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Recent surveys indicate that Pi intake has increased steadily as Pi-containing foods have increased. Our previous study demonstrated that high dietary Pi strongly stimulated lung tumorigeneis. In order to answer the issue whether low Pi may be chemopreventive, we examined the effects of low Pi on lung cancer. Eighteen 5-wk-old male K-*ras*^{LA1} lung cancer model mice were randomly allocated to 2 groups. One group was fed a normal diet (0.5% Pi) and other group was fed low Pi (0.1% Pi) diet for 4 wk. Lung cancer development was evaluated by histopathological examination, Western blot, kinase assay, and immunohistochemistry. Low Pi increased the expression of sodium-dependent phosphate co-transporter 2b, and activated Akt signal with decreased PTEN expression in the lungs of K-*ras*^{LA1} mice. Low Pi increased the Akt/mTOR-mediated protein translation through upregulating the phosphorylation of p70S6K and 4E-BP1. In addition, low Pi stimulated cell cycling as evidenced by altered cell cycle regulators such as cyclin D1 and D3. Finally, low Pi increased lung tumorigenesis in K-*ras*^{LA1} mice compared to the normal diet group. Our results clearly demonstrated that low Pi also promoted lung tumorigenesis, thus suggesting that an appropriate intake of dietary Pi may be critical for lung cancer prevention as well as treatment.

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INTRODUCTION

Phosphate (Pi) is an essential nutrient to all living organisms (1). It is a vital component of phospholipids in membrane and of nucleotides, both of which provide energy and serve as components of DNA, RNA, and phosphorylated intermediates in cellular signaling (1). Many studies that have investigated Pi have focused mainly on bone and kidney. However, recent studies have reported that Pi also plays another important role in several tissues' development including liver, lung, and brain (2–4).

Since Pi cannot be synthesized by animals, the need for this nutrient is satisfied by dietary ingestion (5). However, a high intake of Pi was demonstrated to alter the expression of the sodium-dependent inorganic phosphate co-transporter 2b (NPT-2b) and strongly stimulated lung tumorigenesis through altering Akt signaling (6). NPT-2b is a subtype of a family of Pi transporters, and recent studies have suggested that NPT-2b is closely associated with cancer (7,8). Akt is also known as protein kinase B and has been proposed as a central signaling event in lung tumorigenesis (9). Taketa et al. (10) reported that NPT-2b expression was regulated by dietary Pi, and low Pi diet also

affected the Akt signal pathway (11). Moreover, our previous work has demonstrated that high dietary Pi stimulated lung tumorigenesis (6), thus drawing the question of whether low dietary Pi would be chemopreventive. Based on above reports, we hypothesized that low dietary Pi may also affect lung cancer progression through regulating Akt signals.

To test this hypothesis, we performed a low dietary Pi experiment using K-*ras*^{LA1} lung cancer model mice. K-*ras* is the most frequently mutated member in human tumors, including adenocarcinomas of the lungs (≈25–50%) (12). In K-*ras*^{LA1} mice, K-*ras* allele can be activated by an in vivo spontaneous recombination event (13). K-*ras*^{LA1} mice carrying these mutations were highly predisposed to a range of tumor types, predominantly early onset lung cancer similar to human nonsmall lung cancer (13). Our results support the hypothesis that Pi works as a stimulus capable of increasing or decreasing several pivotal genes for lung cancer development.

MATERIALS AND METHODS

Animal and Experimental Diet

Five-week-old male K-*ras*^{LA1} mice were obtained from Human Cancer Consortium–National Cancer Institute (Frederick, MD) and maintained them on a 12-h light–dark cycle in an ambient temperature of 23 ± 2°C at 50 ± 20% humidity. Eighteen mice were randomly divided into two dietary groups (9 mice/group). One group was fed an AIN93-based diet containing 0.5% Pi (normal Pi), and the other group was fed the same diet containing 0.1% Pi (low Pi; Table 1). All diets were prepared according to the guideline of AIN, and the feeding trial was conducted for 4 wk. At the end of the experiment, all mice were killed, and blood was taken by cardiac puncture for further analysis. During the autopsy procedure, collected the lungs and the lesions were counted carefully on the surface of lungs under microscope. After counting the lesion, a lobe of the left lung was collected from each mouse and fixed in 10% neutral buffered formalin for histopathological examination and immunohistochemistry (IHC). The remaining lungs were stored at –80°C for other studies. The levels of Pi in the serum were determined using a biochemical autoanalyzer (VITALAB, Merch, The Netherlands). All methods used in this study were approved by the Animal Care and Use Committee at Seoul National University (SNU 060804–4).

