

# A Novel Pyruvate Kinase M2 Activator Compound that Suppresses Lung Cancer Cell Viability under Hypoxia

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Pyruvate kinase M2 isoform (PKM2), a rate-limiting enzyme in the final step of glycolysis, is known to be associated with the metabolic rewiring of cancer cells, and considered an important cancer therapeutic target. Herein, we report a novel PKM2 activator, PA-12, which was identified via the molecular docking-based virtual screening. We demonstrate that PA-12 stimulates the pyruvate kinase activity of recombinant PKM2 *in vitro*, with a half-maximal activity concentration of 4.92  $\mu$ M, and effectively suppresses both anchorage-dependent and -independent growth of lung cancer cells in non-essential amino acid-depleted medium. In addition, PA-12 blocked the nuclear translocation of PKM2 in lung cancer cells, resulting in the inhibition of hypoxia response element (HRE)-mediated reporter activity as well as hypoxia-inducible factor 1 (HIF-1) target gene expression, eventually leading to the suppression of cell viability under hypoxia. We also verified that the effects of PA-12 were dependent on PKM2 expression in cancer cells, demonstrating the specificity of PA-12 for PKM2 protein. Taken together, our data suggest that PA-12 is a novel and potent PKM2 activator that has therapeutic implications for lung cancer.

## INTRODUCTION

Cancer cells exhibit a high rate of aerobic glycolysis (the Warburg effect). The metabolic rearrangement in cancer cells is an appropriate means for providing biomaterials as well as energy that are essential for their growth and survival (DeBerardinis et al., 2008; King and Gottlieb, 2009; Vander Heiden et al., 2009). Pyruvate kinases are glycolytic enzymes that convert phos-

phenolpyruvate (PEP) to pyruvate at the final step of glycolysis (Chaneton and Gottlieb, 2012). Among the four pyruvate kinase isozymes PKM2, one of the two splice variants of *PKM* gene, is the one predominantly expressed in various cancers, including those of the bladder, colon, and lung (Bluemlein et al., 2011). Unlike PKM1, which maintains a single activity form, PKM2 can switch between an active tetrameric form and an inactive dimeric form. Fructose 1,6-bisphosphate (FBP), an allosteric PKM2 activator, converts PKM2 to the active tetrameric form that has a high affinity for PEP, resulting in the inhibition of glycolysis and thus diminution of biomaterials for cell growth by diverting glucose from pentose phosphate pathway (Ding et al., 2010; Dombrauckas et al., 2005). On the other hand, various modifications, including phosphorylation (Gao et al., 2012; Yang et al., 2012b), prolyl hydroxylation (Luo et al., 2011), acetylation (Lv et al., 2011), cysteine oxidation (Anastasiou et al., 2011), and demethylation (Wang et al., 2014), induce the conversion of tetrameric PKM2 proteins to a less active dimeric form that has a low affinity for PEP, leading to the intracellular accumulation of glycolytic intermediates for biosynthesis (Gruning et al., 2011).

Recent reports demonstrated that PKM2 activators can induce serine auxotrophy in cancer cells by causing the reduction of serine biosynthesis and promote the expression of high-affinity serine transporters (Kung et al., 2012; Parnell et al., 2013). Serine provides essential precursors for the synthesis of proteins, nucleic acids, and lipids that are crucial for cancer cell growth. Serine is also an allosteric activator of PKM2, promoting the shift of the less active dimeric form to an active tetrameric form (Amelio et al., 2014). Thus, treatment with a PKM2 activator in serine-depleted media induced inhibition of cancer cell growth (Kung et al., 2012; Parnell et al., 2013). Additionally, several studies reported that the inactive dimeric form of PKM2, modified by multiple signaling molecules, is strongly implicated in tumorigenesis, as an active protein kinase phosphorylating specific nuclear proteins (Gao et al., 2012; Yang et al., 2012a) or as a transcriptional cofactor of hypoxia-inducible factor (HIF)-1 $\alpha$  (Luo et al., 2011). These studies suggest a requirement for the therapeutics targeting dimeric PKM2 in cancer treatment.

