinking, eating, etc. or any sign of side effect were observed from LiCl-treated animals. There was no significant difference of animal body weight between treatment and control groups.

In protocol A, PC-3 xenografts were developed around 4 weeks after inoculation in PBS-treated control animals with a 100% intake rate. However, in LiCl-treated animals, palpable xenograft development was delayed until 6–7 weeks after inoculation (2 in week 6 and 2 in week 7, intake rate at 80%). When comparing tumor wet-weight at the end of 8-week treatment between these two groups, LiCl-treated animals showed a significant reduction compared to PBS control (Fig. 1A). In protocol B, when PC-3 xenografts were palpable, animals were treated with LiCl for 2 weeks. Tumor wet-weight at the end of treatment was compared between LiCl-treated and PBS control groups. As shown in Figure 1A, LiCl treatment also significantly reduced tumor wet-weight compared to the PBS control.

Next, since lithium also suppresses other enzymes besides GSK-3 [21–23], we used the ATP-non-competitive GSK-3-specific small molecule inhibitor TDZD-8 to verify GSK-3 inhibition-induced in vivo anti-tumor effect on prostate cancer. Treatment was initialed when PC-3 xenografts were around 30 mm^3 in size for a 4-week period. As shown in Figure 1B,C, TDZD-8 significantly reduced xenograft wet-weight and size compared to the solvent control. In addition, we also tested TDZD-8-induced tumor suppression in C4-2-based xenografts. As shown in Figure 2C, a remarkable suppression of tumor growth was achieved in TDZD-8-treated animals compared to the solvent control. These data clearly demonstrated that GSK-3 inhibition attenuated xenograft tumor formation and growth in vivo.
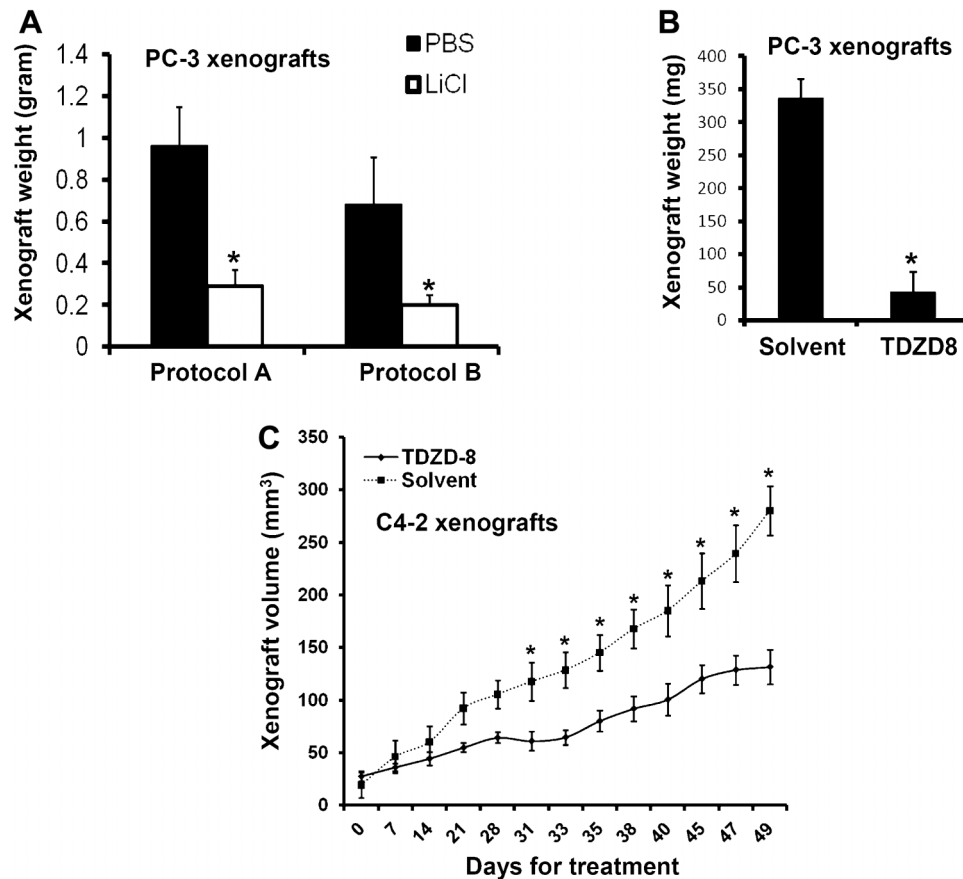
Fig. 1. GSK-3 inhibitors suppress tumor development and growth. To generate xenograft tumors, exponentially growing PC-3 or C4-2 cells (2×10^6) were trypsinized and resuspended in RPMI 1640 media for subcutaneous inoculation into rear flanks of 6-week nude mice. **A**: For protocol A, on next day following PC-3 cell inoculation, animals (n = 5) were divided into two groups and were injected i.p. with 100 μl of PBS as the solvent control or LiCl (2.0 mg/kg body weight dissolved in PBS) daily. Xenograft tumor development and growth were monitored for 8 week. For protocol B, once PC-3 xenografts were established in nude mice (around 30 mm^3), animals were divided into two groups (n = 5) and were treated with PBS or LiCl as above for 2 weeks. **B,C**: For TDZD-8 treatment, animals bearing PC-3 (B) or C4-2 (C) xenograft tumors at a size of around 30 mm^3 were divided into two groups and treated with TDZD-8 (4.0 mg/kg bodyweight, n = 4) or the solvent (n = 5) for 4 (B) or 7 (C) weeks. At the end of experiments, xenograft tumors were dissected and their wet-weights were recorded at necropsy. Data are shown as means ± SEM. The asterisk indicates a significant difference (ANOVA analysis, $P < 0.05$) compared to the control.

