Influence of zinc deficiency on Akt-Mdm2-p53 and Akt-p21 signaling axes in normal and malignant human prostate cells

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Han CT, Schoene NW, Lei KY. Influence of zinc deficiency on Akt-Mdm2-p53 and Akt-p21 signaling axes in normal and malignant human prostate cells. Am J Physiol Cell Physiol 297: C1188–C1199, 2009.—Phosphorylated Akt (p-Akt), a phosphoinositide-3-OH-kinase-activated protein kinase, is highly expressed in prostate tumors. p-Akt can indirectly hinder p53-dependent growth suppression and apoptosis by phosphorylating Mdm2. Alternatively, p-Akt can directly phosphorylate p21 and restrict it to the cytoplasm for degradation. Because the prostate is the highest zinc-accumulating tissue before the onset of cancer, the effects of physiological levels of zinc on Akt-Mdm2-p53 and Akt-p21 signaling axes in human prostate epithelial cells (PrEC) and malignant prostate LNCaP cells were examined in the present study. Cells were cultured for 6 days in low-zinc growth medium supplemented with 0 [zinc-deficient (ZD)], 4 [zinc-normal (ZN)], 16 [zinc-deficient (ZA)], or 32 [zinc-supplemented (ZS)] μM zinc. Zinc status of both cell types was altered in a dose-dependent manner, with LNCaP cells reaching a plateau at >16 μM zinc. For both cell types, p-Akt was higher in the ZD than in the ZN cells and was normalized to that of the ZN cells by treatment with a PI3K inhibitor, LY-294002. PTEN, an endogenous phosphatase targeting Akt dephosphorylation, was hyperphosphorylated (p-PTEN, inactive form) in ZD PrEC. Nuclear p-Mdm2 was raised, whereas nuclear p53 was depressed, by zinc deficiency in PrEC. Nuclear p21 and p53 were lowered by zinc deficiency in LNCaP cells. Higher percentages of ZD, ZA, and ZS than ZN LNCaP cells were found at the G0/G1 phase of the cell cycle, with proportionally lower precentages at the S and G2/M phases. Hence, the increased p-PTEN in ZD PrEC would result in hyperphosphorylation of p-Akt and p-Mdm2, as well as reduction of nuclear p53 accumulation. For ZD LNCaP cells, Akt hyperphosphorylation was probably mediated through p21 phosphorylation and degradation, thus restricting p21 nuclear entry to induce cell cycle arrest. Thus zinc deficiency differentially modulated the Akt-Mdm2-p53 signaling axis in normal prostate cells vs. the Akt-p21 signaling axis in malignant prostate cells.

Akt, or protein kinase B, is known for promoting tumorigenesis by phosphorylating proteins involved in pathways regulating apoptosis and proliferation via phosphoinositide-3-OH-kinase (PI3K) signaling (40). Phosphatase and tensin homolog (PTEN) can dephosphorylate phosphatidylinositol 3,4-bisphosphate (PI(2P)) and phosphatidylinositol 3,4,5-trisphosphate (PI(3P)), thus antagonizing PI3K activity and inhibiting Akt phosphorylation. Phosphorylation on three residues (Ser380, Thr382, and Thr383) of the PTEN tail inactivates its phosphatase activity; therefore, unphosphorylated PTEN is the active form. Hence, PTEN activity is regulated not only by expressed protein levels, but also by the ratio of the phosphorylated to the unphosphorylated form of the protein (41, 44, 45).

The phosphorylated form of Akt (p-Akt) has been shown to interact with Mdm2 and phosphorylate Ser166 and Ser186 on this protein (18). These Akt phosphorylation sites on Mdm2 are close to its nuclear localization signal (NLS) and nuclear export signal (NES) domains, which are critical in determining the import and export, respectively, of Mdm2 between the nucleus and the cytoplasm. In this context, Akt-mediated phosphorylation of Mdm2 (p-Mdm2) leads to its nuclear localization (31, 32, 52).

Mdm2, a ubiquitin E3 ligase, is known for its role in controlling the levels and activity of p53 (32). Once it is phosphorylated by p-Akt, p-Mdm2 translocates to the nucleus and forms a complex with p53 and p300 to promote the ubiquitin-dependent degradation of p53 (30, 31, 52). As a result, p-Akt may indirectly hinder p53-dependent growth suppression and apoptosis, thus leading to cell survival (33). Apart from the role of Akt in the Mdm2-p53 signaling axis, Akt can also promote cell survival through p21, a protein whose gene expression is regulated by p53. p-Akt can phosphorylate p21 within its NLS domain, thus restricting p21 to the cytoplasm for degradation through a p53-independent pathway (52). Furthermore, p-Akt has been shown to promote cell cycle progression by limiting the amount of nuclear p21 that can bind and activate the cyclin D-cyclin-dependent kinase-4 (CDK4) complex (27).

p-Akt has been suggested as a biomarker for prostate cancer (2, 25). Supraphysiologial levels of zinc (50–200 μM) have been shown to stimulate the phosphorylation of Akt in prostate cells, fibroblasts, adipocytes, and lung epithelial cells (3, 24, 39, 50). However, only a few studies have examined the effect of physiological levels of zinc (<32 μM) on Akt phosphorylation in breast cancer cells and adipocytes (7, 34). Yet, the effects of zinc on prostate cells, a zinc-accumulating tissue, have not been investigated in terms of Akt phosphorylation. Previous studies showed a zinc-induced activation of PI3K/Akt

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signaling and PTEN degradation (24, 50), suggesting that zinc may affect PTEN phosphorylation. Because PTEN, the negative regulator of Akt, is functionally inactivated in prostate cancer LNCaP cells, we explored whether zinc-altered Akt phosphorylation was a result of inactivated PTEN in normal and malignant prostate cells.