In view of the evidence that PKM2 activation alters cancer cell metabolism and, consequently, decreases cellular proliferation, PKM2 activators may provide a novel anticancer therapeutic strategy. Several PKM2 activators have been reported, including pyridazinone (Anastasiou et al., 2012) and arylsulfonamides (Boxer et al., 2010; Walsh et al., 2011). Herein, we describe a novel PKM2 activator that shows promising efficacy for lung cancer treatment.

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## MATERIALS AND METHODS

### Cell culture

All cell lines were purchased from American Type Culture Collection (ATCC) and were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. A549 human lung cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic-antimycotic (Gibco, USA). H1299 human lung cancer cells were cultured in RoswellPark Memorial Institute medium 1640 (RPMI1640) medium supplemented with 10% FBS and 1% antibiotic-antimycotic. IMR90 human lung cells were cultured in Eagle's Minimum Essential Medium (EMEM) medium supplemented with 10% FBS and 1% antibiotic-antimycotic. NIH/3T3 mouse embryo fibroblast cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS, Gibco, USA) and 1% antibiotic-antimycotic.

### Reagents

PKM2 activator compounds were purchased from ChemBridge Corporation (USA) and PKM2 activator III (PKIII) from EMD Millipore (USA). An antibody that detects PKM2 was purchased from Cell Signaling Technology (USA). Antibodies against  $\alpha$ -tubulin and lamin B1 were purchased from Santa Cruz Biotechnology (USA). Recombinant human PKM2 protein, for use in the *in-vitro* ATP assay, was obtained from Abnova (USA).

### RNA extraction and reverse transcription PCR (RT-PCR)

Total RNA was isolated using the TRIzol RNA isolation reagent (Invitrogen, USA), and 5  $\mu$ g each RNA sample was reverse transcribed using M-MuLV reverse transcriptase (MBI, St. Leon-Rot, Germany) according to manufacturer's guidelines. The primers used in this study are as follows: PKM2-F, CCGCCG-CCTGGCGCCCATTA; PKM2-R, CGGTCAGCACAATGACC-ACATC;  $\beta$ -Actin-F, CTAGAAGCATTGCGGTGA;  $\beta$ -Actin-R, CTGGAGAAGAGCTACGAGCT; Glut3-F, CCCAGATCTTGG-TCTGGAA; Glut3-R, AACGGCAATGGCAGCTGGAC; Glut1-F, TGACCATCGCGTAGCACTG; Glut1-R, CACAGCATTGAT-CCCAGAGA; VEGF-F, AAGGAGGAGGGCAGAATCAT; VEGF-R, ATCTGCATGGTGATGTTFGA.

### Molecular modeling

Molecular docking-based virtual screening was used to screen the ChemBridge database ( $8.4 \times 10^5$  compounds). The Glide software program (Schrödinger, LLC, USA) was used for virtual docking of compounds and used grid-based ligand docking with an energetics algorithm. The crystal structure of PKM2 with N-(4-[[4-(pyrazin-2-yl)piperazin-1-yl]carbonyl]phenyl)quinoline-8-sulfonamide (Protein Data Bank code: 4G1N) bound in the allosteric binding site was used as a starting model for the virtual screening. The protein structure was corrected by adding hydrogen atoms, bond orders, and formal charges, using the Protein Preparation Wizard tool of the Maestro software package (Version 9.6, Schrödinger, LLC, USA). The protein-ligand structure was then subjected to energy minimization, using the Optimized Potentials for Liquid Simulations-All Atom force field. The binding affinity of the docked molecules can be considered as being directly proportional to the docking score; 85 molecules with a high docking score (Glide gscore < -10) were selected as potential activators of PKM2 (with a potentially high affinity to bind to PKM2) (Supplementary Table). The molecular graphics for the activator binding pocket and refined binding

model for the PKM2 activators were generated using the PyMol molecular graphics package (<http://www.pymol.org>).

### *In vitro* ATP assay for PKM2 activity

To determine pyruvate kinase activity, an ATP assay was carried out using the ATP Colorimetric/Fluorometric Assay Kit, in accordance with the manufacturer's instructions (BioVision, USA). Briefly, assays were carried out in the presence of recombinant PKM2 protein (50 ng) or cell lysate (10  $\mu$ g) in the presence of each compound (30  $\mu$ M). After incubation at room temperature for 30 min, the fluorescence of each sample was measured at excitation and emission wavelengths of 530 nm and 590 nm, respectively.

