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High Dietary Inorganic Phosphate Affects Lung through Altering Protein Translation, Cell Cycle, and Angiogenesis in Developing Mice

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Inorganic phosphate (Pi) plays a key role in diverse physiological functions. Several studies indicate that Pi may affect lung cell development through Na/Pi cotransporter (NPT). Several NPT subtypes have been identified in mammalian lung, and considerable progress has been made in our understanding of their function and regulation. Therefore, current study was performed to elucidate the potential effects of high dietary Pi on lungs of developing mice. Our results clearly demonstrate that high dietary Pi may affect the lung of developing mice through Akt-related cap-dependent protein translation, cell cycle regulation, and angiogenesis. Our results support the hypothesis that Pi works as a critical signal molecule for normal lung growth and suggest that careful restriction of Pi consumption may be important in maintaining a normal development.

Key Words: **Akt; protein translation; cell cycle; angiogenesis; lung; inorganic phosphate.**

Inorganic phosphate (Pi) is an essential nutrient to all living systems. Since phosphate cannot be synthesized, the need for this nutrient is met by ingestion of phosphate in the diet, both naturally and as added phosphate (Weiner *et al.*, 2001). Surveys conducted in various countries indicate that the intake of Pi has increased steadily as Pi-containing foods increased by approximately 17% in the decade leading up to 1993. These surveys also suggested that the use of Pi as a food additive may continue to increase (Calvo, 1993).

Several lines of research indicate that Pi works as a stimulus capable of increasing or decreasing several pivotal genes (Chang *et al.*, 2006; Jin *et al.*, 2006). To date, many studies involving Pi have focused mainly on its effect in bone and kidneys. However, studies have not yet investigated the homeostatic maintenance of normal lung and lung's adaptation to excess Pi. Pi enters into the cells via Na/Pi cotransporter (NPT) (Prie *et al.*, 2005), and the NPT expression is regulated mainly by dietary and serum Pi level (Takeda *et al.*, 2004). Among three classes of NPTs (types I, II, and III), two types (types II and III) have been identified in mammalian lung and considerable progress has been made in our understanding of their function and regulation (Beck *et al.*, 2003). A recent report indicated that elevated Pi could stimulate Akt signaling in human normal lung cells through regulation of NPT expression (Chang *et al.*, 2006).

Akt1/protein kinase B is a serine/threonine kinase, activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components. Akt also has emerged as a crucial regulator of widely divergent cellular processes including apoptosis, proliferation, differentiation, and metabolism (Nicholson and Anderson, 2002). In previous study, we reported that high dietary Pi perturbed normal brain growth through Akt-extracellular signal-regulated kinase (ERK) signaling in developing mice (Jin *et al.*, 2006). In a continuum of works for the elucidation of potential detrimental effects of dietary Pi on normal development, the present study was performed to elucidate the potential effects of high dietary Pi on lungs of developing mice. Our results suggest that high dietary Pi may affect lung through cap-dependent protein translation, cell cycle regulation, and angiogenesis in developing mice.

MATERIALS AND METHODS

Animals and diet. Transgenic mice expressing CMV-LucR-c-myc-IRES-LucF reporter gene are convenient and powerful tools for the confirmation of cap-dependent and cap-independent protein translation since LucR and LucF provide the level of cap-dependent and cap-independent internal ribosome entry site (IRES) protein translation, respectively (Creancier *et al.*, 2001). Such powerful tool for the *in vivo* discrimination between cap-dependent/-independent protein translation has been also confirmed by our previous study demonstrating that high dietary Pi affects brain growth through altering protein translation via Akt signaling (Jin *et al.*, 2006). In a continuing study for the evaluation of potential *in vivo* effects of Pi, we also used same 2-week-old transgenic male mice. The mice were randomly separated into two dietary

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groups (five mice/group): one group on a normal diet containing 0.5% Pi and another group on a high-phosphate diet containing 1% Pi. The mice were on the specified diet for 4 weeks until complete physical maturation (6 weeks after birth). All diets were prepared according to the guideline of American Institute of Nutrition and, thus, fulfill the requirement for normal growth described in Reeves *et al*. (1993). At the end of the experiment, all mice were necropsied and lungs were dissected under stereomicroscope and stored in liquid nitrogen for further use. All animal experiments were performed according to the guideline for the care and use of laboratory animal of Seoul National University.

Luciferase assay. Luciferase activities in the tissue extracts were measured according to the manufacturer's instruction. Briefly, the lungs were homogenized in passive lysis buffer (Promega, Madison, WI). The homogenates were centrifuged for 20 min at 5500 ×g at 4°C, and the supernatant was centrifuged for an additional 15 min at 16,000 ×g at 4°C. LucF and LucR activities were measured using a dual luciferase assay kit (Promega).

Western blot analysis. After measuring the protein concentration of the homogenized lysates using a Bradford kit (Bio-Rad, Hercules, CA), equal amounts (50 μg) of protein were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into nitrocellulose membranes. After the membranes were blocked for 1 h in Tween-Tris Buffered Saline (TTBS) 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, p-Akt (Thr308 and Ser473), Akt1, p-4E-BP1, 4E-BP1, proliferating cell nuclear antigen (PCNA), cyclin D1, cyclinD3, CDK4, p53, p27, p21, matrix metalloproteinase 2 (MMP-2), fibroblast growth factor 2 (FGF-2) and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). mammalian target of rapamycin (mTOR) and phosphor- glycogen synthase kinase 3 were obtained from Cell Signaling (Beverly, MA). Antibody against CD31 and CD34 was purchased from Abcam (Beverly, MA). After washing, 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, Japan, Tokyo). Results were quantified using Multi Gauge version 2.02 program of the LAS-3000.