TABLE 1
Composition of the experimental diet (g/kg diet)[a]

	Normal Diet (0.5% Pi)	Low Pi Diet (0.1% Pi)
Corn starch	513.85	529.49
Casein	200.00	200.00
Sucrose	100.00	100.00
Soybean oil	70.00	70.00
Cellulose	50.00	50.00
Mineral premixture[b]	35.00	35.00
Vitamin mixture	10.00	10.00
Methionine	3.00	3.00
Choline bitartrate	2.50	2.50
Tertiary butylhydroquinone	0.01	0.01
KH_2PO_4	15.64	—

[a]Abbreviations are as follows: Pi, phosphate; KH_2PO_4, monopatassium phosphate.

[b]Pi-free mixture.

Western Blot Analysis and Akt Kinase Assay

After measuring the protein concentration of the homogenized lysates using a Bradford kit (Bio-Rad, Hercules, CA), equal amounts (30 μg) of protein were separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked for 1 h in Tris Buffered Saline + Tween 20 (TBST) containing 5% skim milk; immunoblotting was performed by incubating the membranes overnight with their corresponding primary antibodies at 4°C. Antibodies raised against NPT-2b, phosphatase, and tensin homolog deleted on chromosome 10 (PTEN), p-Akt (Thr308), p-eIF4E binding protein 1 (p-4E-BP1 at Ser 65), the p70 ribosomal protein S6 kinase (p-p70S6K), proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin D3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against phospho-mammalian target of rapamycin (p-mTOR) was obtained from Cell Signaling (Beverly, MA). Monoclonal antibodies against total Akt1 and p-Akt at Ser473 were raised using a general method described elsewhere (14). GAPDH antibodies were obtained from BD Biotechnology (San Jose, CA). After washing in TBST, the membranes were incubated with a horseradish peroxidase (HRP)-labeled secondary antibody, and the bands-of-interest were detected using a luminescent image analyzer, LAS-3000 (Fujifilm, Tokyo, Japan). Results were quantified using Multi Gauge version 2.02 program of the LAS-3000. Kinase activity of Akt was examined with Akt kinase assay kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's instruction.

Histopathological Analysis and IHC

The lung tissues were fixed in 10% neutral buffered formalin, paraffin processed, and sectioned at 4 μm. For histological analysis, the tissue sections were stained with haematoxylin and eosin (H&E). For IHC, formalin-fixed, paraffin-embedded tissue sections (4 μm) were deparaffinized in xylene and rehydrated through alcohol gradients and then incubated in 3% hydrogen peroxide (AppliChem, Darmstadt, Germany) for 30 min to quench endogenous peroxidase activity. After washing in PBS, the tissue sections were incubated with 5% BSA in PBS for 1 h at room temperature to block unspecific binding sites. Primary antibodies were applied on tissue sections overnight at 4°C. The following day, the tissue sections were washed and incubated with secondary HRP-conjugated antibodies (1:50) for

TABLE 2
The average daily gain of body weight of animals fed normal and low phosphate (Pi) diets (g/day)[a]

Group	1 Wk	2 Wk	3 Wk	4 Wk
Normal	0.16 ± 0.024	0.43 ± 0.022	0.20 ± 0.080	0.15 ± 0.034
Low	0.27 ± 0.053	0.44 ± 0.088	0.29 ± 0.037	–0.13 ± 0.008**

[a]Normal, normal Pi diet group; Low, low Pi diet group. Data are expressed as μean ± standard error of the mean; $n = 9$.

**$P < 0.01$, highly significant compared to control.

1 h at room temperature. After careful washing, tissue sections were counterstained with Mayer's Hematoxylin (Dako, Caepinteria, CA) and washed with xylene. Cover slips were mounted using Permount (Fisher, Pittsburgh, PA), and the slides were reviewed using a light microscope (Carl Zeiss, Thornwood, NY). PCNA positive staining was determined by counting 3 randomly chosen fields per section, determining the percentage of DAB-positive cells (nucleus stained cell) per 100 cells at X400 by the method described by Zhang et al. (15).

Statistical Analysis

All results are given as means ± standard error of the mean. Results were analyzed by Student's *t*-test (Graphpad Software, San Diego, CA). A value of $P < 0.05$ was considered significant and $P < 0.01$ highly significant compared to the corresponding control.