Epidemiological evidence showed that high doses of zinc supplements (26) or high dietary zinc intake (15) increased risk for prostate cancer in men. However, some epidemiological studies reported no association or an inverse association between zinc and prostate cancer. The role of zinc in chemotherapy revealed that supplemental zinc sensitized prostate cancer cells to paclitaxel-induced apoptosis. Conversely, this effect of paclitaxel was reduced at lower zinc levels (~8 µM) (43). In view of the prevalence of dietary zinc deficiency and supplementation and the clinical significance of low zinc status in prostate cancer, we designed the present study to examine the influence of low and high zinc levels on cell cycle progression via the PI3K/Akt signaling pathway in human normal prostate epithelial cells (PrEC) and human malignant prostate LNCaP cells. PrEC were selected on the basis of their ability to accumulate zinc and express the wild-type PTEN gene. On the contrary, LNCaP cells were selected because of their loss of the ability to accumulate zinc and their loss of PTEN, in which one allele is deleted and the other allele is mutated. Moreover, LNCaP, an androgen-sensitive cell line with wild-type p53, was chosen to represent an early cancer progression with functional p53 to control the cell cycle. The dependence of zinc-influenced Akt phosphorylation on the modulation of nuclear and cytoplasmic p-Mdm2, p53, and p21 was ascertained by using a PI3K inhibitor, LY-294002. The results of this study contribute to the greater understanding of the mechanism by which zinc exerts its effect on cell cycle progression via the PI3K/Akt signaling cascade and the important role of the transient inhibition of PI3K/Akt signals in modulating zinc-induced cellular processes.

MATERIALS AND METHODS

Cell maintenance. PrEC (lots 3F0854 and 5F1199) were obtained at second passage (Cambrex, Walkersville, MD) and expanded in serum-free prostate epithelial cell growth medium (PrEGM). PrEGM was made from prostate epithelial cell basal medium (PrEBM) supplemented with growth factors, cytokines, and nutrients, namely, bovine pituitary extract, hydrocortisone, human recombinant epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin-amphotericin B, and retinoic acid. Zinc-free PrEBM was specifically formulated without the addition of zinc sulfate heptahydrate (ZnSO₄·7 H₂O, abbreviated as ZnSO₄) by Cambrex. This zinc-free PrEGM, the basal medium, was used as the zinc-deficient (ZD) treatment.

Human prostate malignant LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 0.1 U/l penicillin, and 0.05 µg/ml streptomycin sulfate in a humidified atmosphere of 5% CO₂-95% air at 37°C. Medium was replaced every other day, and 6 days of culture constitute one passage. Nearly (~85%) confluent cells were subcultured using trypsin-EDTA for the initiation of experimental protocols. Chelex 100 resin (Bio-Rad, Hercules, CA), a divalent ion-chelating resin, was used to remove zinc from FBS before the addition of FBS to RPMI 1640 medium (35, 38). The resin was first neutralized to physiological pH with 0.25 M HEPES (pH 7.4) and then mixed with FBS at a 1:4 ratio (wt/vol) at 4°C for 2 h. Chelex 100 resin was separated from FBS by centrifugation and then filtered through a 0.22-µm mesh for sterilization and removal of residual Chelex 100 resin. The RPMI 1640 medium added with Chelex 100-treated 10% FBS, which contains ~0.1 µM zinc, was termed the zinc-deficient (ZD) medium.

Zinc and inhibitor treatment. Zinc was added to the zinc-free PrEGM and zinc-deficient RPMI 1640 medium in the form of ZnSO₄ (Sigma-Aldrich, St. Louis, MO). Therefore, the only difference between these media was the zinc concentration. For the zinc-normal (ZN) medium, 4 µM ZnSO₄ was added to the ZD medium; the zinc-adequate (ZA) medium contained 16 µM ZnSO₄, and the zinc-supplemented (ZS) medium contained 32 µM ZnSO₄. The ZN medium was used as the control for all experiments. The ZA treatment was used as a representative of human plasma zinc levels. The ZS group was used to represent the high end of plasma zinc levels attainable by oral supplementation in humans. After one passage in ZN medium, cells were subcultured and stabilized overnight in ZN medium and then switched to their respective medium, namely, ZD, ZN, ZA, or ZS, for another 6 days (1 passage). Cells were then harvested for assays. The dependence of zinc-induced Akt phosphorylation was ascertained by using the PI3K inhibitor LY-294002 (Calbiochem, San Diego, CA). Cells were treated with 5 µM LY-294002 or 0.05% DMSO (as vehicle control) for 16 h before they were harvested.

DNA content and zinc status. The effect of zinc treatment on cellular zinc uptake in PrEC and LNCaP cells cultured for one passage in medium containing different levels of zinc was determined by atomic absorption spectrophotometry. Cells were scraped from 100-mm petri dishes and collected by centrifugation. Cell pellets were washed twice with PBS. Cells were resuspended into 1.5 ml of PBS and sonicated for 20-s intervals on ice. An aliquot of the sonicated cell suspension was used for the determination of cellular zinc content by a flame atomic absorption spectrophotometer (PerkinElmer, Boston, MA). Zinc standard solutions ranging from 0.05 to 1.0 ppm were used to generate a linear standard curve. The zinc content of the cells was calculated on the basis of these zinc standard solutions. From the same sample, another aliquot of the sonicated cell suspension was used for the determination of cellular DNA content by the diphenylamine method (17), in which diphenylamine is used to develop a blue color with the carbohydrate moiety from the purine nucleotides of the nucleic acid. Since there is a linear relationship between cellular DNA and cell number, as previously established by Wu et al. (49), data are expressed as nanograms of cellular zinc per microgram of DNA.

Cell cycle analysis. The influence of zinc status on cell cycle progression was analyzed by flow cytometry. Cells were collected by trypsinization and centrifugation. Cell pellets were washed twice with PBS and resuspended in 1 ml of PBS. Then 1 × 10⁶ cells were fixed with 70% ethanol, treated with RNase to remove any double-stranded RNA, and labeled with propidium iodide, which binds to double-stranded DNA. A total of 10,000 cell events were collected for DNA analyses on a FACSCalibur cytometer managed with CELLQuestPro software (Becton Dickinson, San Jose, CA). Percentages of stained nuclei, distributed among the three cell cycle phases, were calculated using ModFit LT (version 3.0, Verity Software House, Topsham, ME). Calibration standards, LinearFlow Green (Molecular Probes, Carlsbad, CA) and DNA QC Particle Kit (Becton Dickinson), were used to verify instrument performance. The DNA content of a cell population analyzed by this assay determined the proportion of cells in the G0/G1, S, or G2/M phase of the cell cycle.