Immunoprecipitation and Akt kinase assay. The kinase activity of Akt was measured by using the Akt kinase assay kit according to the instruction of the manufacturer (Cell Signaling Technology, Danvers, MA). Briefly, lung tissues were lysed in ice-cold lysis buffer [20mM Tris (pH 7.5), 150mM NaCl, 1mM ethylenediaminetetraacetic acid, 1mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1 μg/ml leupeptin] plus 1mM phenylmethylsulfonyl fluoride. Tissue homogenates were centrifuged, and 200 μl of supernatants were immunoprecipitated with 20 μl of immobilized antibody bead for overnight at 4°C. One microliter of 10mM adenosine triphosphate and 1 μg of glycogen synthase kinase-3 (GSK-3) fusion protein, an Akt substrate, was added to the suspended pellet with 50 μl of kinase buffer (25mM Tris [pH 7.5], 5mM β-glycerophosphate, 2mM dithiothreitol, 0.1mM Na_3VO_4, and 10mM $MgCl_2$) and incubated for 30 min at 30°C. The reactions were terminated with the addition of 25 μl of 3× SDS sample buffer and boiling at 100°C for 5 min. Reaction samples were resolved by SDS-PAGE and performed by Western blot.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were transferred to plus slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were deparaffinized in xylene and rehydrated through alcohol gradients and incubated in 200 μl of proteinase K and then washed and incubated in 3% hydrogen peroxide (AppliChem, Darmstadt, Germany) for 30 min to quench endogenous peroxidase activity. After washing in phosphate-buffered saline (PBS), the tissue sections were incubated with 5% bovine serum albumin 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 for 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).

Data analysis. Quantification of Western blot analysis was performed using Multi Gauge version 2.02 program (Fujifilm). All results are given as means ± SE. 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

High Pi Increased NPT-2b Expression in the Lungs of Developing Mice

The diverse functional roles of Pi strongly suggest that long-term *in vivo* studies are needed. NPT-2 protein plays an important role in the Pi homeostasis, and the NPT-2 expression was regulated by dietary Pi and serum Pi level (Takeda *et al*., 2004). Therefore, in this study, we analyzed NPT-2b expression by Western blot in the lungs of developing mice. Our results showed that high dietary Pi significantly increased NPT-2b protein expression in the lungs (Fig. 1A), and densitometric analysis clearly confirmed that high Pi increased NPT-1 protein expression in the test group (Fig. 1B).

High Dietary Pi Increased Akt Kinase Activity in the Lungs of Developing Mice

In our previous study, we demonstrated that high dietary Pi changed the pattern of Akt protein expression and

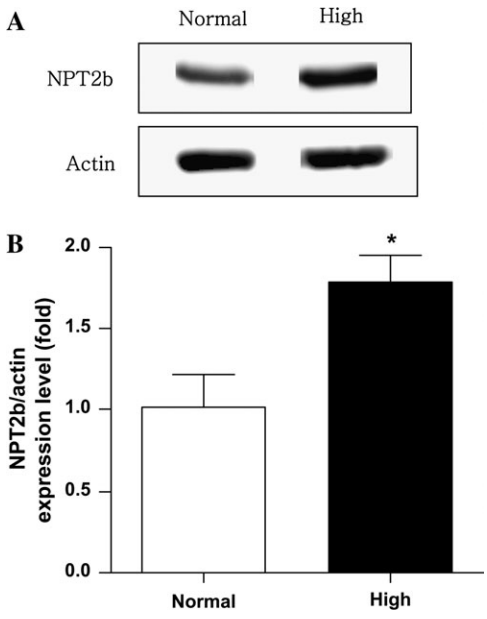

FIG. 1. Western blot analysis of NPT-2b protein in the lungs of transgenic mice expressing CMV-LucR-cMyc-IRES-LucF reporter gene. Two-week-old transgenic mice were fed a normal and high-Pi diet for 4 weeks. Lung homogenates were subjected to Western blot analysis. (A) Expression of NPT-2b in the lungs. (B) The bands of interest were further analyzed by densitometer. $*p < 0.05$ compared with normal diet group (mean ± SE, $n = 3$).

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phosphorylation in mouse brain (Jin *et al*., 2006). Thus, in the present study, we also examined the effects of high Pi on Akt expression and phosphorylation in the lungs of mice. As shown in Figures 2A and 2B, high dietary Pi significantly increased Akt phosphorylation both at Ser473 and Thr308; however, no significant changes were observed in total Akt expression. Such increased Akt phosphorylation at both critical sites caused high Akt kinase activity in the high-Pi group (Fig. 2C). Moreover, such changes of Akt phosphorylation were further confirmed by immunohistochemistry (IHC) (Fig. 2D) as well as assays of labeling indices (Fig. 2E).