RESULTS

Low Dietary Pi Decreased the Body Weight Gain and Stimulated Lung Tumorigenesis in K-*ras*^{LA1} Mice

Results showed that low dietary Pi significantly decreased the body weight after 3 wk of feeding the low Pi diet (Table 2). We also examined the potential effect of low dietary Pi on lung tumorigenesis in K-*ras*^{LA1} lung cancer mouse. Our results clearly demonstrated that low dietary Pi significantly increased the lung tumor lesions and the tumor size of lesions at least 1.5 mm in diameter (Figs. 1A, 1B). Histopathological examination also showed that low dietary Pi significantly stimulated pulmonary tumor progression (arrows in Fig. 1C). The results are summarized in Table 3.

Low Dietary Pi Decreased Serum Pi Concentration and Increased NPT-2b Expression in the Lungs of K-*ras*^{LA1} Mice

NPT-2b is known to be closely related with cancer, and NPT expression is regulated by dietary and serum Pi values (1); thus, we evaluated the effect of low dietary Pi on serum Pi level and NPT-2b expression in the lungs. As shown Fig. 2, low dietary Pi resulted in a significant decrease of serum Pi level (Fig. 2A); however, NPT-2b expression was significantly increased in the lungs of K-*ras*^{LA1} mice (Fig. 2B). Further, the increased NPT-2b protein expression was clearly demonstrated by densitometric analysis (Fig. 2C).

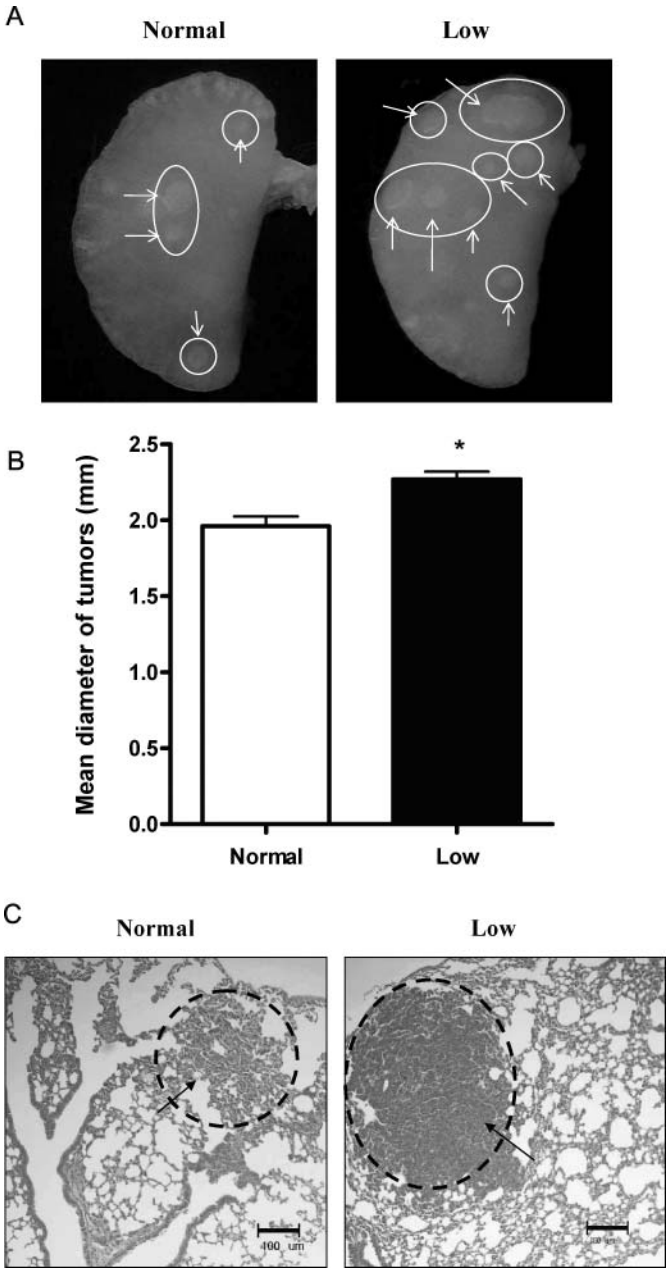

FIG. 1. Tumor pathology of the lungs in K-*ras*^{LA1} mice. A**:** K-*ras*^{LA1} mice lungs showing numerous visible lesions (arrows and dotted circles). B: The mean diameter of tumors of at least 1.5 mm in diameter in K-*ras*^{LA1} mice lung. *, $P < 0.05$ compared to normal diet group (mean ± standard error of the mean; $n = 9$). C: Histological characteristics of K-*ras*^{LA1} mice lungs. Arrows and dotted circles indicate the tumor lesion in the lungs of K-*ras*^{LA1} mice. Normal, normal Pi diet group; Low, low Pi diet group. Magnification ×100; scale bar = 100 μm.