### Anchorage-independent cell growth

Cells ( $8 \times 10^3$ ) suspended in complete growth medium were added to 0.6% agar, together with different doses of each compound, in a base layer and overlaid with a top layer of 0.3% agar. The cultures were maintained at 37°C in a 5% CO<sub>2</sub> incubator for 3 weeks and colony images were captured under a microscope using the DP Controller software (version 2.1, Olympus).

### Cell viability assay

Cells were seeded ( $1 \times 10^3$  cells/well) in 96-well plates, incubated for 24 h, and then treated with PKM2 activator compounds or PKIII in BME. After incubation for 48 h under normoxia or hypoxia, cell viability was determined by counting cell numbers or by a fluorescence assay using CellTiter-Blue® reagent (Promega, USA). For the fluorescence assay, the cells were incubated with 20  $\mu$ l of CellTiter-Blue® reagent for 2 h at 37°C in a 5% CO<sub>2</sub> incubator. The fluorescence in each well was measured at excitation and emission wavelengths of 560 and 590 nm, respectively.

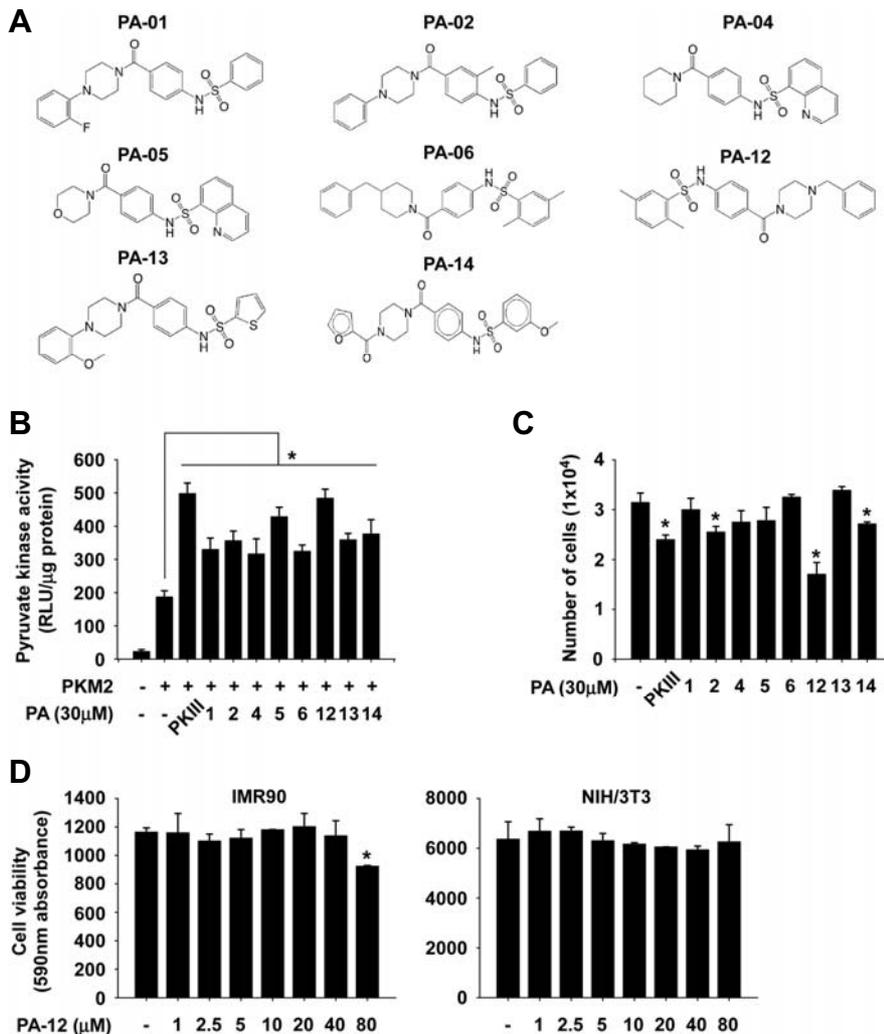
### Luciferase assay for hypoxia response element (HRE) reporter activity

Transient transfection was conducted using Lipofectamine™ (Invitrogen, USA) and Enhancer Q (JBI, Korea), and assays to determine firefly luciferase and *Renilla* luciferase activities were carried out according to the manufacturer's manual (Dual-Luciferase Reporter Assay System, Promega, USA). Cells ( $1 \times 10^4$  cells/well) were seeded the day before transfection in 12-well culture plates. Cells were co-transfected with an HRE reporter plasmid (250 ng) and an internal control vector (pCMV-*Renilla*; 50 ng) and incubated for 24 h. PA-12- or PKIII-treated cells were incubated for 2 h and then cultured for 48 h under hypoxia. Cells were harvested using the lysis buffer and luciferase activities measured using the substrates provided in the reporter assay system. The firefly luciferase activity was normalized to *Renilla* luciferase activity.