Nuclear and cytoplasmic extractions. Nuclear and cytoplasmic extracts were obtained by using NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL). Briefly, cells were washed with PBS, scraped off the dish, and collected by centrifugation at 1,500 g for 5 min. The pellet was resuspended in cytoplasmic extraction reagent (CER) I and incubated on ice. Then CER II was added, and the extracts were further incubated on ice. The

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extracts were centrifuged for 15 min, and the supernatant (cytoplasmic fraction) was transferred to a new tube. Nuclear extraction reagent (NER) was added to the pellet, and the nuclear pellet was incubated on ice for 40 min, with vortexing every 10 min. The extract was centrifuged for 15 min, and the supernatant (nuclear fraction) was transferred to a new tube. In addition to the standard HALT protease inhibitor cocktail (Pierce Biotechnology), a phosphatase inhibitor cocktail (Pierce Biotechnology) was added to CER I and NER to prevent dephosphorylation. The isolated fractions were stored at -80°C until analysis. Protein concentration was determined by using bicinchoninic acid protein assay reagents (Pierce Biotechnology) with BSA used as the standard.

**Western blot analysis.** Approximately 40 µg of protein from the cell extracts were mixed with an equal volume of Laemmli sample loading buffer, which consisted of 62.5 mM Tris-HCl (pH 6.8), 25% glyceral, 2% SDS, 0.01% bromphenol blue, and 35.5 mM β-mercaptoethanol. These extracts were separated according to size by 10% SDS-PAGE under reduced conditions and then electrotransferred onto an ECL nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL). A blocking buffer consisting of 5% (wt/vol) nonfat dry milk in 10 mM Tris-HCl (pH 7.4) with 0.1% Tween-20 (TBS-T) was used to block nonspecific proteins on the membrane for 1 h at room temperature. After several washes with TBS-T, the membrane was probed with p-Akt (Ser473) antibody (Cell Signaling Technology, Beverly, MA) diluted in 1% (wt/vol) BSA in TBS-T overnight at 4°C. Bound primary antibody was detected with horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate SuperSignal WestPico Chemiluminescence reagent (Pierce Biotechnology). Immunoreactive bands were visualized by exposure of membranes to X-OMAT film (Eastman Kodak, Rochester, NY), which was developed by an X-ray film processor (AFP Imaging, Elmsford, NY). Membranes were then stripped and reprobed with Akt antibody (Cell Signaling Technology) for verification of equal protein loading. Relative band intensities were digitally quantified by using the FluorChem 8900 imaging system with AlphaEase FC Stand Alone Software (Alpha Innotech, San Leandro, CA). Other primary antibodies were as follows: p-PTEN (Ser380), PTEN (catalog no. 9552), and p-Mdm2 (Ser166) from Cell Signaling Technology; Mdm2 (C-18), p53 (DO-1), and p300 (N-15) from Santa Cruz Biotechnology (Santa Cruz, CA); and p21 (Ab-1) from Calbiochem (San Diego, CA). Membranes were reprobed with nucleoparin (p62) or GAPDH (FL-335) antibodies (Santa Cruz Biotechnology) to ensure equal loading of nuclear or cytoplasmic protein, respectively. Cross contaminations of nuclear and cytoplasmic extracts as determined by probing with GAPDH and nucleoparin, respectively, have been routinely established to be <5%.

**Statistical analysis.** Values are means ± SE from three independent experiments. Statistical analyses were performed using SPSS (version 10.0, SPSS, Chicago, IL). Data were analyzed using one-way ANOVA, and the means were further compared by least significant differences post hoc test, with \( P < 0.05 \) regarded as significant.

**RESULTS**

**PrEC were more susceptible than LNCaP cells to zinc treatments.** Since zinc is essential for cell growth and is enriched in the prostate, the influence of zinc on growth of prostate cells was examined. PrEC were cultured for one passage (6 days) in basal PrEGM supplemented with different levels of zinc. This basal PrEGM was specifically formulated by Clonetics (Cambrex) without the normal addition of zinc. After addition of the growth factors, cytokines, and nutrient supplements, zinc concentration of the basal medium increased to 0.9 µM as determined by atomic absorption spectrophotometry (data not shown). When PrEC were cultured directly in basal PrEGM containing 0.9 µM zinc, these ZD cells reached confluency at a slightly lower rate than cells cultured in the ZN medium. Cell growth, measured as DNA content per plate, was significantly lower (by 34%) in ZD than in ZN PrEC (Fig. 1A). However, no differences in cell growth were observed among ZA, ZS, and ZN PrEC. Thus cell growth appeared to be adversely affected by zinc deficiency. This might be due to the direct involvement of zinc in DNA synthesis. Zinc appears to regulate the transcription of thymidine kinase through zinc-dependent protein binding to the promoter region of the gene (5). Higher-than-adequate levels of zinc (16 and 32 µM), however, had no further effect on cell growth. Apoptosis was not observed in cell culture medium. The absence of apoptosis was supported by the lack of DNA fragments detected in the sub-G0/G1 phase with any of the treatments.

LNCaP cells were treated with various concentrations of zinc for one passage. After zinc was removed from FBS by Chelex 100, final zinc concentration of the basal medium consisting of RPMI 1640 medium + 10% FBS was <0.1 µM (data not shown). Cell growth was significantly lower (39%) in ZD than in ZN LNCaP cells (Fig. 1B). However, cell growth was similar for the ZN, ZA, and ZS LNCaP cells.

The amount of zinc taken up by prostate cells was determined by the zinc content of the cells. Cellular zinc status was expressed relative to DNA content to correct for any differences in cell numbers between plates. As shown in Fig. 1C, cellular zinc status of PrEC was dose dependently increased as the level of zinc increased in the medium. In PrEC, ZD treatment was able to deplete cellular zinc status to 59% of ZN controls. Zinc status was significantly higher in ZA and ZS (by 86% and 137%, respectively) than in ZN PrEC. Moreover, cellular zinc levels were significantly different between ZA and ZS PrEC. In LNCaP cells, ZD treatment was able to deplete cellular zinc status to 75% of ZN controls as shown in Fig. 1D. LNCaP cells cultured in ZA medium had no further effect on zinc status, whereas zinc status was only 16% higher in the ZS than in the ZN group. These data suggest that cellular zinc status of PrEC was highly susceptible to the availability of zinc in the medium in a dose-dependent manner. In contrast to LNCaP cells, PrEC showed greater ability to retain the highest concentration of zinc (32 µM).