Low Dietary Pi Decreased the PTEN Expression and Increased Akt Activity in the Lungs of K-*ras*^{LA1} Mice

Results showed that low dietary Pi significantly increased Akt phosphorylation at Ser473, whereas PTEN expression was decreased in the lungs of K-*ras*^{LA1} mice (Figs. 3A, 3B). Since Akt phosphorylation is associated with Akt kinase activity (16),

TABLE 3
Summary of tumor incidences in the lungs of K-*ras*^{LA1} mice[a]

Group	No. Mice	Tumor Number/Mouse			Hyperplasia Incidence	Adenoma Incidence
		Total	>1.5 mm[b]	<1.5 mm[c]		
Normal	9	16 ± 0.9	4 ± 0.3	12 ± 0.7	69%	44%
Low	9	$31 \pm 2.2^{**}$	$7 \pm 0.6^{**}$	$24 \pm 1.8^{**}$	44%	100%

[a]Normal, normal diet group; Low, low phosphate diet group. Data are expressed as μean $\pm$ standard error of the mean.
$^{**}P < 0.01$, highly significant compared to control.
[b]Number of tumors of at least 1.5 μm in diameter.
[c]Number of tumors of smaller than 1.5 μm in diameter.

we performed an Akt kinase assay. As shown in Figs. 3C and 3D, low dietary Pi significantly increased Akt kinase activity in the lungs of K-*ras*^{LA1} mice.

Low Dietary Pi Altered Protein Translation in the Lungs of K-*ras*^{LA1} Mice

To investigate whether low dietary Pi affects Akt-downstream signal pathway, we analyzed the changes of mTOR phosphorylation and its downstream protein by Western blot. As shown Fig. 4A, low dietary Pi significantly increased the phosphorylation of mTOR, p70S6K, and 4E-BP1 in the lungs of K-*ras*^{LA1} mice. The increased phosphorylation was clearly demonstrated by densitometric analysis (Fig. 4B).

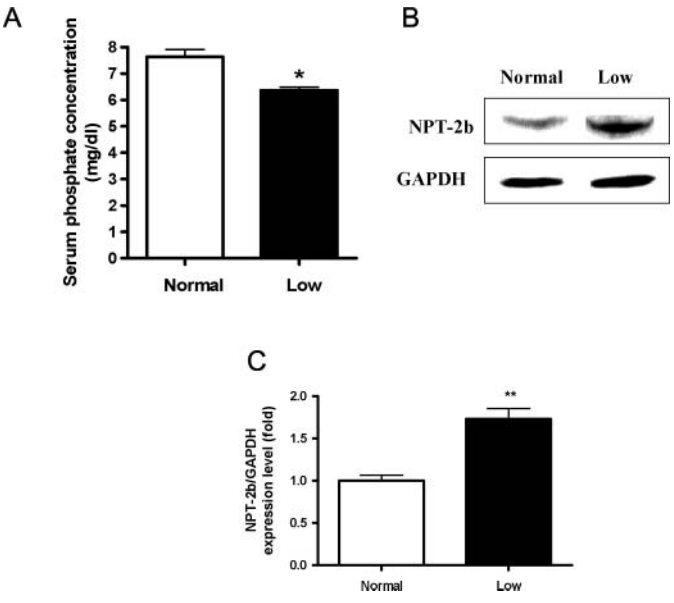

FIG. 2. Serum phosphate level and sodium-dependent inorganic phosphate co-transporter 2b (NPT-2b) protein expression in the lungs of K-*ras*^{LA1} mice fed low and normal phosphate diet. A: Concentration of serum phosphate. B: Expression of NPT-2b protein in the lungs. C**:** The band-of-NPT-2b was further analyzed by densitometer. *, $P < 0.05$, considered significant; **, $P < 0.01$, highly significant compared to control (mean $\pm$ standard error of the mean; $n = 9$). GAPDH, glyderaldehyde-3-phosphate dehydrogenase.