## RESULTS

### Identification of a potent PKM2 activator, PA-12

To identify the initial group of candidate compounds targeting PKM2, we performed molecular docking-based virtual screening using published PKM2 structure (Kung, Hixon et al., 2012) and  $8.4 \times 10^5$  compounds in the ChemBridge database, and identified 85 compounds that exhibited significant binding potentials to the allosteric binding site of tetrameric PKM2 protein (Supplementary Table and Supplementary Fig. 1A). We then experimentally evaluated these compounds for their effects on the pyruvate kinase activity in an *in-vitro* enzyme assay using recombinant PKM2 protein, and identified eight compounds



**Fig. 1.** Identification of PA-12 as a potent PKM2 activator. (A) Structure of potential PKM2 activator candidates. (B) Effects of the candidate compounds on the pyruvate kinase activity of PKM2. The activity was assessed by an *in-vitro* ATP assay using PKIII as the reference. (C) Effects of the candidate compounds on A549 lung cancer cell viability in an NEAA-depleted medium. (D) Cytotoxic effects of PA-12 on IMR-90 human lung fibroblast and NIH/3T3 mouse embryonic fibroblast cells. For B-D, all data are represented as the mean  $\pm$  S.D. of values from three independent experiments with duplicate samples. The asterisk (\*) indicates a significant difference ( $p < 0.05$ ) of PA-12-treated samples or cells compared to untreated controls.

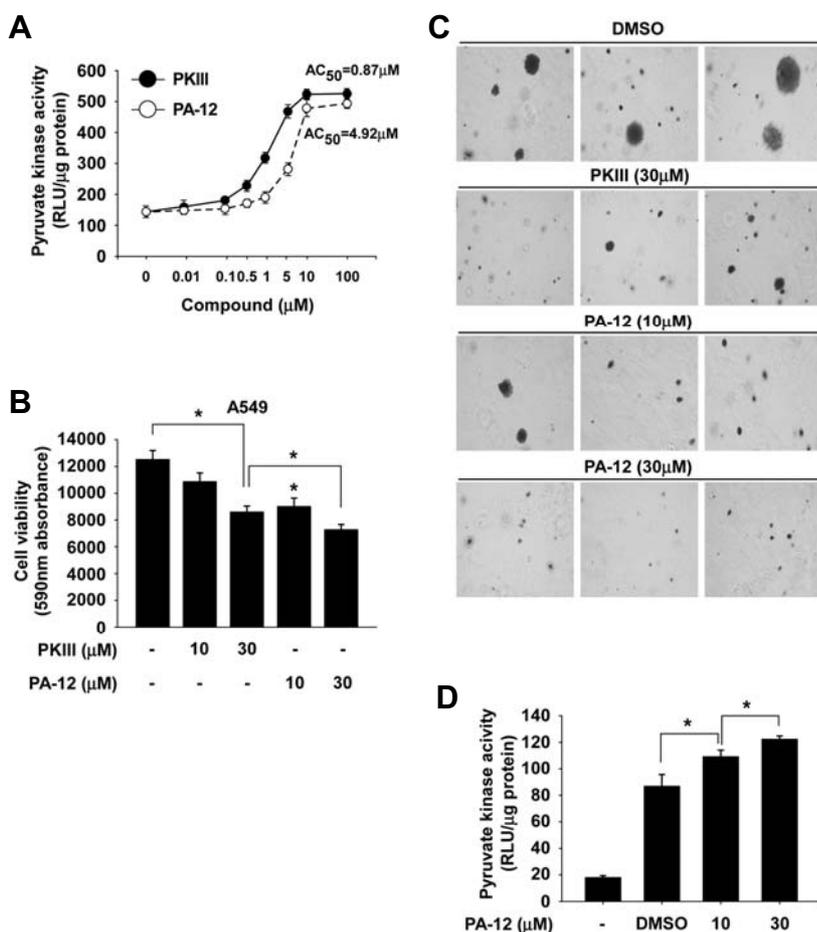
showing > 2-fold activation compared to PKM2 enzyme alone as the candidate PKM2 activators (Fig. 1A and Supplementary Fig. 1B). The effects of these eight compounds in activating the pyruvate kinase activity of PKM2 were confirmed by repeating the *in-vitro* pyruvate kinase assay with PKIII included as the reference compound (Fig. 1B).