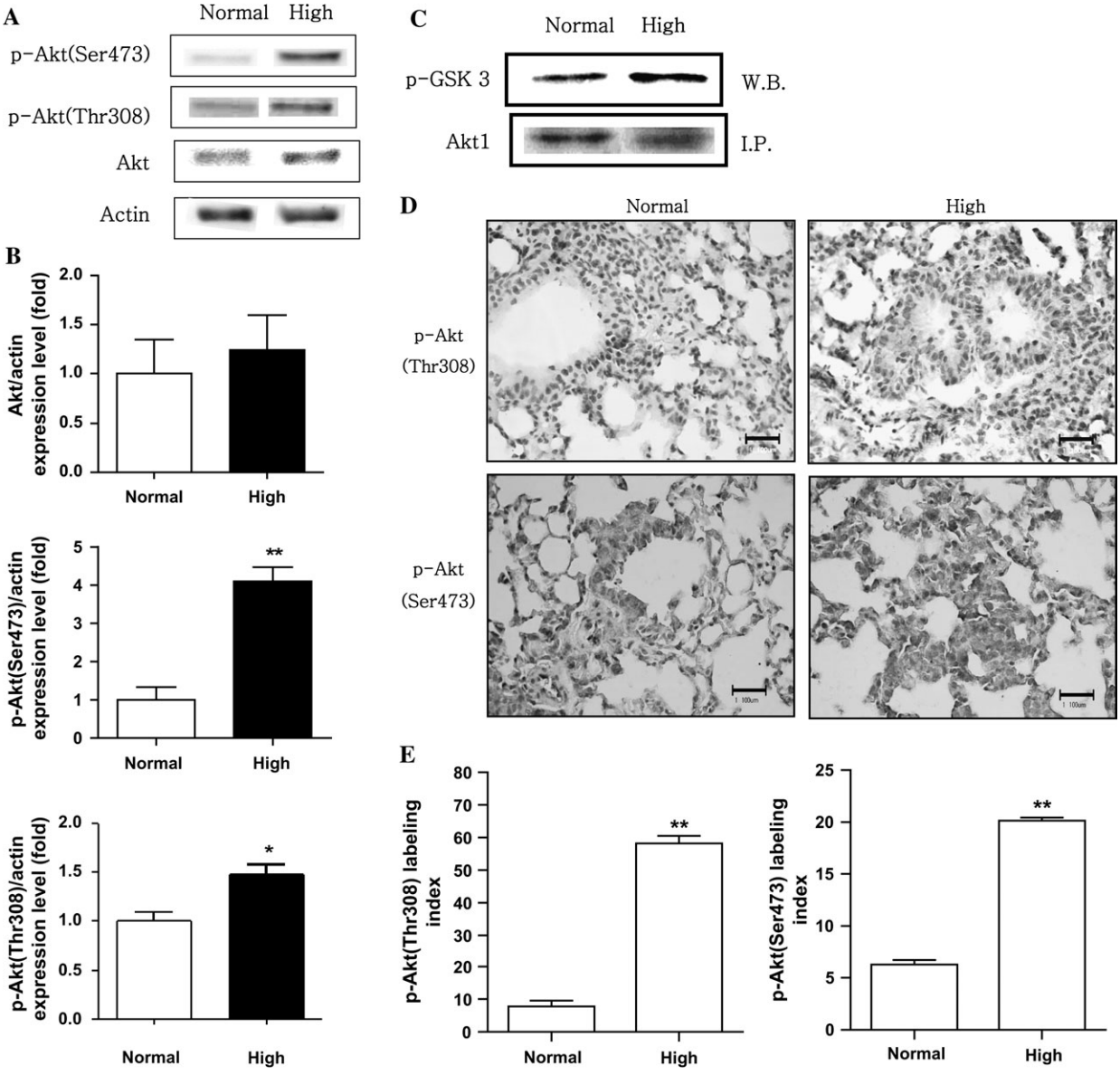

FIG. 2. Western blot, IHC, and kinase assay of Akt in the lungs of transgenic mice expressing CMV-LucR-cMyc-IRES-LucF reporter gene. Two-week-old transgenic mice were fed a normal and high-Pi diet for 4 weeks. Lung homogenates were subjected to Western blot analysis. (A) Expression of Akt and phospho-Akt protein in lungs. (B) The bands of interest were further analyzed by densitometer. (C) Akt-kinase activity was measured in the lung homogenates. Protein was immunoprecipitated with Akt-specific antibody, and GSK-3 protein was used as a substrate. The immunoblotting of Akt1 was used as a control (mean ± SE, $n = 3$). (D) IHC of phospho-Akt. For IHC, lung tissues were fixed and incubated with phospho-Akt antibodies. Dark brown color represents the phosphorylated Akt. The high dietary Pi increased the expression of Akt phosphorylation at Ser473 and Thr308 in lungs. Original magnification was × 200. Scale bar = 100 μm. (E) Comparison of Akt phosphorylation at Thr308 and Ser473 labeling indices in lungs. Positive stainings of p-Akt (Thr308) and p-Akt (Ser473) were determined by counting 10 randomly chosen fields per section, determining the percentage of diaminobenzidene-positive cells per 100 cells at × 200 magnification (mean ± SE, $n = 3$). $^*p < 0.05$, $^{**}p < 0.01$ compared with normal diet group.