Low Dietary Pi Stimulates Cell Proliferation-Related Protein Expression in the Lungs of K-*ras*^{LA1} Mice

We evaluated the effects of low dietary Pi on cell cycle in the lungs of K-*ras*^{LA1} mice. As shown Figs. 5A and 5B, low dietary Pi significantly increased the expression of the cell proliferation maker protein PCNA and cell cycle regulators such as cyclin D1 and cyclin D3. The altered pattern of PCNA was clearly reconfirmed by IHC analysis (Figs. 5C, 5D).

DISCUSSION

Pi plays a critical role in animal organ development including skeletal (1) and bone formation (17). Therefore, dietary Pi restriction causes the significant decreases of average daily body weight gain (Table 2). Such results have also been observed by other research groups. Czarnogorski et al. (18) reported that low Pi diets significantly decrease average daily body weight gain in rat. Marks et al. (19) also observed significantly decreased body weight in mice by feeding low Pi diet. Body growth and feed intake is closely related, however. Ohnishi et al. (20) demonstrated that Pi restriction-induced decreases in body weight was not due to a decrease in food intake.

Changes in dietary Pi may alter cell function by a number of mechanisms through transportation into the cell. Regulation of Pi transport is accomplished by the family members of NPT (21). Several studies have reported that dietary Pi restriction led to increase the intestines and kidney renal tubular Pi reabsorption to maintain the Pi level within a narrow range along with increased NPT-2b expression (22,23). However, long-term restriction of dietary Pi causes low serum Pi (24). NPT-2b is also expressed in the lungs (7). However, Traebert et al. (25) reported that expression of NPT-2b was not changed by feeding a low Pi diet in the normal lungs of rat. In this study, our results showed that low dietary Pi led to a significant increase in the expression of NPT-2b protein in the lungs of lung cancer model mice (Fig. 2). The increased NPT-2b expression in the lungs of K-*ras*^{LA1} mice may be related with the poor progressed lung cancer because NPT-2b might also play other important roles in cancer. Yin et al. (8) reported that NPT-2b is a potential target for immunotherapy of cancer with antibodies and vaccines. Hashimoto