Meanwhile, we determined if LiCl- or TDZD-8-induced tumor suppression was associated with reduced DNA synthesis and cell proliferation, as shown in our recent publication [18]. DNA synthesis was measured by an in vivo BrdU incorporation assay. Meanwhile, the cell proliferation hallmark Ki-67 was included and the apoptotic event was analyzed using the TUNEL assay. BrdU was injected 2 hr before animal was sacrificed. As shown in Figure 2A, both LiCl and TDZD-8 treatment dramatically reduced the BrdU labeling in PC-3 xenograft tumors compared to the solvent control (panels b and c vs. a). Similarly, Ki-67 positive cells decreased dramatically in treated tumors compared to that in the control (panels e and f vs. d). However, TUNEL staining did not shown any noticeable difference between the treatment and the control groups (panels g–i). Quantitative data from each group

was shown in Figure 2B. These data suggest that suppression of GSK-3 activity reduced tumor cell proliferation in vivo, which is in agreement with our previous in vitro data [18].

GSK-3 Inhibition Reduces Prostate Cancer Development and Growth in TRAMP Mice

Next, we assessed GSK-3 inhibition-induced antitumor effect in the autochthonous prostate cancer TRAMP model [34]. In addition to the small molecule TDZD-8, the peptide inhibitor L803-mts was also included to confirm that GSK-3 specific inhibition suppresses tumor development and growth in vivo as observed in the xenograft experiments (Fig. 1). At the age of 10 weeks when the transgene SV-40 large-T antigen is fully activated [34], TRAMP animals were
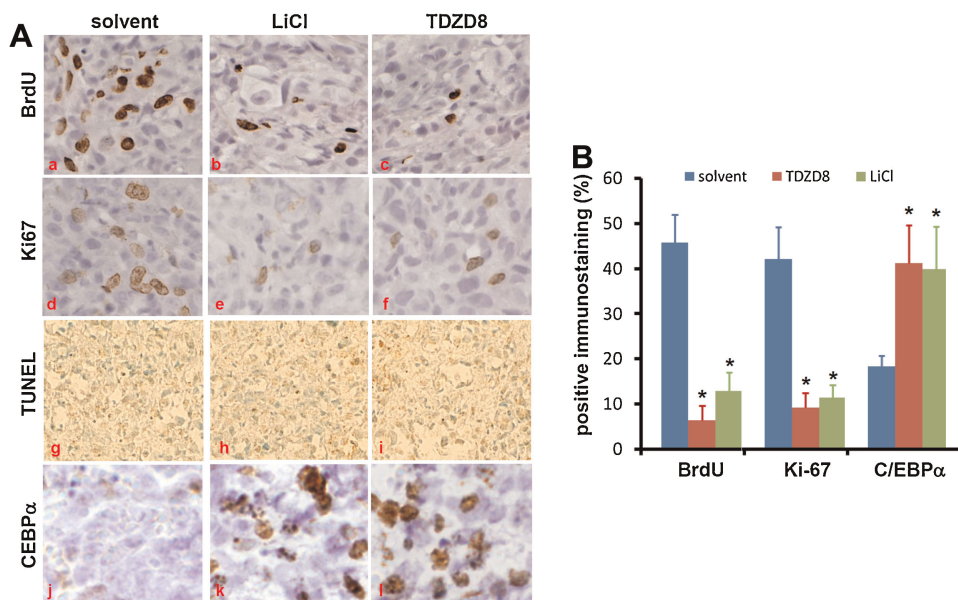
Fig. 2. GSK-3 inhibitors reduce tumor cell proliferation and cause C/EBPα accumulation. **A**: The in vivo BrdU labeling assay was conducted by injection of 500 μl of l0 mM BrdU solution 2 hr before necropsy. After treatment as described in Figure IA (protocol A) and Figure IB, tumor specimens were harvested and paraffin-embedded. Tissue sections were made at 4 μM thickness for immunostaining. BrdU-labeled cells (panels a−c), Ki-67 expression (panels d−f), TUNEL assay for apoptotic events (panels g−i), and C/EBPα expression (panels j−l) were examined with standard immunostaining protocols and positive labeling was developed with DAB. After immunostaining, tissue sections were counter-stained with hematoxylin except in the TUNEL assay with methyl green. Microscopic images were taken at magnification of 400×. **B**: Quantitative data for all immunostaining and TUNEL assay from a total of l0 microscopic fields are shown as means ± SEM as described in our previous publication [30]. The asterisk indicates a significant difference (Student's t-test, $P < 0.0l$) compared to the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