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High Dietary Pi Facilitates Cap-Dependent Protein Translation in the Lungs of Developing Mice

We also examined the potential effects of high Pi on protein translation in the developing murine lung. Results showed that high Pi significantly changed the Akt downstream proteins such as mTOR and 4E-BP1. High dietary Pi increased the protein expression of mTOR, decreased 4E-BP1 protein expression, and increased the phosphorylation of 4E-BP1 significantly (Figs. 3A and 3B). To determine the differential level of cap-independent and cap-dependent protein translation, we performed firefly and renilla luciferase assays. Our results indicated that high Pi enhanced cap-dependent protein translation in developing mice lung (Fig. 3C).

High Dietary Pi Stimulates Cell Cycle in the Lungs of Developing Mice

We evaluated the effects of high Pi on lung cell proliferation through measuring the expressions of cell cycle–related marker proteins, motivated not only by critical roles of Akt in cell cycle (Nicholson and Anderson, 2002) but also by our current findings of upregulated Akt kinase activity (Fig. 2). Our results showed that high dietary Pi significantly decreased the protein

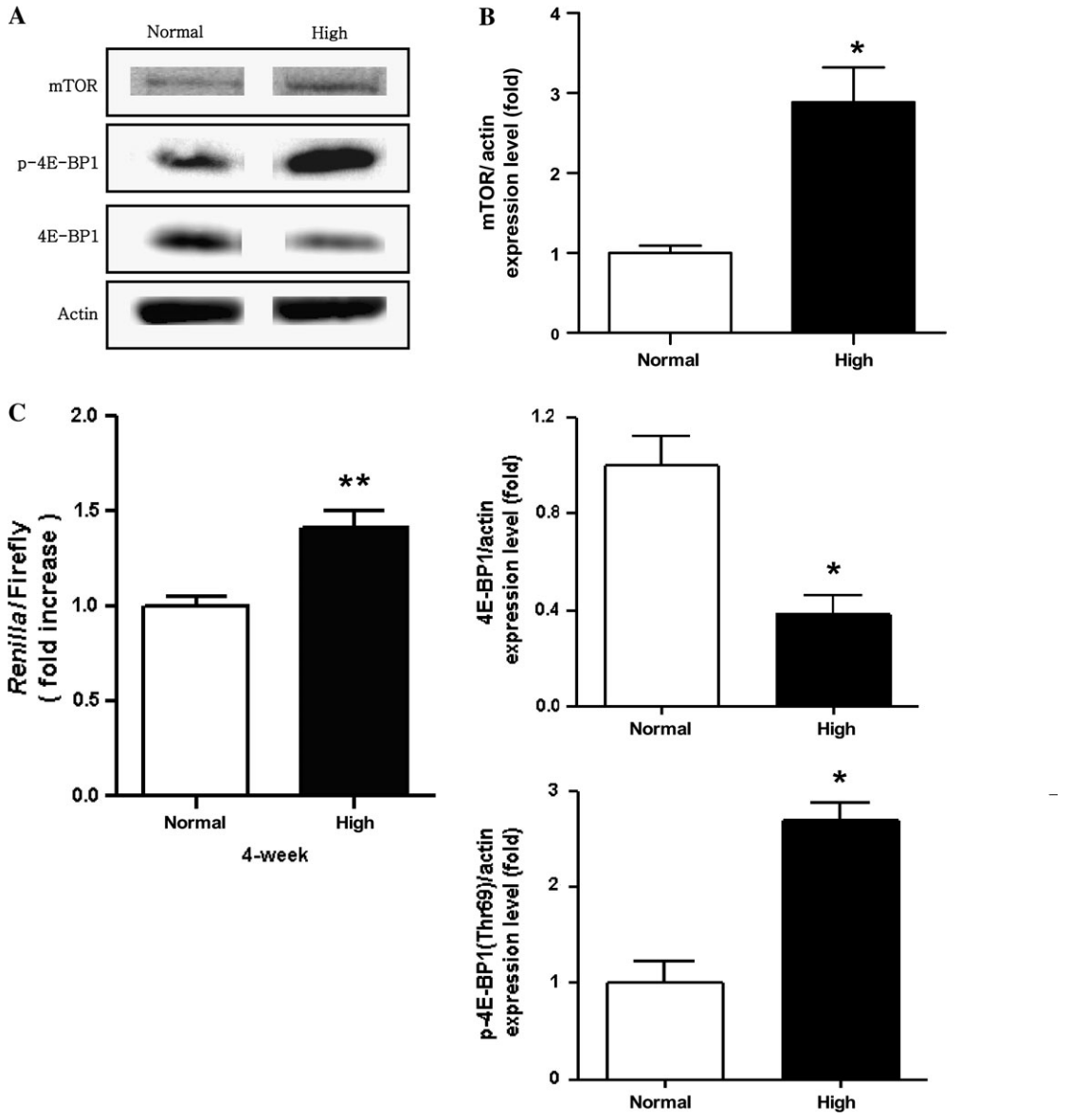

FIG. 3. Western blot analysis of Akt downstream signals and luciferase assay in the lungs of transgenic mice expressing CMV-LucR-cMyc-IRES-LucF reporter gene. Two-week-old transgenic mice were fed a normal and high-Pi diet for 4 weeks. Lung homogenates were subjected to Western blot analysis. (A) Expressions of mTOR, p-eIF4E-BP1, and eIF4E-BP1 protein in the lungs. (B) The bands of interest were further analyzed by densitometer. (C) Luciferase ratios in the lungs. *$p < 0.05$ compared with normal diet group (mean ± SE, $n = 3$).