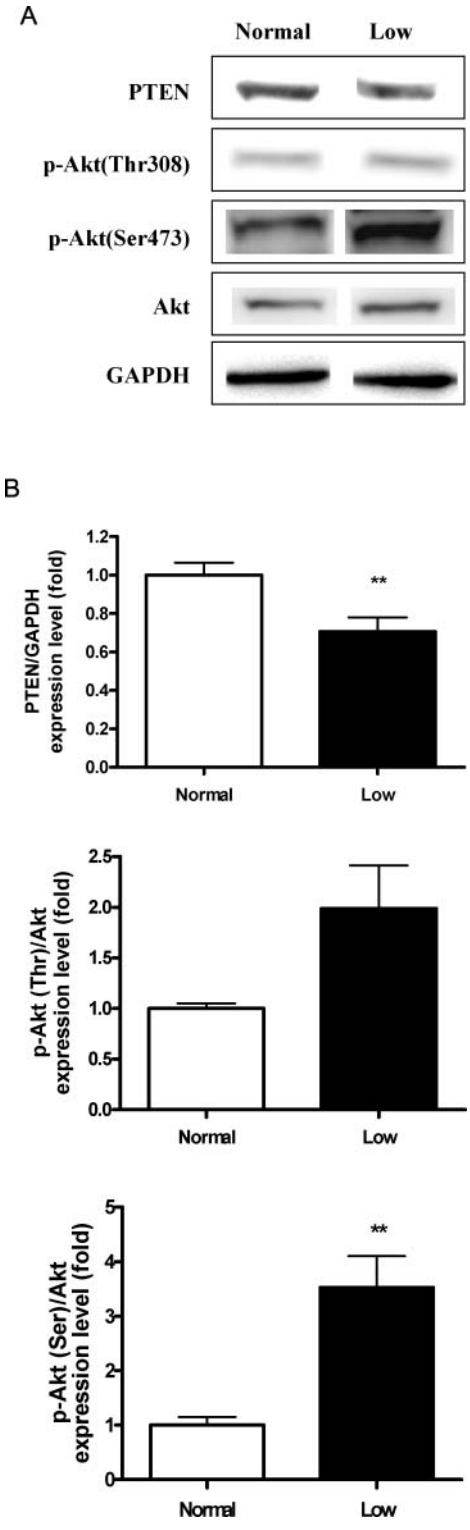

FIG. 3. Akt kinase assay and Western blot (W.B.) analysis of PTEN, Akt, and phospho-Akt proteins in the lungs of K-*ras*^{LA1} mice. A: Expression of PTEN, Akt, and phospho-Akt proteins in the lungs. B: The bands of interests were further analyzed by densitometer. C: Akt kinase activity was measured in the lung homogenates. D: The bands of interest were further analyzed by densitometer. *, $P < 0.05$; **, $P < 0.01$ compared to normal diet group (mean ± standard error of the mean; $n = 9$). GAPDH, glyderaldehyde-3-phosphate dehydrogenase; IP = immunoprecipitation; p-GSK 3 = phosphor-glycogen synthase kinase.

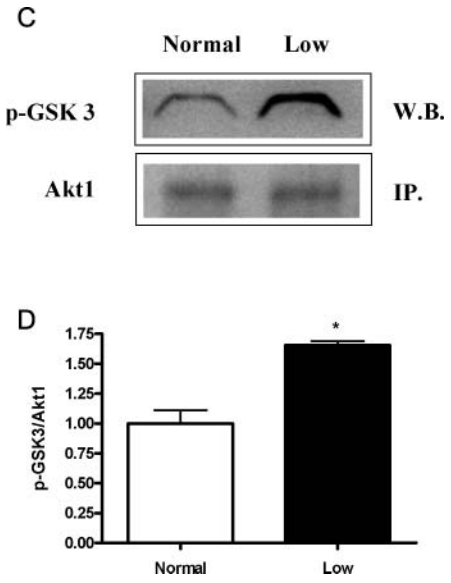

FIG. 3. (Continued)

et al. (7) reported that NPT-2b may be a useful marker to analyze the histopathogenesis of lung cancer, and characterization of human adenocarcinoma by this transporter is also expected.

Akt is a potent survival-promoting molecule that has been proposed as a central signaling event in tumor growth and progression (26). Our results clearly showed that feeding a low Pi diet significantly stimulates lung tumorigenesis through increasing Akt activity while suppressing tumor suppressor protein PTEN expression (Fig. 1 and Fig. 3). Our results are supported by several recent studies. Our previous study clearly demonstrated that low dietary Pi may activate Akt signals in murine normal lungs (27), and West et al. (16) reported that redundant activation of Akt can accelerate K-*ras* mutation initiated lung tumorigenesis. Iwanaga et al. (28) reported PTEN inactivation also can accelerate K-*ras*-initiated lung tumorigenesis, and the K-*ras* mutation caused lung tumorigenesis suppressed by PTEN delivery (29). In addition, Tang et al. (9) reported that overexpression of phosphorylated Akt and loss of PTEN expression in nonsmall cell lung cancer confers poor prognosis.

The Akt-mediated protein kinase controls cellular protein translation, and protein translation is closely related with cancer cell growth (30). Akt can activate mTOR by phosphorylation of mTOR at Ser-2448 (31). Mamane et al. (30) reported that activated Akt/mTOR signaling profoundly stimulates protein translation through increased phosphorylation of its downstream targets 4E-BP1 and p70S6K. Our results have suggested that low dietary Pi strongly stimulates the lung tumorigenesis through facilitating Akt/mTOR signal-mediated protein translation (Fig. 4). Wislez et al. (32) demonstrated that phosphor-p70S6K increased with malignant progression in the lungs, and inhibition mTOR reduced the phosphor-p70S6K expression as well as neoplastic lesion number and size. In addition, Fumarola et al. (33) reported that inhibition of mTOR/S6K signaling reduced