treated with TDZD-8 and L803-mts for a 4-week period. The relative prostate weight, a ratio of prostate wet-weight versus body weight, was compared between the treatment and the control groups. At necropsy, there was no obvious difference in other organs including testis, liver, spleen, kidney, etc., but the genital organs including seminal vesicle and prostate lobes were noticeably smaller in L803-mts-treated animals than that in age-matched control animals. A representative photo was shown in Figure 3A. While there was no statistic difference in animal body weight among the control or treatment groups (Fig. 3B), the relative prostate wet-weight in L803-mts or TDZD-8-treated animals was significantly reduced compared to the control (Fig. 3C). Histological examination of the prostate lobes according to a previous approach [35] revealed that the incidences of prostatic intraepithelial neoplasia (PIN) and carcinoma (PCa) were significantly lower in L803-mts or TDZD-8-treated animals than that in control group (Fig. 3D).

We then examined the effect of GSK-3 inhibition on DNA synthesis (BrdU labeling assay) in the prostate tissues from TRAMP mice. As shown in Figure 4A, BrdU positive cells in PIN and PCa tissues were dramatically reduced in L803-mts or TDZD-8-treated prostates compared to the control animals. Quantita-tive data were shown in Figure 4B. Taken together, these data suggest that suppression of GSK-3 activity reduced prostate cancer development and growth in vivo by suppressing tumor cell proliferation.

GSK-3 Inhibition Results in C/EBPα Protein Accumulation in Prostate Cancer Cells

C/EBPα is a member of the basic leucine zipper transcription factor family and has been reported to induce cell cycle arrest by increasing CDK inhibitor p21^{cip1} expression and by blocking S-phase factor E2F-mediated gene transcription [36]. In our previous report [18], we showed that GSK-3 inhibition disrupted E2F1–DNA interaction and up-regulated p21^{cip1} expression. Since C/EBPα is a GSK-3 substrate [37,38] and GSK-3 phosphorylation usually results in its sub-strate degradation [9], we hypothesized that C/EBPα is accumulated in GSK-3 inhibitor-treated prostate cancer cells.