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expressions of p53, p21, and p27 (Figs. 4A and 4B). High dietary Pi also increased the protein expressions of cyclin D1 and cyclin D3, CDK4, and cell proliferation marker PCNA (Figs. 4C and 4D).

High Dietary Pi Facilitates Angiogenesis in the Lungs of Developing Mice

To investigate the potential effects of high dietary Pi on the angiogenesis, we measured the expression levels of MMP-2 and FGF-2 proteins by Western blot and IHC. As shown in Figures 5A and 5B, high Pi significantly increased the protein expressions of MMP-2 and FGF-2 in the lungs of developing mice. Labeling index assay of FGF-2 in the lung also clearly demonstrated that high Pi caused the FGF-2 protein expression (Fig. 5C). Angiogenesis, the sprouting of new blood vessels, plays a role in diverse pathologies including neoplastic, inflammatory, and degenerative conditions (Cherrington *et al*., 2000). Thus, further confirmatory study of angiogenesis

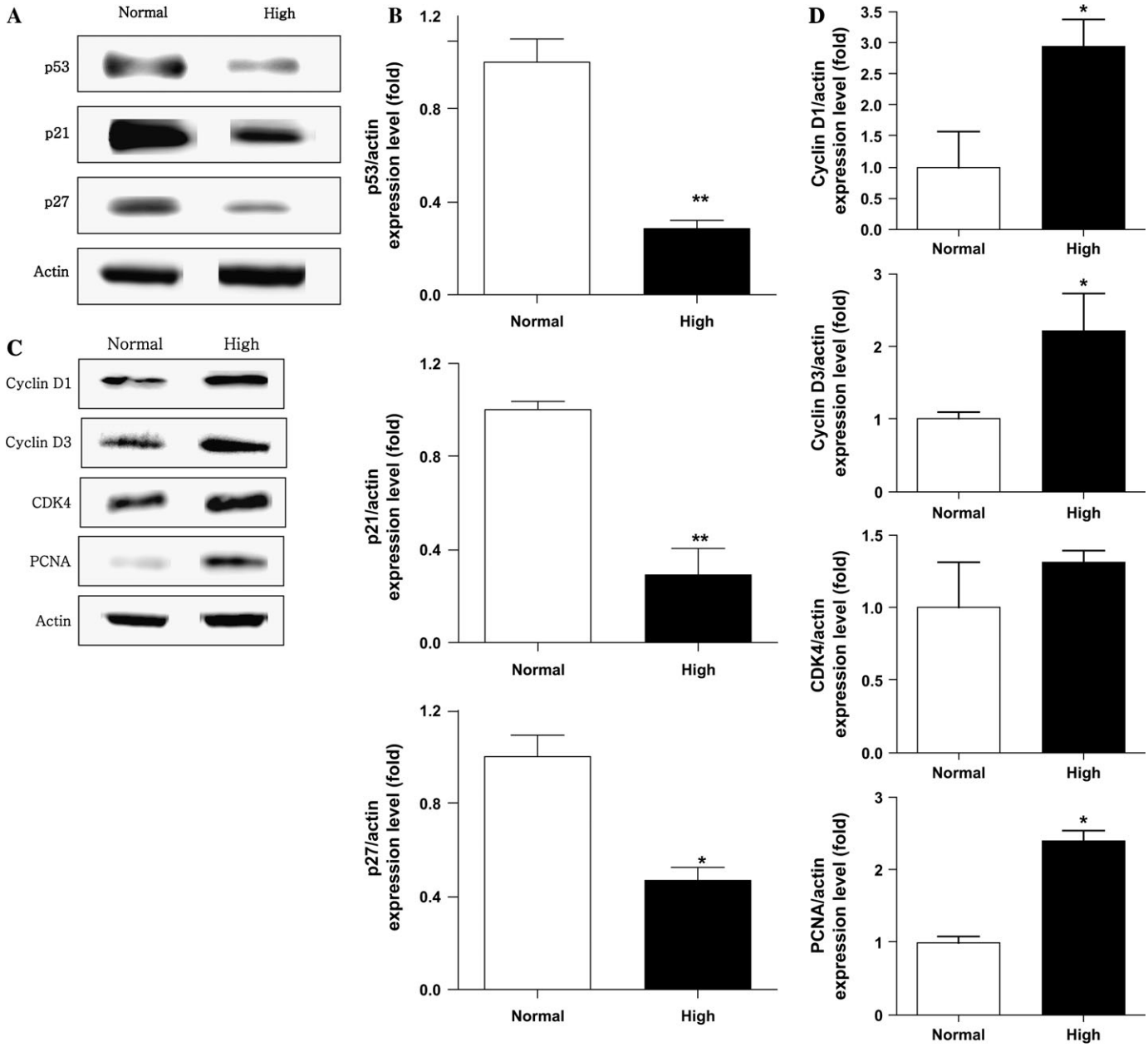

FIG. 4. Western blot analysis of cell cycle signal proteins in the lungs of transgenic mice expressing CMV-LucR-cMyc-IRES-LucF reporter gene. Two-week-old transgenic mice were fed a normal and high-Pi diet for 4 weeks. Lung homogenates were subjected to Western blot analysis. (A) Expressions of p53, p27, and p21 protein. (C) Expressions of PCNA, cyclin D1, cyclin D3, and CDK4 proteins in lungs. (B, D) The bands of interest were further analyzed by densitometer. *$p < 0.05$ compared with normal diet group (mean ± SE, $n = 4$).