**Effect of zinc on cell cycle distribution.** Previous observations indicate that G1/S and G2/M progression is depressed in zinc-deficient hepatoblastoma Hep G2 cells and zinc-supplemented human normal bronchial epithelial cells, respectively (37, 48). However, the effect of zinc status on cell cycle progression in PrEC and LNCaP cells has not been investigated. As shown in Fig. 2A, flow cytometric analysis of PrEC indicates that low- and high-zinc treatments have little or no effect on the G0/G1 and S phases of the cell cycle. The percentage of cells at the G2/M phase of the cell cycle was significantly reduced (~13%) in the ZA and ZS groups compared with the ZD and ZN groups (Fig. 2A). These results suggest that G2/M progression was depressed by high-zinc treatments.

At all zinc treatments in PrEC, the percent distributions of LY-294002-treated cells in the G0/G1, S, and G2/M phases were significantly altered from their untreated counterparts [vehicle control (VC)]. Compared with the untreated ZN group, LY-294002 treatment resulted in a 12–18% increase in the percentage of cells detected at the G0/G1 phase among all zinc groups (Fig. 2A). In addition, LY-294002 treatment resulted in a 6–14% reduction of the percentage of cells in the S
phase. In particular, the percentages of LY-294002-treated ZD cells in the S phase were only 57% of their untreated counterparts (Fig. 2A). Interestingly, the percentages of LY-294002-treated cells in the G0/G1 and S phases were highest and lowest in ZD cells, respectively, and these levels were significantly different from the other zinc treatment groups. Moreover, compared with untreated cells, LY-294002 treatment markedly reduced the percentage of cells in the G2/M phase to similar levels across the zinc groups (Fig. 2A). Overall, LY-294002 markedly increased the fractions of cells at the G0/G1 phase. Thus a reduction of cells in the S and G2/M phases was concomitantly observed. A large blockage of G1-to-S progression (expressed as G1-to-S ratio) was only seen in LY-294002-treated ZD PrEC (Fig. 2A). The G1-to-S ratios were 127%, 60%, 63%, and 71% higher in LY-294002-treated ZD, ZN, ZA, and ZS groups, respectively, than in the non-LY-294002-treated ZN group. Overall, ZD PrEC were more sensitive to the PI3K inhibitor LY-294002 during G1-to-S progression than other zinc groups.

In the cell cycle distribution shown in Fig. 2B, retention of LNCaP cells at the G0/G1 phase (as a percentage of total LNCaP cells) was 10% greater after one passage of ZD treatment than after ZN treatment and 6% greater after ZA and ZS treatments. Retention at the S phase was 8% less in the ZD than in the ZN group and 4% less in the ZA and ZS groups than in the ZN group. The effect of zinc was less pronounced at the G2/M phase, with 1–2% less retention of ZD, ZA, and ZS than ZN cells. Briefly, the number of ZN cells that progressed to the S phase was >2.6-fold higher than the number of ZD cells. Across the zinc groups, treatment of LNCaP cells with the PI3K inhibitor LY-294002 uniformly affected each phase of the cell cycle to a similar extent (Fig. 2B). Compared with non-LY-294002-treated cells, cell retention was higher at the G0/G1 phase, lower at the S phase, and about the same at the G2/M phase. After LY-294002 treatment, retention of ZD, ZN, ZA, and ZS cells was 5.3%, 12.5%, 8.5%, and 6.7% greater (as a percentage of total LNCaP cells), respectively, at the G0/G1 phase than non-LY-294002 treated cells. At the S phase, retention of ZD, ZN, ZA, and ZS cells was significantly reduced 4.0%, 10.1%, 6.5%, and 6.0% (as a percentage of total LNCaP cells), respectively, with LY-294002 treatment compared with their untreated counterparts. Retention at the G2/M phase was ~2% less in ZD, ZA, and ZS than in ZN cells. These results suggest that, other than normal zinc treatment (ZN), LNCaP cells respond to low (ZD) and high zinc treatment (ZA and ZS) in a similar fashion at each stage of the cell cycle.

The G1-to-S ratio was 2.8-fold higher in ZD than in ZN LNCaP cells and ~1.7-fold higher in ZA and ZS than in ZN cells (Fig. 2B). Thus zinc depletion significantly delayed the progression of ZD cells from the G1 to the S phase. A significant blockage of G1-to-S progression by LY-294002 treatment was evident (Fig. 2B). The ratio was highest in LY-294002-treated ZD cells, followed by ZS, ZA, and ZN cells. Overall, LY-294002 had a significant effect on increasing fractions of cells retained at the G1 phase, which is consistent with the previous observations that LY-294002 caused a blockage at the G1-to-S transition in LNCaP cells (19) and other prostate cancer cell lines, DU145 and PC-3 (16).

Fig. 1. Effect of zinc on cellular zinc status and DNA content of normal human prostate epithelial cells (PrEC; A and C) and malignant human prostate (LNCaP) cells (B and D). Cells were treated with zinc-deficient (ZD), zinc-normal (ZN), zinc-adequate (ZA), and zinc-supplemented (ZS) media for 6 days. Cellular zinc status was determined by atomic absorption spectrophotometry. A and B: DNA content measured by the diphenylamine method. C and D: measurement of zinc status to account for differences in cell numbers between plates. Values are means ± SE, which represent an average of triplicate results from LNCaP cells or 2 different donors of PrEC. Different letters (a, b, c, d) indicate significant differences among groups (P < 0.05); treatments with the same letters indicate no significant difference.
Hyperphosphorylation of Akt was induced by zinc deficiency and reversed by LY-294002 in PrEC and LNCaP cells. The effects of supraphysiological levels of zinc (50 – 200 μM) on PI3K/Akt signaling have been extensively studied in prostate cells, fibroblasts, adipocytes, breast cells, and lung epithelial cells (3, 24, 34, 39, 50). However, the effect of physiological levels of zinc (<32 μM) on Akt phosphorylation has not been examined, in particular, in prostate cells. Moreover, a high level of p-Akt has been suggested as a biomarker for prostate cancer (2). To explore the influence of zinc on the phosphorylation of Akt, we used Western blot analysis with anti-p-Akt (Ser473) antibody to detect phospho-specific protein expression level in various zinc-treated PrEC and LNCaP cells. Data were normalized to total Akt and presented as a ratio of p-Akt to total Akt. In addition, data are expressed as percentage of the control group (i.e., cells treated with ZN medium then with VC). As shown in Fig. 3A, cytoplasmic protein levels of p-Akt were 42% higher in ZD than in ZN PrEC. However, the levels of p-Akt were similar among ZN, ZA, and ZS cells. Although the Western blot may appear to show p-Akt in ZA and ZS cells to be slightly lower than in ZN cells, the overall analysis of data from three experiments indicates that the level of p-Akt was similar among ZN, ZA, and ZS cells. Since the phosphorylation of Akt is dependent on PI3K (9), its inhibitor LY-294002 was used to examine the role of PI3K in zinc deficiency-induced Akt phosphorylation. The elevated p-Akt level observed in ZD cells was normalized to that of ZN, ZA, and ZS cells after 16 h of treatment with LY-294002 (Fig. 3A). For ZD cells, LY-294002 treatment produced a significant 55% reduction in p-Akt compared with the untreated counterpart. These results suggest that zinc deficiency induced hyperphosphorylation of Akt and sensitized normal prostate cells to LY-294002.