To test this hypothesis, we examined C/EBPα protein level after GSK-3 inhibition. We first examined the tissue expression of C/EBPα protein in PC-3 xenograft tumors after LiCl treatment. As shown in Figure 2A (panels j and i) and Figure 2B, C/EBPα protein levels increased dramatically in LiCl- or TDZD-
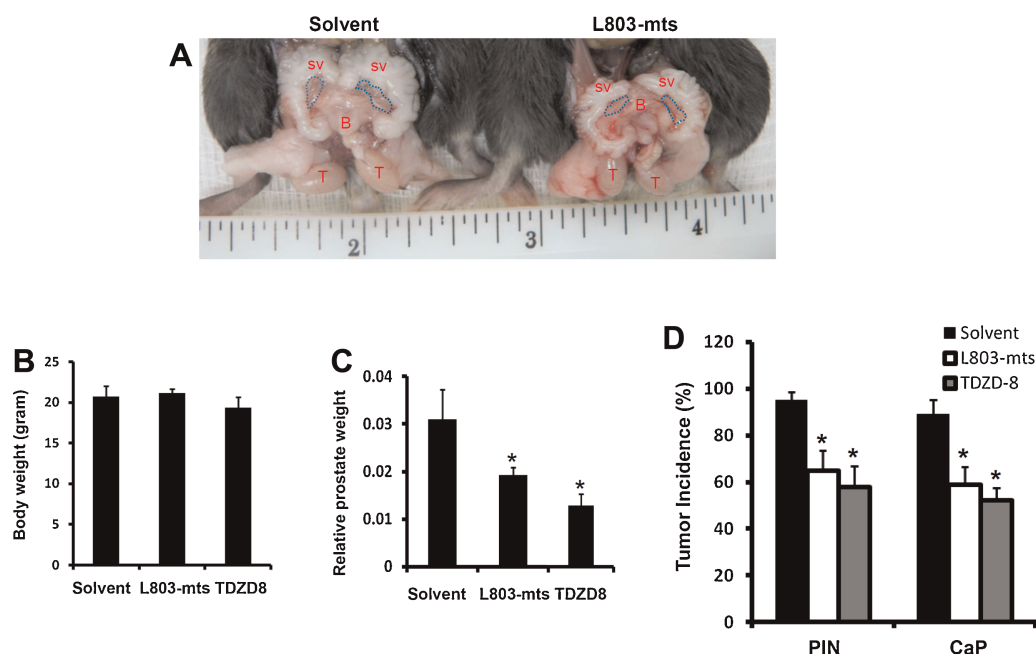
Fig. 3. GSK-3 inhibitors suppress prostate cancer development and growth in TRAMP mice. Male TRAMP mice at age of 10 weeks were randomly assigned into three groups (n = 10) and received i.p. injection of the solvent, L803-mts (1.0 μM/injection) or TDZD-8 (4.0 mg/kg body weight) three times a week for 4 weeks. Animals were sacrificed and prostate lobes were dissected. A representative photo for a side-by-side comparison purpose was taken from two mice received the solvent or L803-mts treatment, respectively (**A**). Please note that the glossy mass of the genital organs, especially the seminal vesicle and anterior prostate lobes, are largely reduced in L803-mts-treated animal as compared to the solvent-treated animal (A). Notes: circled area with dotted line indicates anterior prostate lobe (AP); B, urinary bladder; SV, seminal vesicle; T, testis. Quantitative data for animal body weight (**B**), the relative prostate wet-weight (prostate lobes/body weight, **C**), and tumor incidence (**D**) were shown as means ± SEM. The asterisk indicates a significant difference (ANOVA analysis, $P < 0.05$) compared to the solvent control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

8-treated tumor compared to the solvent control. Then, we examined C/EBPα protein level in prostate cancer cells following GSK-3 inhibition. PC-3 cells were treated with TDZD-8 for 2–4 hr. The levels of C/EBPα and C/EBPβ proteins were assessed in Western blotting assay. The basal level of C/EBPα protein was very low. However, TDZD-8 addition dramatically increased C/EBPα protein level as early as 2 hr (Fig. 5A). In a sharp contrast, C/EBPβ protein was expressed at a relatively higher level under the basal condition and remained unchanged after TDZD-8 addition. Similarly, L803-mts treatment also dramatically increased C/EBPα but not C/EBPβ protein level at a dose-dependent manner (Fig. 5B). Consistently, all these inhibitors also increased C/EBPα but not C/EBPβ protein levels in C4-2 cells (Fig. 5C). However, there was no alteration at the mRNA levels for the expression of C/EBPα gene after GSK-3 inhibition (Fig. 5D), suggesting that GSK-3 inhibition-induced C/EBPα accumulation is due to reduced protein degradation.