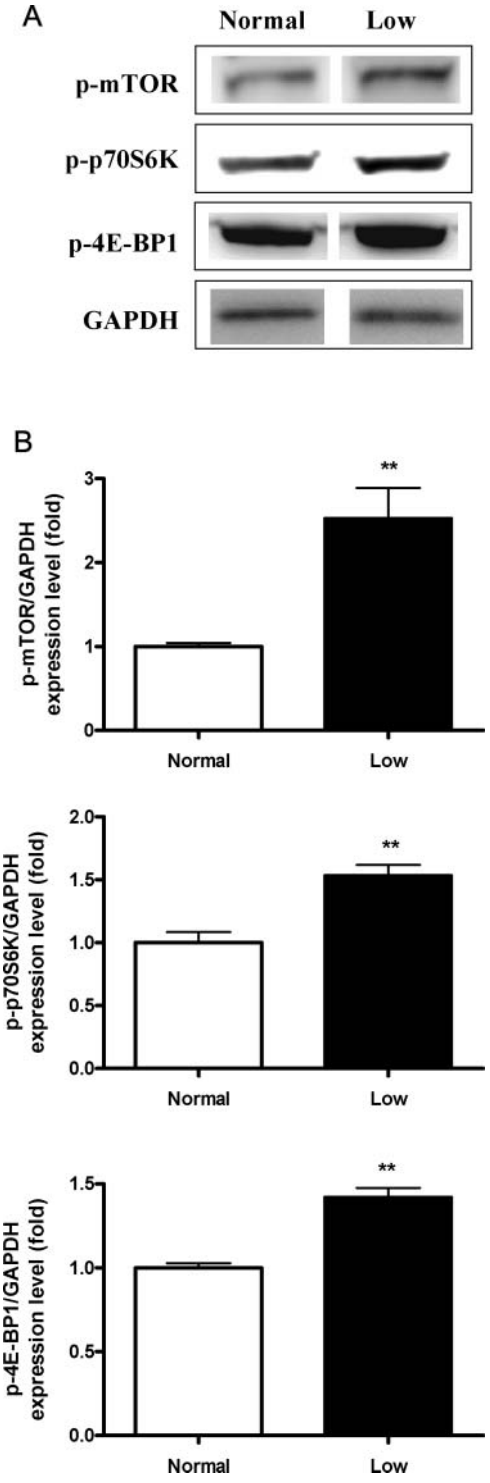

FIG. 4. Western blot analysis of phosphor-mTOR, phosphor-p70S6K, and phosphor-4E-BP1 proteins in the lungs of K-*ras*^{LA1} mice. A: Expression of phosphor-mTOR, phosphor-p70S6K, and phosphor-4E-BP1 proteins in the lungs. B: The bands of interests were further analyzed by densitometer. **, $P < 0.01$ compared to normal diet group (mean ± standard error of the mean; $n = 9$). GAPDH, glyderaldehyde-3-phosphate dehydrogenase.