Cytoplasmic protein levels of p-Akt were 75%, 81%, and 81% higher in ZD, ZA, and ZS, respectively, than in ZN LNCaP cells (Fig. 3B). After 16 h of LY-294002 treatment, the elevated p-Akt levels in ZD, ZA, and ZS cells were normalized to a level similar to that in ZN cells (Fig. 3B). These results suggest that physiological, either lower- or higher-than-normal, levels of zinc would induce hyperphosphorylation of Akt in LNCaP cells. Furthermore, these zinc-altered malignant prostate cells were responsive to LY-294002 in inhibiting Akt phosphorylation.

In summary, the present data indicate that the PI3K signaling pathway is a key mediator in the activation of Akt by zinc, rather than direct activation of Akt by zinc.

Hyperphosphorylation of PTEN in ZD PrEC. PTEN catalyzes the dephosphorylation of PIP2 and PIP3, thus antagonizing PI3K activity and inhibiting its downstream effector p-Akt. PTEN activity is regulated not only by expressed protein levels but also by the ratio of phosphorylated to unphosphorylated forms of the protein (41, 44, 45). Phosphorylation of three residues (Ser380, Thr382, and Thr383) of the PTEN tail inactivates its phosphatase activity. To prevent an untimely or unregulated activation of PTEN, dephosphorylation of the tail would result in an increase in PTEN activity and its rapid
degradation by the proteasome. Since the magnitude of PTEN phosphorylation reflects the capacity of PI3K/Akt signaling, whether PTEN plays a role in zinc deficiency-induced Akt phosphorylation can be determined by the ratio of phosphorylated (inactive) to unphosphorylated (active) PTEN. Because PTEN, the negative regulator of Akt, is functionally inactivated in prostate cancer (47), we explored whether increased Akt phosphorylation by zinc deficiency was a result of inactivated PTEN. Moreover, previous studies showed that supraphysiological levels of zinc induced PI3K/Akt signaling pathway in fibroblasts (24) and PTEN degradation in human airway epithelial cells (50), suggesting that zinc may affect PTEN phosphorylation.

To test this assumption, we used Western blotting to measure phospho-specific and total PTEN protein levels in PrEC exposed to various concentrations of zinc. A phospho-specific PTEN antibody to Ser380, one of the three residues that can be phosphorylated on the tail, was used to measure the extent of phosphorylation. Data are expressed as the ratio of p-PTEN to total PTEN to indicate PTEN activity. In addition, data are expressed as percentage of the control group (ZN-VC, i.e., cells treated with ZD medium and then with VC without LY-294002). As shown in Fig. 4, cytoplasmic levels of p-PTEN were 41% higher in ZD than ZN cells. However, the levels of p-PTEN were similar among ZN, ZA, and ZS cells. Since the phosphorylation of PTEN at Ser380 is indicative of a loss of PTEN protein stability and activity, the zinc deficiency-induced phosphorylation of PTEN at Ser380 may contribute to more inactive and less active PTEN to dephosphorylate Akt, which then leads to our observed increased phosphorylation of Akt in zinc deficiency. In addition, because PTEN antagonizes the PI3K/Akt pathway, these data suggest that the zinc deficiency-induced PI3K/Akt signaling was due to PTEN inactivation.

Hyperphosphorylation of Mdm2 was induced by zinc deficiency and abrogated by LY-294002 in PrEC. p-Akt has been shown to interact with Mdm2 and phosphorylate Mdm2 at Ser166 and Ser186 (18). These Akt phosphorylation sites in Mdm2 are close to its NLS and NES domains, which are critical in determining the trafficking of Mdm2 between the nucleus and the cytoplasm. Therefore, Akt-mediated phosphorylation of Mdm2 (p-Mdm2) leads to its nuclear localization (31, 33, 52). In the present study, we investigated whether zinc deficiency-induced activated Akt would phosphorylate its downstream effector Mdm2 and mediate nuclear translocation of Mdm2.

Fig. 3. Effect of zinc on phosphorylation of Akt protein in PrEC (A) and LNCaP cells (B). Cells were treated with ZD, ZN, ZA, and ZS media for 6 days. Cells were treated with VC or LY for 16 h and then harvested. Cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p-Akt (Ser473). To ensure equal loading, the blot was reprobed with anti-Akt. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Data are expressed as ratio of phosphorylated Akt (p-Akt) to total Akt and presented as percentage of the control group (cells treated with ZN medium and then with VC). Values are means ± SE, which is an average of triplicate experiments. Blot is representative of results from 3 independent experiments that showed similar results. Different letters indicate significant differences among groups (P < 0.05); treatments with the same letters indicate no significant difference.

Fig. 4. Effect of zinc on phosphorylation of PTEN protein in PrEC. Cells were treated with ZD, ZN, ZA, and ZS media for 6 days. Cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p-PTEN (Ser380) antibody. To ensure equal loading, the blot was reprobed with anti-PTEN antibody. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Data are expressed as ratio of phosphorylated PTEN (p-PTEN) to total PTEN and presented as percentage of the control group (cells treated with ZN medium). Values are means ± SE, which is an average of triplicate experiments. Blot is representative of 3 independent experiments that showed similar results. Different letters indicate significant differences among groups (P < 0.05); treatments with the same letters indicate no significant difference.