Then, to test this hypothesis, we conducted a protein pulse-chase experiment using cycloheximide (CHX), a protein translation inhibitor, to assess the effect of L803-mts on C/EBPα protein stability. PC-3 cells were treated with CHX in the presence or absence of L803-mts for up to 8 hr. As shown in Figure 5E, C/EBPα protein level decreased rapidly in CHX-treated cells, while L803-mts addition dramatically slowed down the decent of C/EBPα protein level compared to CHX alone. These data strongly suggest that suppression of GSK-3 activity increased C/EBPα protein stability.

Since we have previously shown that E2F1 activity is suppressed by GSK-3 inhibitors in prostate cancer cells [18], we then examined if C/EBPα is involved in GSK-3 inhibitor-induced E2F1 suppression. E2F1 transactivation was assessed in a reporter gene assay, as described [18]. Gene expression was knocked down using gene-specific siRNA approach as described in our previous publications [17,18]. PC-3 cells were transfected with the control or siRNAs specifically to C/EBPα or β genes for 2 days and then transfected again with E2F-LUC/CMV-SEAP reporters overnight. Cells were then treated with the solvent or L803-mts for 24 hr. As shown in Figure 6A, L803-mts treatment induced C/EBPα protein accumulation in control siRNA-transfected cells, which was abolished by C/EBPα siRNA transfection (lane 4 vs. 2 in C/EBPα panel). Consistent with our previous report [18], L803-mts significantly suppressed E2F-LUC activity (Fig. 6B). However, transfection of the C/EBPα siRNAs but not the control
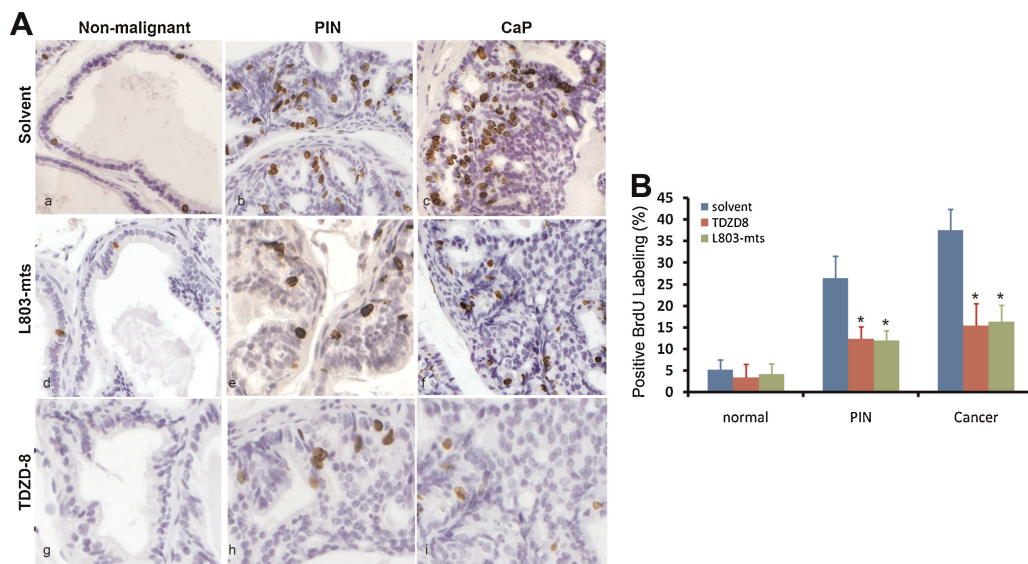
Fig. 4. GSK-3 inhibitors suppress cell proliferation of prostate cancer inTRAMP mice. **A**: Tissue sections of ventral prostate lobes harvested from the animals treated with the solvent, L803-mts or TDZD-8 as described in Figure 3 were subjected to anti-BrdU immunostaining. The in vivo BrdU labeling assay was conducted as described in the legend of Figure 2. The representative microscopic images were taken from ventral prostate lobes with non-malignant, PIN lesion, and carcinoma at magnification of 200×. **B**: Quantitative data for BrdU labeled cells from a total of 10 microscopic fields are shown as means ± SEM. The asterisk indicates a significant difference (ANOVA analysis, $P < 0.05$) compared to the solvent control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