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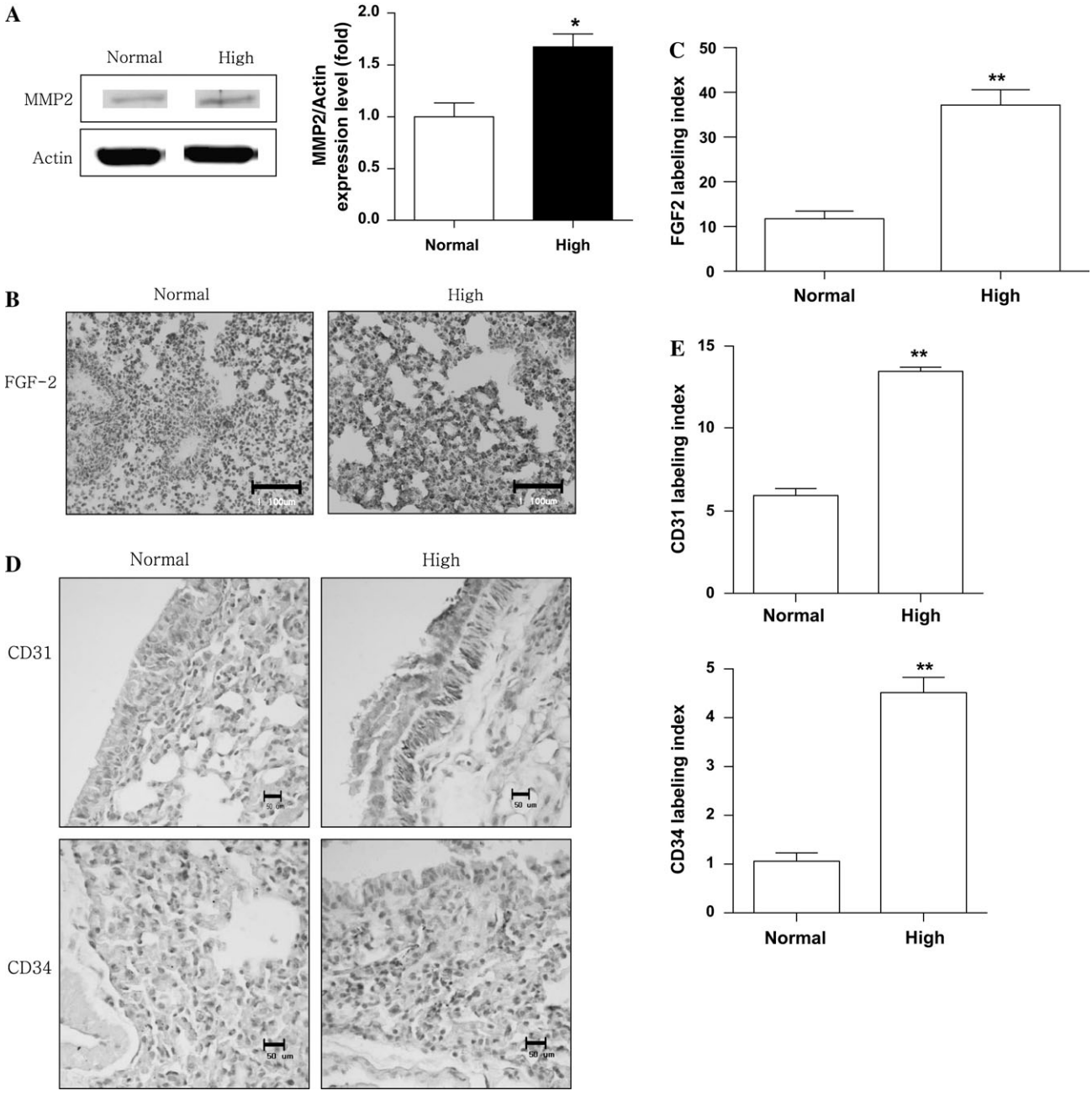

FIG. 5. Western blot analysis of MMP-2 and immunohistochemical analysis of FGF-2, CD31 and CD34 in the lungs of expressing CMV-LucR-cMyc-IRES-LucF reporter gene transgenic mice. Two-week-old transgenic mice were fed a normal or high-Pi diet for 4 weeks. Lungs homogenates were subjected to Western blot. (A) Expression of MMP-2 in the lungs. (B) IHC of FGF-2 in lung tissues. For IHC, lung tissues were fixed and incubated with FGF-2 antibody. Dark brown color represents the FGF-2. The high-Pi diet increased the expression of FGF-2 protein in lungs. Original magnification was × 200. Scale bar = 100 μm. (C) Comparison of FGF-2 labeling index in lungs. Positive staining of FGF-2 was determined by counting 10 randomly chosen fields per section, determining the percentage of diaminobenzidene (DAB)-positive cells per 100 cells at × 200 magnification (mean ± SE, $n = 3$). Scale bar = 100 μm. (D) IHC of CD31 and CD34 in lung tissues. Dark brown color represents the CD31 and CD34, respectively. The high-Pi diet increased the expression of CD31 and CD34 proteins in lungs. Original magnification was × 400. Scale bar = 50 μm. (E) Comparison of CD31 and CD34 labeling indices in lungs. Positive staining of CD31 and CD34 was determined by counting 10 randomly chosen fields per section, determining the percentage of DAB-positive cells per 100 cells at × 400 magnification (mean ± SE, $n = 3$). *$p < 0.05$, **$p < 0.01$ compared with normal diet group.