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Using a phospho-specific Mdm2 antibody, we determined Mdm2 phosphorylation at Ser\(^{166}\) in the nuclear fraction. As shown in Fig. 5, the level of p-Mdm2 was significantly higher (47%) in ZD than in ZN PrEC. However, there was no difference among ZN, ZA, and ZS cells. With LY-294202 treatment, hyperphosphorylation of Mdm2 induced by zinc deficiency was normalized to that of non-LY-294002-treated ZN cells. This significant inhibition by LY-294002 lowered the level of p-Mdm2 in ZD cells to 67% of that in non-LY-294002-treated ZD cells. However, LY-294002 had no inhibitory effect on ZN, ZA, and ZS cells. Cytoplasmic levels of Mdm2 were unchanged by zinc and/or LY-294002 treatment (data not shown). Thus, in ZD PrEC, the marked increase in nuclear, but not cytoplasmic, p-Mdm2, as measured by the phosphorylation of Mdm2 at Ser\(^{166}\), is indicative of increased nuclear localization of p-Mdm2.

Nuclear levels of p-Mdm2 were essentially equivalent in ZD, ZN, ZA, and ZS in LNCaP cells (data not shown). The PI3K inhibitor LY-294002 had no effect on this protein. Cytoplasmic levels of Mdm2 were also unaffected by zinc and/or LY-294002 in LNCaP cells. These data indicate that the zinc-altered Akt phosphorylation had no subsequent effect on nuclear Mdm2 phosphorylation or the cytoplasmic level in LNCaP cells.

However, the data demonstrated that Mdm2 was more susceptible to phosphorylation by Akt with zinc deficiency in normal than in malignant prostate cells. In addition, because Mdm2 can only be phosphorylated by Akt at Ser\(^{166}\) and Ser\(^{186}\) and no other kinase can phosphorylate Mdm2 at these two residues, the effect through Akt is specific. Moreover, the normalization of the zinc deficiency-induced hyperphosphorylation of Mdm2, by the use of the Akt inhibitor LY-294004, to that of the control group (ZN cells treated with VC) level also indicates that the effect through Akt is specific. Furthermore, phosphorylation of Mdm2 was not influenced by LY-294002 in cells with higher zinc status.

**Nuclear accumulation of p53 was suppressed by zinc deficiency in PrEC and LNCaP cells.** Mdm2, a ubiquitin E3 ligase, is known for its role in controlling p53 activity (32). Once Mdm2 is phosphorylated by p-Akt, p-Mdm2 translocates to the nucleus and forms a complex with p53 and p300 to promote the ubiquitin-dependent degradation of p53 (30, 31, 52). As a result, p-Akt may indirectly hinder p53-dependent growth suppression and apoptosis, leading to cell survival (33). Since we observed hyperphosphorylation of Akt and Mdm2 in ZD PrEC, we next investigated whether p53 was affected through this signaling pathway. As shown in Fig. 6A, nuclear and cytoplasmic accumulations of p53 were significantly lower (32% and 39%, respectively) in ZD than in ZN cells. There was no difference among ZN, ZA, and ZS cells. Nuclear p53 accumulation was significantly reduced by 38% and 32% in LY-294002-treated ZN and ZA cells, respectively, compared with their untreated counterparts (Fig. 6A). However, LY-294002 had no effect on p53 nuclear accumulation in ZD and ZS groups. On the contrary, cytoplasmic p53 levels were significantly lower, by 45% and 32%, in LY-294002-treated ZN and ZS cells, respectively, than in their untreated counterparts (Fig. 6A). These data indicate that zinc deficiency has a suppressive effect on nuclear and cytoplasmic accumulations of p53.

**Fig. 5. Effect of zinc on nuclear level of phosphorylated Mdm2 (p-Mdm2) protein in PrEC.** PrEC were cultured in ZD, ZN, ZA, and ZS media for 6 days. Cells were treated with VC or LY for 16 h and then harvested. Nuclear extracts were subjected to SDS-PAGE and immunoblotting using anti-p-Mdm2 (Ser\(^{166}\)) antibody and reprobed with anti-Mdm2 (C-18) antibody to ensure equal loading. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Values are means ± SE of triplicate results from 2 different donors of PrEC. Blot is representative of 3 independent experiments that showed similar results. Different letters indicate significant differences among groups (P < 0.05); treatments with the same letters indicate no significant difference.

The nuclear level of p53 in LNCaP cells was 41% lower in ZD than in ZN cells (Fig. 6B). However, this level was similar among ZN, ZA, and ZS cells. LY-294002 treatment exerted little or no effect on the nuclear p53 levels compared with untreated (VC) groups, except there was a 30% reduction in ZA cells. The cytoplasmic level of p53 was unaffected by zinc and/or LY-294002 treatment (data not shown).

**Nuclear p21 level was depressed by zinc deficiency in LNCaP cells.** Akt can phosphorylate p21 within its NLS domain and, thus, restrict it to the cytoplasm for degradation (52). Furthermore, p-Akt has been shown to promote cell cycle progression by limiting nuclear p21 protein levels that bind and activate the cyclin D-CDK4 complex (27). Since Akt was hyperphosphorylated in ZD cells, we tested whether the activation of Akt could affect cellular localization of p21.

The nuclear level of p21 was significantly higher (72% and 87%, respectively) in ZA and ZS than in ZN and ZD PrEC (Fig. 7A). LY-294002 significantly elevated nuclear p21 for all groups. The cytoplasmic level of p21 was unaffected by zinc and/or LY-294002 treatment (data not shown).

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reduced by LY-294002 treatments across the zinc groups. The cytoplasmic level of p21 was 45%, 61%, and 45% lower in the ZD, ZA, and ZS groups than in the ZN group. Across the zinc groups, LY-294002 depressed cytoplasmic p21 to a similar extent. These observations indicate that zinc deficiency depressed nuclear p21 of LNCaP cells. Cytoplasmic p21 was able to respond as expected, since p-Akt levels were higher in ZD, ZA, and ZS than in ZN cells. Thus the zinc deficiency-induced Akt phosphorylation in LNCaP cells might have an effect on limiting nuclear and cytoplasmic p21 levels through phosphorylation and degradation of p21. In ZA and ZS cells, only cytoplasmic p21 might be affected by Akt phosphorylation.

