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Original Paper

PKM2 Knockdown Induces Autophagic Cell Death via AKT/mTOR Pathway in Human Prostate Cancer Cells

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Key Words
Pyruvate kinase M2 • Prostate cancer • Cancer metabolism • mTOR • Autophagy

Abstract
Background/Aims: Pyruvate kinase M2 (PKM2) is essential for aerobic glycolysis. Although high PKM2 expression is observed in various cancer tissues, its functional role in cancer metabolism is unclear. Here, we investigated the role of PKM2 in regulating autophagy and its associated pathways in prostate cancer cells. ***Methods:*** Immunohistochemistry was performed to compare the expression level of PKM2 in prostate cancer patients and normal human, whereas expression of PKM2 in several cell lines was also examined by using western blot. PKM2 expression was silenced using various small interfering RNAs (siRNAs). Cell viability was examined using IncuCyte ZOOM™ live cell imaging system. Western blotting and immunofluorescence were performed to investigate the PKM2 knockdown on other cellular signaling molecules. Acridine orange and Monodansylcadaverine staining was performed to check effect of PKM2 knockdown on autophagy induction. High performance thin layer chromatography was carried out to quantify the level of different cellular metabolites (pyruvate and lactate). Colony formation assay was performed to determine the ability of a cells to form large colonies. ***Results:*** PKM2 was highly expressed in prostate cancer patients as compared to normal human. PKM2 siRNA-transfected prostate cancer cells showed significantly reduced viability. Acridine orange, Monodansylcadaverine staining and western blotting analysis showed that PKM2 downregulation markedly increased autophagic cell death. Results of western blotting analysis showed that PKM2 knockdown affected protein kinase B/mechanistic target of rapamycin 1 pathway, which consequently downregulated the expression of glycolytic enzymes lactate dehydrogenase A and glucose transporter 1.

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Knockdown of PKM2 also reduced the colony formation ability of human prostate cancer cell DU145. ***Conclusion:*** To the best of our knowledge, this is the first study to show that PKM2 inhibition alters prostate cancer cell metabolism and induces autophagy, thus providing new perspectives for developing PKM2-targeting anticancer therapies for treating prostate cancer.

Introduction

Prostate cancer is characterized by an abnormal growth of cells in the prostate gland and metastasis of these cancerous cells to other parts of the body, such as the lymph nodes and bone marrow. Prostate cancer generally develops in men aged over 50 years [1]. Prostate cancer is the sixth leading cause of cancer-related deaths worldwide, and >200, 000 new prostate cancer cases are predicted in the United States in 2013 [2]. According to Siegel et al. 2018, 164, 690 new case has been registered in USA and 29, 430 males has been died due to prostate cancer [3].

Tumor cells need sufficient amount of energy and biosynthetic precursors for survival and proliferation [4]. Tumor cells generally use large amounts of glucose and secrete large amounts of lactate in the presence of oxygen, a phenomenon referred to as aerobic glycolysis or "Warburg effect" [5, 6]. Glycolytic intermediates produced through the Warburg effect promote the biosynthesis of ATP and cellular macromolecular building blocks, including nucleotides, amino acids, proteins, and lipids [7, 8]. Pyruvate kinase M2 (PKM2), a rate-limiting terminal glycolytic enzyme, is a major player in aerobic glycolysis in cancer cells and catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate and releases energy, thus providing a selective growth advantage to cancer cells [9-13]. Pyruvate kinase has four isoforms, namely, PKM1, PKM2 (encoded by *PKM*), PKL, and PKR (encoded by *PKLR*). PKM2 is produced by the alternative splicing of *PKM* pre-mRNA by heterogeneous nuclear ribonucleoproteins A1 and A2 and polypyrimidine tract-binding protein splicing factor (which is upregulated by oncogenic transcription factor c-MYC) that promotes the inclusion of exon 10 and exclusion of exon 9 [14-16]. PKM1 is highly expressed in normal tissues [16], whereas PKM2 is predominantly expressed in various cancers such as colon cancer [9, 17], hepatocellular carcinoma [18], lung cancer [18], breast cancer [19], renal cell carcinoma [20-22], and gastrointestinal and cervical cancers [23], thus making it a potential hallmark of cancer metabolism.