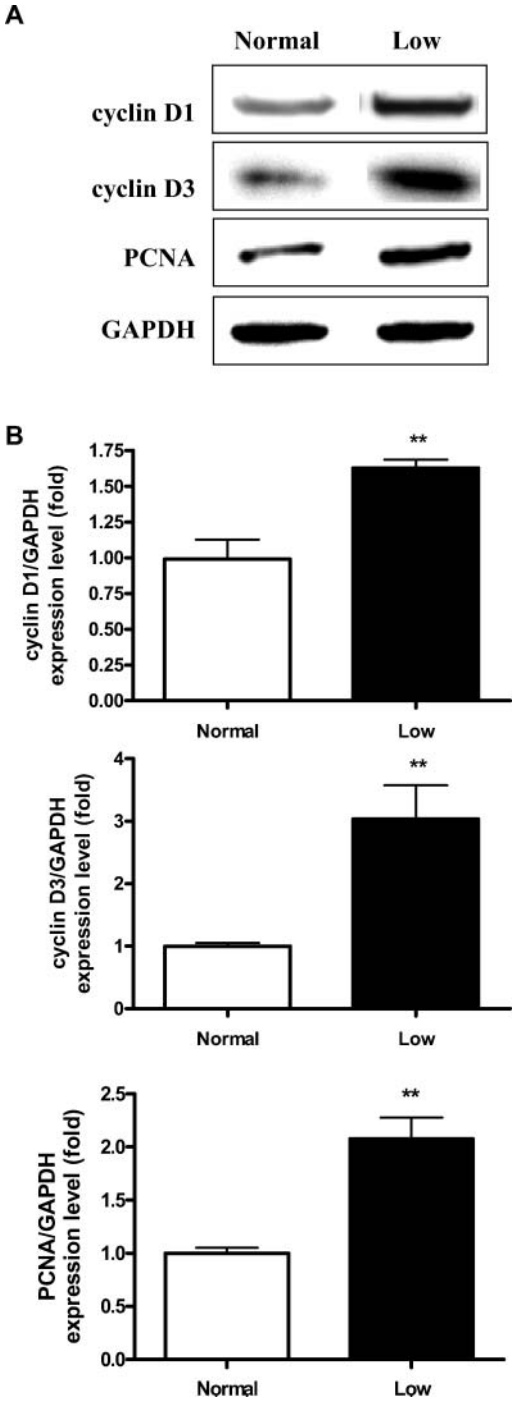

FIG. 5. Western blot analysis of cell cycle signaling proteins in the lungs of K-*ras*^{LA1} mice. A: Expression of cyclin D1, cyclin D3, and PCNA proteins in the lungs. B: The bands of interests were further analyzed by densitometer. C: Immunohistochemical measurement of PCNA in the lungs. Dark brown color indicates the PCNA expression (original magnification, ×400). D: Comparison of PCNA labeling index in lungs of K-*ras*^{LA1} mice. PCNA-positive staining was determined by counting 3 randomly chosen fields per section, determining the percentage of DAB-positive cells per 100 cells at ×400 magnification (scale bar = 100 μm). **, $P < 0.01$ highly significant compared to control (mean ± standard error of the mean; $n = 9$). GAPDH, glyderaldehyde-3-phosphate dehydrogenase.

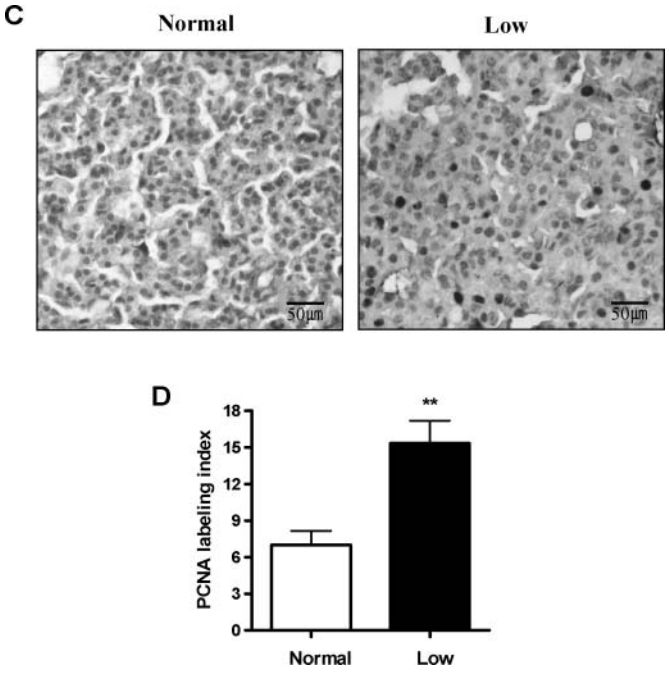

FIG. 5. (Continued)

cell size. Highly expressed p-4E-BP1 has been also detected in tumors, and the p-4E-BP1 level has been associated with a poor prognosis (34). Mutation of the 4E-BP1 phosphorylation site could decrease cell size through inhibiting translation initiation (35).

Akt/mTOR pathway is involved in the regulation of cell cycle progression and cellular proliferation through controlling cyclin D translation (36). D-type cyclins serve as cellular sensors and integrators of extracellular signals during the early to mid G1 cell cycle phase (37). Lin et al. (38) reported that upregulation of cyclin D3 mRNA was associated with carcinogen induced murine lung adenocarcinoma. Cyclin D1 also is a key driver of malignant transformation in nonsmall lung cancer (39), and overexpression of cyclin D1 is a frequent and early step in lung carcinogenesis (40). Gautschi et al. (39) reviewed that smoking induced nuclear accumulation of cyclin D1 in human bronchial epithelium in situ and that accumulation of cyclin D1 caused uncontrolled proliferation in normal human cells, which might facilitate the development of invasive cancer. Taken together, these results suggest that low dietary Pi promotes lung cancer development through stimulating lung cell progression (Fig. 5).

In summary, our previous work indicated that high dietary Pi strongly stimulates lung tumorigenesis; however, this study revealed that excessive dietary Pi restriction also stimulates lung tumorigenesis. Dietary Pi restriction increased the expression of NPT-2b and activated Akt pathway in the lungs of K-*ras*^{LA1} mice. Consequently, the activated Akt pathway facilitated the lung cancer development through increasing protein translation and cell cycling in K-*ras*^{LA1} mice. Therefore, optimal regulation of Pi consumption may be important for part of the treatment of lung cancer patients.

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