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was performed using CD31 and CD34 as angiogenic marker proteins because CD31 and CD34 stainings are widely used to predict tumor formation as they show the extent of angiogenesis (Pusztaszeri *et al.*, 2006). As shown in Figures 5D and 5E, high dietary Pi increased the angiogenesis in the lungs of developing mice.

DISCUSSION

Nononcogenic as well as neoplastic lung tissues often display alterations of gene expression in signal transduction pathways responsible for homeostasis; however, the exact mechanisms by which the gene modulates abnormal cell growth/differentiation have yet to be determined. Such alterations are likely associated with cellular changes that involve an imbalance between cell proliferation, DNA repair, and cell death. Additionally, these alterations may result from cellular aging and/or insults from endogenous or exogenous chemical exposure (Chang *et al.*, 2006).

Pi is abundant in the diet, and the intestinal absorption of Pi is efficient and well regulated. The kidney is a major regulator of Pi homeostasis and can increase or decrease its Pi reabsorptive capacity to accommodate Pi need. The bulk of filtered Pi is reabsorbed in the proximal tubule where sodium-dependent Pi transport system in the brush-border membrane mediates the rate-limiting step in the overall Pi reabsorptive process (Takeda *et al.*, 2000). As mentioned earlier, Pi plays a key role in diverse physiological functions with the aids of NPT subtypes expressed in mammalian lung. Several lines of research indicate that Pi works as a stimulus capable of increasing or decreasing several pivotal genes such as transcriptional regulator, signal transduction, and cell cycle regulator (Beck *et al.*, 2003; Chang *et al.*, 2006, Jin *et al.*, 2006). Together, the potential importance of Pi as a novel signaling molecule and pulmonary expression of NPTs with poor prognosis of diverse oncogenic or nononcogenic lung diseases have prompted us to begin to define the pathways by which Pi regulates lung of developing mice.

Our study demonstrated that high dietary Pi significantly increased the protein expression of NPT-2b in the lungs of developing mice (Fig. 1). This result suggests that high Pi may increase the amount of Pi entrance into lung through increasing NPT-2b expression. Such high Pi entrance may be responsible for regulation of signals important for maintaining cellular homeostasis. Our finding is supported by reports from other works. Recently, Beck *et al.* (2003) reported that phosphate might be an important global signaling molecule capable of regulating the expression of a variety of gene in multiple cell type and multiple tissue types. In fact, our group also demonstrated that elevated inorganic phosphate significantly increased Akt-related signal protein expressions in normal lung cells (Chang *et al.*, 2006).

Akt/protein kinase B has emerged as a crucial regulator of widely divergent cellular processes including apoptosis, proliferation, differentiation, and metabolism (Nicholson and Anderson, 2002). For full activity, Akt requires phosphorylation both at Thr308 and Ser473 (West *et al.*, 2003). In present study, we demonstrated that high Pi significantly increased the activity of Akt through increasing the phosphorylation of Akt both at Thr308 and Ser473 in the lungs of developing mice (Fig. 2). These results suggest that high Pi can regulate the Akt downstream signal pathways through activated Akt. The mTOR protein belongs to PI3K pathway (Asnaghi *et al.*, 2004), directly activated by Akt (Sekulic *et al.*, 2000), and acts as a regulator of protein synthesis and translation initiation (Gingras *et al.*, 2001). Such important function of mTOR was confirmed by the current study because high Pi induced mTOR activation and increased 4E-BP1 phosphorylation significantly (Fig. 3). The mTOR kinase is known to phosphorylate 4E-BP1, including its dissociation from eIF4E, which can bind the cap structure at the 5′ terminal of mRNAs, thereby allowing cap-dependent translation (Schmelzle and Hall, 2000). Our luciferase result (Fig. 3C) also demonstrated that high Pi increased the cap-dependent protein translation in murine lungs. Current findings are supported by our previous studies that elevated Pi can stimulate the cap-dependent protein translation through activation of Akt signaling in normal lung cells (Chang *et al.*, 2006) and brain (Jin *et al.*, 2006).

The mTOR integrates signals from nutrients and growth factors leading to cell growth (Nave *et al.*, 1999). Several studies demonstrated that Akt/mTOR pathway might promote cell cycle progression through control S6K1, 4E-BP1 (Fingar *et al.*, 2004), p27, p21 (Mayo and Donner, 2002), and cyclin D3 proteins (Feng *et al.*, 2000). In present study, we also observed that high dietary Pi increased cell proliferation marker PCNA expression through decreasing p53, p21 and p27 and increased cyclin D expression in the lungs of mice (Fig. 4). The p53 protein is a major tumor suppressor protein and plays an important role on cell cycle, and activity of p53 is regulated by Akt signal pathway (Mayo and Donner, 2002). According to El-Deiry (1998), p53 could increase p21 expression, a cell cycle inhibitor. Taken together, our data suggest that high Pi may affect lung by controlling the cell cycle function in developing mice lungs.