Besides its well-established role in aerobic glycolysis, PKM2 performs various non-metabolic functions in cancer cells, including gene expression and cell cycle progression. Extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated nuclear translocation of PKM2 activates β-catenin, which in turn upregulates the expression of c-MYC and induces the expression of glycolytic enzymes, including glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA). This upregulation also induces PKM2 expression by splicing the *PKM* pre-mRNA into PKM2 mRNA [24-27]. Nuclear PKM2 act as a transcriptional co-activator of hypoxia-inducible factor 1-alpha (HIF1α) to reprogram cancer cell metabolism [28]. Moreover, PKM2 promotes the phosphorylation and activation of STAT3 and ERK1/2 to enhance their transcriptional activity and to promote cell proliferation [29, 30]. Upregulation of these glycolytic genes increases glucose consumption and lactate production rates, thus promoting tumorigenesis [31, 32].

In the cellular environment, PKM2 is associated with protein kinase B (Akt)/mechanistic or mammalian target of rapamycin (mTOR) signaling pathway [33], which regulates numerous cellular processes, including cell growth and survival, cell cycle progression, protein synthesis, and angiogenesis [34]. Activation of the Akt/mTOR pathway impairs autophagy in prostate cancer cells [34]. Autophagy is a well-regulated homeostatic mechanism characterized by the collection of the cytoplasmic machinery into autophagosomes, followed by its lysosomal proteolytic digestion and recycling to maintain cellular growth [35]. Under metabolic stress, autophagy promotes cancer cell survival; however, unrestricted autophagy can lead to uncontrolled cellular consumption and ultimately cell death [36]. Autophagy is

highly regulated by the kinase mTOR. In cancer cells, overexpressed PKM2 activates mTORC1 by phosphorylating its substrate AKT1 substrate 1 (AKT1S1), which in turn accelerates autophagy inhibition and oncogenic growth, leading to poor patient outcomes [34, 37].

However, the exact oncogenic role of PKM2 in prostate cancer is unclear. Different studies have used different strategies involving PKM2 silencing and activation to determine the tumorigenic role of PKM2 and the therapeutic potential of PKM2-targeting strategies. In the present study, we investigated PKM2 expression in prostate cancer cells and observed a correlation between PKM2 expression and cancer cell survival. Moreover, we found that PKM2 was an upstream regulator of the Akt/mTOR oncogenic pathway in prostate cancer cells and that *PKM2* knockdown hampered the growth of prostate cancer DU145 cells by inducing autophagy by inhibiting the Akt/mTOR pathway. These results suggest that PKM2 targeting can expedite the reduction of the "Warburg effect" and provide a basis for developing PKM2-targeting therapies for treating prostate cancer.