We next investigated the effects of the 8 candidate PKM2 activators and PKIII on the viability of A549 lung cancer cells in non-essential amino acid (NEAA)-depleted medium. Cell viability was determined by counting cell numbers after incubating A549 cells with 30  $\mu$ M of each compound for 48 h. Three compounds (PA-2, PA-12, and PA-14) among the 8 candidates significantly suppressed cell viability. In particular, PA-12 was more effective in suppressing cell viability than the reference compound, PKIII (Fig. 1C). To determine the cancer specificity of the effect of PA-12 on cell viability, we treated two non-transformed cell lines, IMR-90 (human fetal lung fibroblast) and NIH/3T3 (mouse embryonic fibroblast), with PA-12 at various concentrations. Cell viability was unaffected up to 40  $\mu$ M of PA-12 in both cell lines (Fig. 1D), suggesting that the suppressive effect of PA-12 might be more selective towards the characteristics of cancer cells.

### PA-12 effectively suppresses cancer cell growth by activating PKM2 *in vivo*

For further validation of the efficacy of PA-12 as a PKM2 activator suppressing cancer cell growth, we examined the binding characteristics between PA-12 and PKM2 protein. We predicted a binding model for the two molecules by docking PA-12 *in silico* to a selected allosteric pocket in the PKM2 protein structure. The results indicated that PA-12 forms favorable interactions at numerous points and docked precisely within the allosteric site of tetrameric PKM2 (Supplementary Fig. 2A). We then quantitatively determined the PKM2 activating activity of PA-12 by a dose-responsive pyruvate kinase assay *in vitro*. PA-12 showed an effective PKM2 activation with a half-maximal activity concentration of 4.92  $\mu$ M, which was comparable to that of the reference compound (Fig. 2A). We also confirmed that PA-12 increases pyruvate kinase activity in cancer cell lysates (Supplementary Fig. 2B). These data indicate that PA-12 is a potent activator of PKM2.

We then characterized the inhibitory effect of PA-12 on cancer cell growth in more detail. A549 and H1299 lung cancer cell lines treated with two different concentrations of PA-12 in NEAA-depleted medium indicated that PA-12 treatment can



**Fig. 2.** PA-12 suppresses lung cancer cell growth. (A) *In-vitro* pyruvate kinase activity assay of PKM2 in the presence of PA-12. The activity was assessed by an *in-vitro* ATP assay. (B) Effect of PA-12 on lung cancer cell growth in an NEAA-depleted medium. Cell growth was analyzed using the CellTiter-Blue® assay. (C) PA-12 inhibits anchorage-independent growth of lung cancer cells in an NEAA-depleted medium. Data shown are three different areas randomly selected to represent the total population; similar results were obtained from three independent experiments. (D) PA-12 induces pyruvate kinase activity in A549 lung cancer cells.

cause a strong inhibition of cell growth in a dose-dependent manner (Fig. 2B and Supplementary Fig. 2C). The growth inhibitory effect of PA-12 was superior to that of the reference compound, even at the concentration of 10 μM. We also investigated the effect of PA-12 on anchorage-independent cell growth in soft agar medium and found a strong suppression of colony formation (Fig. 2C). We then investigated whether the inhibitory effect of PA-12 on cancer cell growth is associated with the activation of PKM2 activity inside the cell *in vivo*. Assay of pyruvate kinase activity in cell lysates prepared after treating A549 cells with PA-12 in culture indicated that PA-12 could significantly induce pyruvate kinase activity in intact cells in a dose-dependent manner (Fig. 2D). Therefore, these results suggest that PA-12 might be an effective anti-tumor compound, activating PKM2 both *in vitro* and *in vivo*.