DISCUSSION

After treatment of PrEC and LNCaP cells with various concentrations of zinc for 5 days, the zinc status of each cell type was shown to be differentially dependent on the zinc concentration in the medium (Fig. 1). As expected, PrEC were dose dependently responsive to zinc concentration in the medium. In contrast to the zinc status of LNCaP cells, which tended to reach a plateau at >4 μM, the zinc uptake of PrEC was evident even at the highest concentration tested (32 μM) (Fig. 1). These observations highlight the propensity of normal prostate cells to sequester zinc and the inability of malignant prostate cells to accumulate zinc. Downregulation of the zinc uptake transporter ZIP during cancer initiation and/or progression was proposed as the responsible mechanism (10, 14). Our zinc-deficient treatment was able to deplete the zinc status of PrEC and LNCaP cells to 59% and 25%, respectively, of their ZN controls (Fig. 1). Similarly, cell growth was inhibited by zinc deficiency but remained unchanged in ZN, ZA, and ZS cells. Our ZD PrEC and LNCaP cells did not undergo apoptosis, inasmuch as no DNA fragments were observed in the sub-G0/G1 region after staining (data not shown). The progression of these cells through the G0/G1 and S phases of the cell cycle was similar to that of the other zinc-treated groups. This might be a result of hyperphosphorylation of Akt, which is known for its prosurvival activity, as suggested by our findings of zinc deficiency-induced phosphorylation of Akt in PrEC and LNCaP cells.

Previous studies focused on acute (from 0.5 to 72 h) reduction of zinc levels (23, 28, 42, 43). Even though these previous studies included a group of cells treated with 0–1.5 μM zinc, none was able to show successful depletion of cellular zinc status. Liang et al. (28) reported that the zinc status of cells treated with 1.5 μM zinc was even higher than that of cells growing under normal conditions. This suggests that their 48-h depletion process (vs. 5 days in the present study) might not be long enough to induce zinc deficiency. Furthermore, a substantial amount of zinc from FBS was taken into account in several studies (23, 42, 43). Using our chelating strategy, we were able to deplete zinc from FBS-containing medium to <0.1 μM without eliminating other growth components in FBS. Moreover, Liang et al. switched the culture condition from medium containing 10% FBS to serum-free medium 24 h before various zinc treatments.

Fig. 6. Effect of zinc on nuclear and cytoplasmic p53 in PrEC (A) and LNCaP cells (B). Cells were cultured in ZD, ZN, ZA, and ZS media for 6 days. Cells were treated with VC or LY for 16 h and then harvested. Nuclear and cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p53 (DO-1) antibody. To ensure equal loading, blots of nuclear and cytoplasmic extract were reprobed with anti-nucleoporin (N-19) and anti-GAPDH (FL-335) antibody, respectively. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Values are means ± SE, which is an average of triplicate experiments. Blot is representative of 3 independent experiments that showed similar results. Different letters indicate significant differences among groups (P < 0.05); treatments with the same letters indicate no significant difference.
According to standard LNCaP cell culture protocol, the number of cells would slowly decline in 0.01% FBS. Consequently, whether the serum deprivation approach adapted by Liang et al. would induce the growth inhibitory effect of zinc deficiency warrants further investigation.

We found that Akt phosphorylation was 42% higher in ZD (0.9 μM) normal prostate cells (PrEC), whereas Akt phosphorylation status of ZA (16 μM) and ZS (32 μM) cells was equal to that of ZN (4 μM) cells (Fig. 3A). The magnitude of Akt hyperphosphorylation in ZD (<0.1 μM) malignant prostate LNCaP cells (Fig. 3B) and PrEC was 75% vs. 42% greater, respectively, than in their respective ZN controls. This might be due to the fact that normal prostate cells retain wild-type PTEN, which can negatively regulate the phosphorylation of Akt to keep it at basal level. However, the PTEN gene in malignant prostate cells, similar to LNCaP cells in this case, is deleted and mutated and, thus, incapable of inhibiting Akt phosphorylation. Therefore, the present data indicate that ZD, ZA, and ZS malignant prostate cells were more susceptible to Akt phosphorylation, probably because of a nonfunctional PTEN. The use of a specific inhibitor of the PI3K/Akt pathway, LY-294002, normalized Akt hyperphosphorylation in ZD, ZA, and ZS cells to that of ZN LNCaP cells (Fig. 3B). These data confirm that the zinc-induced Akt hyperphosphorylation was indeed caused by PI3K signaling.

When a wild-type PTEN is phosphorylated, it is inactive and unable to prevent Akt phosphorylation and, thus, serves as an endogenous negative regulator of the Akt signaling pathway (45). Since PrEC contain wild-type PTEN, the present study also examined the extent of PTEN phosphorylation with various zinc treatments. Again, PTEN phosphorylation was higher in zinc deficiency (Fig. 4). This validated our assumptions that the zinc deficiency-induced Akt phosphorylation was due to inhibited PTEN activity.

Following the Akt-Mdm2-p53 signaling axis, we examined the degree of Mdm2 phosphorylation and p53 accumulation in the nucleus. In accordance with zinc deficiency-induced Akt phosphorylation, Mdm2 phosphorylation was higher and p53 accumulation was lower in zinc deficiency for all PrEC treatment groups but not for the LNCaP groups (Figs. 5 and 6). Treatment of PrEC with the inhibitor LY-294002 reversed zinc deficiency-induced Mdm2 phosphorylation. This finding was consistent with the notion that p-Akt could subsequently phosphorylate Mdm2 and promote its nuclear translocation (31). Consequently, nuclear Mdm2 could complex with p53 and p300 to form polyubiquitinated p53, which was then shuttled to the proteasome for degradation (20, 31). Thus cytoplasmic p53 was also lower in ZD PrEC than in all other treatment groups. Despite our previous finding that nuclear p300 was markedly depressed by zinc deficiency in Hep G2 cells (1), it was unchanged in PrEC and treatment conditions of the present study (data not shown). This suggests that, in zinc-deficient PrEC, p300 was maintained in the nucleus at the normal level capable of forming a functional complex with p53 and Mdm2, necessary for the normal p53 ubiquitination and degradation.

Fig. 7. Effect of zinc on p21 in PrEC (A) and LNCaP cells (B). Cells were cultured in ZD, ZN, ZA, and ZS media for 6 days. Cells were treated with VC or LY for 16 h and then harvested. Nuclear and cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p21 (Ab-1) antibody. To ensure equal loading, blot was reprobed with anti-nucleoporin (N-19) antibody or anti-GAPDH (FL-335) antibody. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Values are means ± SE, which is an average of duplicate experiments. Blot is representative of 2 independent experiments that showed similar results. Different letters indicate significant differences among groups (P < 0.05); treatments with the same letters indicate no significant difference.
As a result, Akt may exert an indirect effect on inhibiting apoptotic function of p53 tumor suppressor protein through Mdm2 in zinc deficiency.