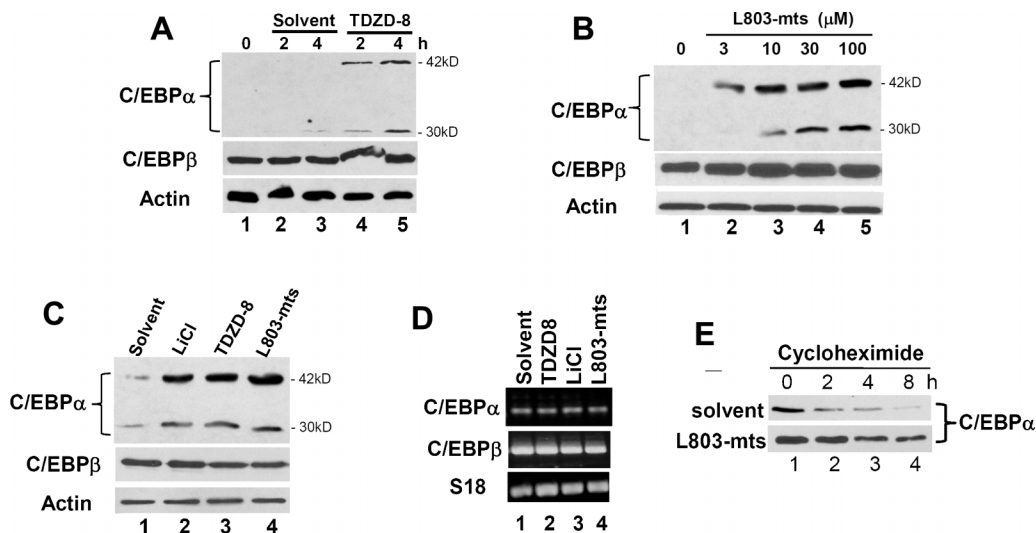
Fig. 5. GSK-3 inhibition increases C/EBPα protein stability. **A**,**B**: PC-3 cells were serum-starved for 24 hr and then treated with the solvent, TDZD-8 at 10 μM (A), or L803-mts at increasing doses for 8 hr (B) in 10% FBS-supplied media. Equal amount of protein extracts was subjected for Western blotting with the antibodies as indicated on the left side of the panels. **C**: Serum-starved C4-2 cells were treated with the solvent, LiCl (10 mM), TDZD-8 (10 μM), or L803-mts (100 μM) for 8 hr. Cells were harvested and equal amount of protein extracts were subjected to Western blotting assays. Actin blots were used as protein loading control. **D**: After treatment, PC-3 cells were harvested and total cellular RNAs were prepared using TriZol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) assay was conducted as described in our previous publication [18] to assess gene expression at the mRNA level. After amplification, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide. **E**: Exponentially growing PC-3 cells were treated with cycloheximide (2.0 μg/ml) in the presence or absence of L803-mts (100 μM) for 8 hr in 10% FBS-supplied media. C/EBPα protein levels were assessed with Western blotting assay. All data represent three independent experiments.
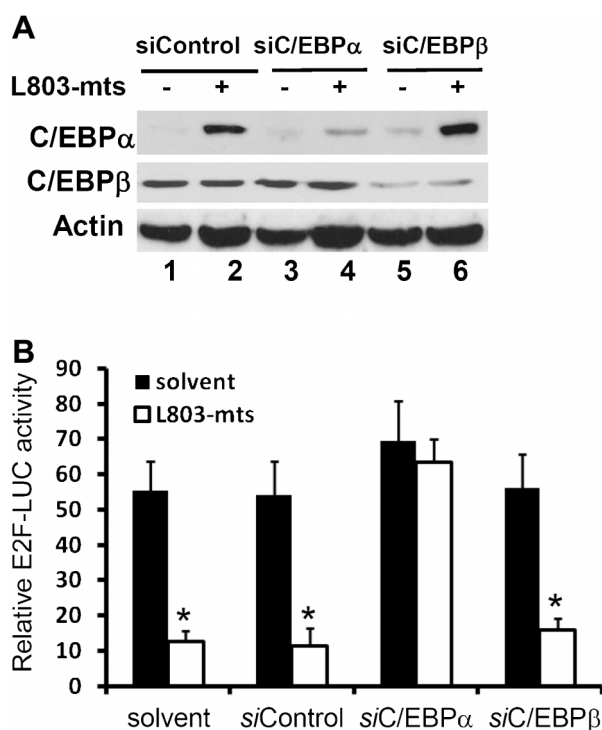
Fig. 6. C/EBPα is involved in GSK-3 inhibitor-induced E2F suppression in prostate cancer cells. PC-3 cells were transfected with the siRNAs (50 nM in the media) as indicated for 48 hr, followed by another transfection with E2F-LUC/CMV-SEAP reporters overnight. Cells were then treated with the solvent or L803-mts (100 μM) for 24 hr. Equal amount of protein extracts was subjected to Western blotting assay with the antibodies as indicated (**A**) or was used for E2F-LUC reporter assays as described [18]. The relative E2F-LUC activities were shown as means ± SEM (**B**). Data represents three independent experiments. The asterisk indicates a significant difference (ANOVA analysis, $P < 0.01$) compared to the solvent control.