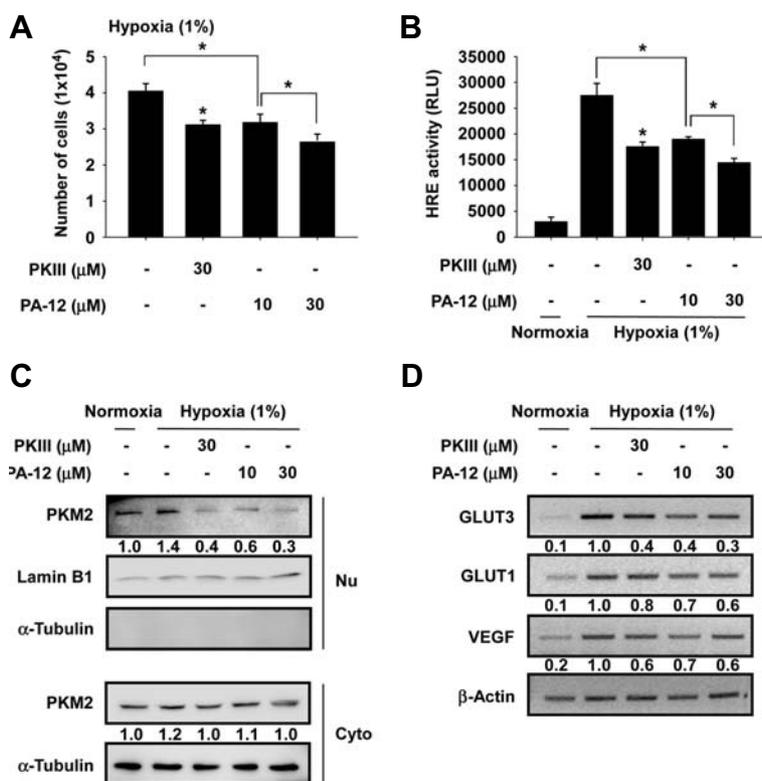
#### PA-12 suppresses hypoxia-induced lung cancer cell viability via inhibition of HRE activity

A previous study reported that PKM2 activators suppressed the growth of xenografted tumors and prolonged cancer cell doubling time under hypoxia (Anastasiou et al., 2012). Therefore, we investigated whether PA-12 could suppress cancer cell viability under hypoxia. Cells were pretreated with PA-12 or PKIII for 2 h under normoxia and then incubated further for 48 h under hypoxia. The cell viability rate under hypoxia, measured by counting cell numbers, was significantly decreased by PA-12 in a dose-dependent manner (Fig. 3A). It was reported that

dimeric PKM2 enhanced HIF-1α activity in the nucleus to facilitate the reprogramming of glucose metabolism and promote tumorigenesis (Luo et al., 2011), while PKM2 activators changed the dimeric form of PKM2 to a tetrameric form (Anastasiou et al., 2012). We, therefore, first investigated the effects of PA-12 on the nuclear localization of PKM2 under hypoxia. Cells were pretreated with PA-12 or PKIII for 2 h, cultured further for 12 h under hypoxia, and then the nuclear fraction was isolated for the immunodetection of PKM2. The nuclear PKM2 level was slightly increased under hypoxia compared to normoxia but PA-12 strongly suppressed the nuclear translocation of PKM2 (Fig. 3B). We then investigated whether PA-12 can also regulate HIF activity in A549 cells under hypoxia. At 24 h post-transfection with an HRE reporter, the cells were treated with PA-12 or PKIII for 2 h and then cultured further for 12 h under hypoxia. HRE reporter activity was strongly suppressed by PA-12 in a dose-dependent manner (Fig. 3C). In accordance, PA-12 significantly inhibited the expression of HIF target genes, including GLUT3, GLUT1 and VEGF (Fig. 3D). These results indicate that PA-12 can abolish the PKM2-mediated transcriptional activation of HIF-1 by suppressing the nuclear translocation of PKM2.