More recently, another antiapoptotic role of Akt was described in p21 phosphorylation. p21 is a CDK inhibitor that can arrest the cell cycle in response to DNA damage, generally in a p53-dependent manner. The antiproliferative effects of p21 are assisted by its ability to bind to proliferating cell nuclear antigen (PCNA) and block DNA synthesis required for the S phase of the cell cycle. However, in opposition to its antiproliferative functions, p21 can be proproliferative when localized in the cytoplasm. When p21 is phosphorylated by Akt at Thr145, p-p21 is retained in the cytosol and loses the ability to interact with PCNA, because the phosphorylation of p21 at Thr145 results in two modifications of p21. 1) The Thr145 site lies adjacent to the NLS domain, which prevents interaction of p21 with importins and, hence, blockade of nuclear translocation (21). 2) The Thr145 residue lies within the consensus binding sequence for PCNA. Thus Thr145 phosphorylation of p21 by Akt can disrupt the hydrogen bonding between p21 and PCNA, thereby destabilizing the complex (46). A further consequence of cytoplasmic location of p21 is facilitation of the assembly and activity of the cyclin D-CDK4 complex, which promotes cell cycle progression through the G1 phase into the S phase (4). We observed a high Akt phosphorylation, low nuclear p21, and high G1-to-S ratio in ZD LNCaP cells (Figs. 2B, 3B, and 7B). These results led us to postulate that p21 might be phosphorylated by Akt in zinc deficiency, so less cytoplasmic p21 was translocated into the nucleus. This, in turn, maintained the cell cycle progression from the G1 to the S phase. Future study should be designed to determine whether zinc deficiency-inhibited nuclear p21 accumulation has an effect on the expression of cyclins and the activity of the cyclin D-CDK4 complex in LNCaP cells.

In contrast to previous findings of zinc deficiency-induced p53 nuclear accumulation in Hep G2 cells (1, 35), normal human bronchial epithelial cells (12), and human aortic endothelial cells (11), we did not observe p53 accumulation in PrEC or LNCaP cells. Nuclear p53 was 32% lower in ZD PrEC and 41% lower in ZD LNCaP cells (Fig. 6). Under cellular stress, p21 expression is increased through a p53-dependent or -independent pathway. Previously, our laboratory demonstrated that nuclear p21 level was lower in zinc-deficient than in zinc-normal Hep G2 cells, despite p53 nuclear accumulation (1). In the present study, zinc deficiency-suppressed p53 nuclear accumulation was associated with markedly suppressed nuclear p21 levels in ZD LNCaP cells but not PrEC (Figs. 6 and 7). The difference in p21 level between PrEC and LNCaP cells might be due to the zinc status of ZD PrEC; i.e., zinc was not sufficiently depleted from the originally high cellular zinc level in these zinc-accumulating cells to exert a suppressive effect on nuclear p21 level. For LNCaP cell culture, we used Chelex-100 to remove the zinc from FBS. Thus in the growth medium containing 10% FBS, zinc concentration was <0.1 μM. In contrast, for PrEC culture conditions, zinc was omitted during the formulation of basal medium. After addition of growth factors, cytokines, and nutrient supplements to the basal medium, this growth medium contained 0.9 μM zinc. Consequently, the zinc status of ZD PrEC might not have been as depleted as in ZD LNCaP cells. Therefore, we did not observe a depressive effect on nuclear p21 level by zinc deficiency in PrEC.

Alternatively, depressed nuclear p21 in LNCaP cells might be explained by a p53-independent mechanism, such as the phosphorylation, retention, and degradation of p21 in the cytoplasm by Akt phosphorylation, thus inhibiting translocation of p21 into the nucleus. This, in turn, slows the rate at which the cell cycle progresses from the G1 to the S phase, as shown by a high G1-to-S ratio in ZD LNCaP cells (Fig. 2B). Future studies should be designed to determine whether zinc deficiency-inhibited nuclear p21 accumulation has an effect on
the expression of cyclins and the activity of the cyclin D-CDK4 complex in LNCaP cells.

Lower percentages of ZA and ZS PrEC were detected at the G2/M phase (Fig. 2A). This observation coincides with the elevated nuclear p21 levels in ZA and ZS PrEC (Fig. 7A). In contrast, a previous study showed that zinc-induced arrest of the cell cycle at the G2/M phase was accompanied by increased mRNA levels of p21 in malignant prostate LNCaP (p53+/−) and PC3 (p53−/−) cells, which indicated that the impaired cell cycle progression might be independent of p53 (28). Differences in culture condition and zinc treatment may explain the difference between our present results and the results reported by Liang et al. (28). However, the protein level of p21 was not reported by Liang et al. Also, cells were subjected to serum starvation before and during zinc treatment in the study of Liang et al. Furthermore, growth of LNCaP and PC-3 cells has been shown to be suppressed by 200 and 100 μM zinc, respectively, which are supraphysiologically (22). Although we did not observe growth inhibition in PrEC with the highest zinc treatment (32 μM), the concentration chosen was based on physiological relevance.

Together, the results of this study suggest that the zinc deficiency-induced Akt phosphorylation in PrEC was a result of PTEN phosphorylation and less nuclear accumulation of p53 (Fig. 8), thus exerting less of the tumor-suppressive effect of p53 and promoting cell survival. Therefore, the progression of the cell cycle was maintained through an Akt-Mdm2-p53 signaling axis in ZD PrEC. In contrast, in LNCaP cells lacking an active PTEN, Akt phosphorylation was maintained in cells with low or high zinc status. In zinc deficiency, suppressed nuclear p53 accumulation and unchanged nuclear Mdm2 phosphorylation indicate a non-Akt-Mdm2-p53 signaling axis in LNCaP cells. Suppressed nuclear and cytoplasmic p21 levels in ZD LNCaP cells might be due to p21 phosphorylation by Akt, which inhibits p21 nuclear entry and promotes cytoplasmic degradation. As a result, more ZD LNCaP cells survived and progressed through the G0/G1 phase of the cell cycle. In summary, these results suggest that zinc deficiency has an effect on the Akt-Mdm2-p53 signaling axis in normal prostate cells and the Akt-p21 signaling axis in malignant prostate cells.

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