or C/EBPβ siRNAs abrogated L803-mts-induced E2F1 suppression (Fig. 6B). These data indicate that C/EBPα protein is involved in GSK-3 inhibition-induced E2F1 suppression in prostate cancer cells.

DISCUSSION

The goal of this study was to establish a proof of concept that suppression of GSK-3 activity leads to reduced tumor development and growth of prostate cancer in vivo. We used two animal models, subcutaneous xenograft and spontaneous TRAMP tumor. In mouse xenograft models, GSK-3 inhibition suppressed xenograft tumor development and growth. Again, with the TRAMP model, GSK-3 inhibitors reduced the tumor burden of cancerous prostate tissues in mice. These anti-tumor effects were associated with impaired DNA synthesis and reduced cell proliferation as

assessed by BrdU labeling and Ki-67 immunostaining. These data are in agreement with our previous findings that GSK-3 inhibitors reduced prostate cancer cell proliferation in vitro by blocking S-phase gene expression [18], as well as other reports from different groups [19,20]. In conjunction with other reports that GSK-3 inhibitors have therapeutic potential for human cancers through various mechanisms [39,40], our results provided additional support to the notion that GSK-3 inhibitors might be developed as new agents for prostate cancer intervention, particularly for late-stage advanced diseases.

Most importantly, in this study, we documented that GSK-3 inhibition resulted in accumulation of C/EBPα protein. Sequential analysis revealed that C/EBPα accumulation is responsible for GSK-3 inhibitor-induced suppression of E2F1 transactivation. Although most of previous studies regarding C/EBPα function are related to adipocyte differentiation, emerging data suggest that it might also act as a tumor suppressor [41]. In prostate cancer cells, C/EBPα has been found to negatively regulate AR-mediated gene expression and cell proliferation in prostate cancer cells [42–44]. In this study, we found that C/EBPα protein levels increased rapidly but the mRNA levels remained unchanged following GSK-3 inhibition, suggesting that GSK-3 inhibition reduced C/EBPα protein degradation. Since GSK-3 has been shown to phosphorylate C/EBPα [38] and GSK-3-mediated phosphorylation usually results in its substrate degradation via ubiquitin-proteasome pathway [9], it is plausible that GSK-3 inhibition resulted in reduced C/EBPa phosphorylation and subsequently proteasome-mediated degradation. On the other hand, a previous report showed that lithium-induced C/EBPα accumulation in a mouse keratinocyte model was due to reduced proteasome-dependent proteolytic degradation, but GSK-3's role in C/EBPα stability was not clearly defined in their study [45]. This inconsistency suggests a cell-specific difference in C/EBPα regulation. Nonetheless, our data indicated C/EBPα accumulation is involved in GSK-3 inhibition-induced cytostatic effect in prostate cancer cells, although the detailed mechanism of GSK-3-dependent regulation of C/EBPα protein stability requires further investigation.

Currently, advanced prostate cancers after escaping androgen ablation therapy are virtually no means to cure. Therefore, agents that target unique cellular signal pathways such as GSK-3 might represent novel therapeutic targets for these patients. Furthermore, considering that most of prostate cancer patients with advanced disease are at their late stage of life, kinase-targeted inhibitors like GSK-3 inhibitors TDZD-8 and L803-mts that can slow down tumor growth, might be able to reduce mortality and to improve quality of life.

In conclusion, we documented in this study that suppression of GSK-3 activity reduced prostate cancer development and growth in vivo, and increased C/EBPα protein stability might be involved in GSK-3 inhibitors-induced suppression of tumor cell proliferation. Our results indicated that GSK-3 inhibition might be a potential strategy in managing advanced prostate cancers although further pre-clinical and clinical testing are desirable as described in a recent overview paper [46], and a clinical trial evaluating the effects of lithium in the prostate in patients being treated for prostate cancer is currently being planned by our group.

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