#### Anticancer activity of PA-12 is dependent on the abundance of PKM2

In order to study the influence of PKM2 expression on the PA-12-mediated inhibition of lung cancer cell viability, we con-



**Fig. 3.** Effects of PA-12 on lung cancer cell viability under hypoxia. (A) PA-12 suppresses lung cancer cell viability under hypoxia. (B) Effect of PA-12 on intracellular localization of PKM2 under hypoxia. For A-B, A549 lung cancer cells were pretreated with PA-12 or PKIII for 2 h and then cultured for a further 48 h (A) or 12 h (B) under hypoxia. The cytoplasmic or nuclear protein was fractionated from PA-12 or PKIII-pretreated or untreated cells, and analyzed by Western blotting. Expression of lamin B1 (nuclear protein) and  $\alpha$ -tubulin (cytoplasmic protein) was used to verify sample loading. (C) PA-12 suppresses hypoxia-induced hypoxia response element (HRE) activity. HRE activity was analyzed by a luciferase assay. (D) PA-12 inhibits the expression of HIF target genes. A549 cells were pretreated with PA-12 or PKIII for 2 h and then cultured for 12 h under hypoxia. Expression of HIF target genes was analyzed by RT-PCR with  $\beta$ -actin as the loading control. For B and D, the numbers underneath the graphic western blotting and RT-PCR data are quantitative estimations of the band intensity made using Image J program (NIH, USA). Similar results were obtained from three independent experiments, and representative data are shown.

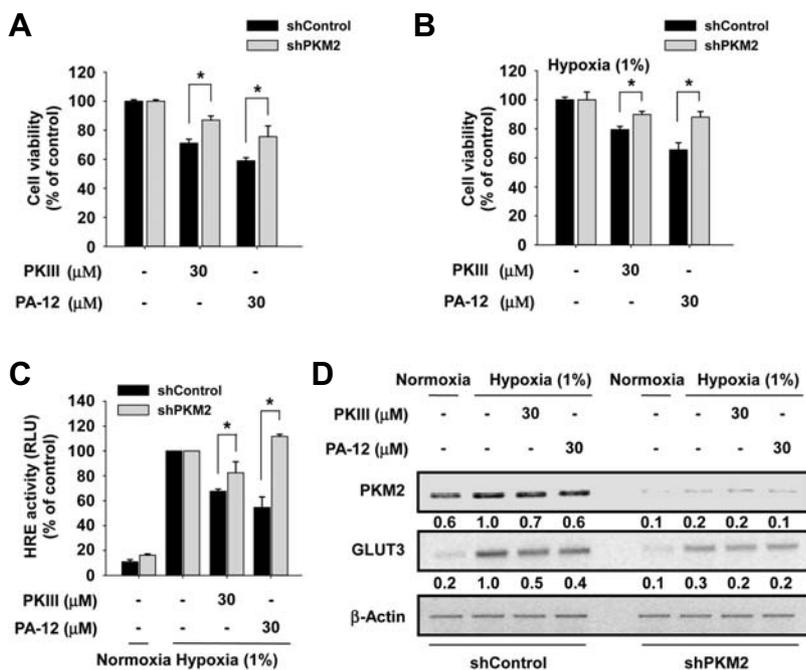
structured A549 cell lines stably expressing short hairpin RNA (shRNA) for PKM2 (*shPKM2*) or mock (*shControl*). The cells were treated with PA-12 or PKIII in NEAA-depleted medium for 48 h in normoxia, and cell viability was assessed by counting cell numbers. Results indicated that *shPKM2* cells were less sensitive to the inhibitory effect of PA-12 (30  $\mu\text{M}$ ) than *shControl* cells (Fig. 4A). Since PA-12 could inhibit HIF signaling, we also determined the effect of PA-12 on cell viability under hypoxia. A549 lung cancer cells were pretreated with PA-12 or PKIII for 2 h and cultured further for 48 h under hypoxia. Results showed that the PA-12-mediated inhibitory effect on cell viability was significantly blocked in the PKM2-depleted cells (Fig. 4B). In accordance, the hypoxic HRE reporter activity suppressed by PA-12 was dramatically restored in the PKM2-depleted cells (Fig. 4C). We then examined the effect of PA-12 on the hypoxia-induced expression of the HIF-1 $\alpha$  target gene, GLUT3. Cells were pretreated with PA-12 or PKIII for 2 h and then incubated under hypoxia for 12 h. The hypoxic induction of GLUT3 was much weaker in *shPKM2* cells than in control cells (Fig. 4D). In addition, the inhibitory effect of PA-12 on the hypoxia-induced expression of GLUT3 seemed to be relatively weaker in *shPKM2* cells compared to that in *shControl* cells. Taken together, these results imply that the inhibitory effect of PA-12 on lung cancer cell viability is dependent on PKM2 protein expression.