The MMP-2 has been recognized as a major mediator of basement membrane degradation, angiogenesis, tumor invasion, and metastasis (Kurizaki *et al.*, 1998). The FGF-2 is one of the most important angiogenic factors and is known to be upregulated in angiogenic lesions (Bremnes *et al.*, 2006). Recently, several studies reported that expressions of MMP-2 (Zhang *et al.*, 2004) and FGF-2 (Newman *et al.*, 2004) were stimulated by Akt pathway. Such findings are well matched with our result that FGF-2 and MMP-2 expressions were increased (Fig. 5). As mentioned earlier, angiogenesis plays a role in diverse pathologies including neoplastic, inflammatory, and degenerative conditions (Cherrington *et al.*, 2000). In current study, we used two representative angiogenesis marker proteins such as CD31 and CD34. CD31 (platelet/endothelial

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cell adhesion moleculae-1) is a single-chain type-1 transmembrane protein that plays a role in adhesive interactions between adjacent endothelial cells as well as between leukocytes and endothelial cells (Pusztaszeri *et al*., 2006). Recently, usefulness of CD31 as a progressive inflammatory angiogenesis marker has been reconfirmed in mouse liver model (Straub *et al*., 2007). On the other hand, CD34, a member of the immunoglobulin superfamily, is frequently used as an angiogenic marker protein because CD34 staining shows the extent of angiogenesis. CD34 plays a major role in the adhesion cascade between endothelial cells during angiogenesis (Pusztaszeri *et al*., 2006). In our study, it has been clear that high dietary Pi has induced the angiogenesis in the lungs of developing mice. Combined with abnormal Akt activity (Fig. 2), alteration of cell cycle control (Fig. 4), and increased expression of MMP-2, FGF-2, and CD31/34 (Fig. 5), these data suggest that high Pi may contribute to the formation of angiogenesis in the developing mice lung. Further works to elucidate detailed relationship between high dietary Pi and angiogenesis in terms of abnormal lung growth and/or function are needed.

The changes observed in current study, however, may be a part of systemic toxicity because our previous work has also demonstrated that high dietary Pi also has caused adverse effects on brain (Jin *et al*., 2006). In this study, the high-Pi diet contains 1% phosphorus, which is about 40 mg/day of daily intake. Such daily intake is about 1.4–1.6 times of dietary reference intakes (Anderson and Garner, 1996). Therefore, concentration of Pi used in current study is not very high, rather the concentration seems to be over the normal intake level. At this concentration, tissues may demonstrate some adaptive activity such as apoptosis because some degree of apoptosis has occurred in the lung exposed to high dietary Pi (data not shown). However, the overall balance looks to be tilted toward abnormal toxic responses because increased Akt activity (Fig. 2), altered cell cycle regulation (Fig. 4), and increased angiogenesis (Fig. 5) have been clearly demonstrated. Moreover, decrease of body weight gain was obvious and critical unbalance of serum Ca^{++} and Pi was observed in our previous work (Jin *et al*., 2006). Significant reductions in serum Ca^{++} levels in response to high dietary Pi may shed light on the detrimental effects of high Pi on normal growth. In fact, Ca^{++} is responsible for the tight regulation of the secretion of parathyroid hormone (PTH), an excess of which results in hyperparathyroidism. To maintain an appropriate level of PTH, PTH degradation normally occurs within the parathyroid gland prior to secretion (Shoback *et al*., 2003). In our study, it is hypothesized that high Pi–induced reduction of serum Ca^{++} interferes with the normal degradation of PTH and in turn affects growth. This hypothesis is further supported by a recent study demonstrating how low concentrations of extracellular Ca^{++} downregulate degradation of PTH through the suppression of (1–84)hPTH generation, which is responsible for N-terminal truncation of PTH in parathyroid cells (Kawata *et al*., 2005). Therefore, it can be concluded that high Pi–induced reductions of serum Ca^{++} levels may lead to harmful effects. Recent study performed by Takeda *et al*. (2004) also has demonstrated that excess Pi for a long time may stimulate aging process. Another study by us using normal human lung cells also has clearly demonstrated that high dietary Pi controls lung cell growth by activating ERK cascades and by facilitating the translocation of Mnk1 from cytosol into nucleus through Akt-mediated mitogen-activated protein kinase/extracellular signal-regulated kinase kinase pathway. Sequentially, translocated Mnk1 increases eIF4E-BP1 phosphorylation. As a result, Pi stimulates cap-dependent protein translation (Chang *et al*., 2006). Together, our studies suggest that excess Pi (not very high level, but just over normal level) may be associated with detrimental health effects.

In conclusion, our results suggest that high dietary Pi may have detrimental effects on the normal development of young mouse lung through protein translation, cell cycle, and angiogenesis. The effects of dietary Pi on such important signaling pathways may be involved in numerous pulmonary function during development; thus, careful restriction of Pi consumption may be important in maintaining a normal development.

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