## DISCUSSION

Recent advances in the discovery of anticancer drugs include an emphasis on molecular target-based therapeutic agents (Higgins and Baselga, 2011; Imai and Takaoka, 2006). Structural and computational technique-based virtual screening, especially the application of molecular modeling, molecular

docking, and virtual high-throughput and targeted drug screening, has emerged as a reliable, cost-effective, and time-saving approach for the discovery of lead compounds (Sliwoski et al., 2014). In the present study, we applied a virtual screening technique to select potential PKM2 activators, and identified PA-12 as a potent PKM2 activator. Although the effect of PA-12 on the activation of PKM2 activity was weaker than that of PKIII in an *in-vitro* pyruvate kinase assay, PA-12 more strongly inhibited cancer cell viability than PKIII. In contrast, examination of the inhibitory effect of PA-12 on the viability of non-transformed lung and embryonic fibroblast cell lines indicated that PA-12 has only a minimal general cytotoxicity. In addition, since the inhibitory effect of PA-12 on cancer cell growth was significantly alleviated by PKM2 knock-down, and since the inhibition of HRE reporter activity by PA-12 was completely restored by PKM2 knock-down, it is likely that PA-12 action has a high specificity towards PKM2. However, the question why PA-12 exerts more effective anticancer activities than PKIII remains unanswered, and this will be investigated in further studies.

Cancer cells exhibit considerably different metabolic requirements compared to normal cells, especially with respect to glycolysis, glutaminolysis, and fatty acid synthesis (DeBerardinis, Lum et al., 2008), whose alterations and adaptations are known to be essential for supporting tumor cell growth and survival (Tennant et al., 2009). Thus, many reports have described how targeting of cellular metabolism may help increase the response to cancer therapy (Dang, 2012; Kroemer and Pouyssegur, 2008; Tennant et al., 2010). Recently, the role of PKM2 in hypoxia and the mechanism of its regulation of cancer cell metabolism have been proposed (Christofk et al., 2008; Luo and Semenza, 2011). Additionally, small molecules increasing PKM2 activity have been reported to suppress tumor growth *in vivo* (Anastasiou et al., 2012; Kung et al., 2012; Parnell et al., 2013). However, it is



**Fig. 4.** The anticancer effects of PA-12 are dependent on PKM2 expression. (A and B) PKM2 depletion desensitizes cancer cells to PA-12 treatment in an NEAA-depleted medium in normoxia (A) and under hypoxia (B). (C) The inhibitory effect of PA-12 on HRE reporter activity is dependent on PKM2 expression. The cells were pretreated with PA-12 or PKIII for 2 h and then cultured for 12 h under hypoxia. HRE reporter activity was measured by a luciferase assay. (D) The inhibitory effect of PA-12 on the hypoxic expression of HIF target gene, GLUT3, is dependent on PKM2 expression. Stable *shPKM2* and *shControl* cells were pretreated with PA-12 or PKIII for 2 h and then cultured for 12 h under hypoxia. Gene expression was analyzed by RT-PCR. Numbers under the graphic RT-PCR data are quantitative estimations of the band intensity made using Image J program. Similar results were obtained from three independent experiments, and representative data are shown.

still not known how PKM2 activators inhibit cancer cell viability under hypoxia. Herein, we identified that a PKM2 activator, PA-12 can block nuclear translocation of PKM2 protein, suppressing the HRE-mediated transcriptional activity and concomitantly the expression of glycolysis-related HIF target genes under hypoxia (Fig. 3D). In conclusion, we provide evidence for the anti-cancer activity of a potent PKM2 activator, PA-12, identified by a virtual screening and *in-vitro* pyruvate kinase assay. We verified its cancer therapeutic effects via cell-based assays, revealing the cancer-specific suppression of cell viability in a NEAA-depleted condition, and the inhibition of HRE-mediated transcriptional activity under hypoxia. These findings might provide a basis for the development of therapeutics preventing cancer progression via targeting the cancer metabolism.

Note: Supplementary information is available on the *Molecules and Cells* website ([www.molcells.org](http://www.molcells.org)).

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