Materials and Methods

Chemicals and reagents

Roswell Park Memorial Institute-1640 (RPMI-1640) medium (cat. no. LM011-01) and penicillin and streptomycin solutions (cat. no. LS202-02) were purchased from Welgene (South Korea). Fetal bovine serum (FBS; cat. no. 10099-141), Dulbecco's phosphate-buffered saline (DPBS; cat. no. 31600-026), trypsin (cat. no. 25300-054), and reduced serum medium OPTI-MEM (cat. no. 31985070) were obtained from Gibco, Life Technologies (Carlsbad, CA, USA). Lipofectamine RNAiMAX (cat. no. 13778150) was obtained from Invitrogen. NE-PER subcellular fractionation kit (cat. no. 78833) and Pierce bicinchoninic acid (BCA) protein assay reagent A (cat. no. 23228) and B (cat. no. 1859078) were obtained from Thermo Fisher Scientific. FITC Annexin V apoptosis detection kit (cat. no. 556547) was purchased from BD Pharmingen. Acridine orange solution (cat. no. A8097), monodansylcadaverine (MDC) (cat. no. D4008), acetone (cat. no. 270725), goat serum (cat. no. G9023), Triton X-100 (cat. no. T8787), 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI; cat. no. D9542), and thiamine (cat. no. T1270) were obtained from Sigma-Aldrich. Bovine serum albumin (BSA; cat. no. BSAS 0.1) and crystal violet (cat. no. C1035) were purchased from Bovogen and Biosesang Inc., respectively. Protein extraction solution PRO-PREP (cat. no. 17081) was purchased from Intron Biotechnology. Luminata™ Crescendo Western Horseradish Peroxidase (HRP) Substrate and polyvinylidene difluoride (PVDF) membrane were purchased from Millipore. HPLC-grade water (resistivity, 18 MΩ-cm) was obtained using a water purification system (Puris), and lactic acid (cat. no. L0226) was obtained from Tokyo Chemical Industry Co, Ltd. Rabbit monoclonal antibodies against PKM1 (cat. no. D30G6), PKM2 (cat. no. 4053), mTOR (cat. no. 2983), glycogen synthase kinase 3 beta (GSK3β) (cat. no. 12456), phosphorylated GSK3β (p-GSK3β; S9) (cat. no. 5558), phosphorylated ERK1/2 (p-ERK1/2; T202/Y204) (cat. no. 4370), LDHA (cat. no. C4B5), GLUT1 (cat. no. 12939), β-catenin (cat. no. 8480), phosphorylated STAT3 (p-STAT3) (Y705) (cat. no. 9145), Beclin 1 (cat. no. 3495), ATG7 (cat. no. 8558), LC3A/B (cat. no. 12741), p53 (cat. no. 2527), phosphorylated p53 (cat. no. 9286) and GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit polyclonal antibodies against Akt (cat. no. 9272), phosphorylated Akt (p-Akt; S473) (cat. no. 9271), phosphorylated mTOR (p-mTOR; S2448) (cat. no. 2971), AMPKα (cat. no. 2532), phosphorylated AMPKα (T172) (2531), and ribosomal protein S6 kinase beta-1 (P70S6K) (cat. no. 9202) were obtained from Cell Signaling Technology, Inc. Goat polyclonal antibody against Stat3 (cat. no. ab5073) were purchased from Abcam. Rabbit polyclonal antibodies against c-MYC (cat. no. sc-789), mouse monoclonal antibody against HIF1α (cat. no. SC-13515) and MCT-4 (cat. no. SC-376140) were purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal antibodies against phosphorylated P70S6K (p-P70S6K; T389) (cat. no. 9206) were purchased from Cell Signaling Technology, Inc.,. A rabbit polyclonal antibody against PDK1 (cat. no. NB100-2383) was purchased from Novus Biologicals. Alexa Fluor 488-conjugated goat anti-rabbit antibody (cat. no. ab150077) was purchased from Abcam.

Prostate tissue samples

Tissue microarray (TMA) slides of 5 normal human prostate tissue samples and 35 prostate adenocarcinoma tissue samples (duplicate per cases) were obtained from US Biomax Inc. (Rockville, MD, USA). Detailed information about the TMA samples is mentioned in Table 1.

Immunohistochemical analysis

Immunohistochemical analysis was performed to investigate PKM2 expression in the normal prostate and prostate adenocarcinoma tissue samples. The TMA slides were transferred to a xylene chamber and were dipped in a graded alcohol series and water before transferring in 3% H_2O_2 to quench endogenous peroxidase activity. Next, the slides were incubated in a buffer containing 4% BSA and 5% horse serum at

Table 1. Clinicopathological features of the experimental samples (prostate adenocarcinoma tissue and normal prostate tissue samples). T: Primary tumor, Tx: Primary tumor cannot be assessed, T0: No evidence of primary tumor, Tis: Carcinoma in situ; intraepithelial or invasion of the lamina propria, T1: Tumor invading the submucosa, T2: Tumor invading the muscularis propria, T3: Tumor invading through the muscularis propria into the subserosa or into non-peritonealized pericolic or perirectal tissues, T4: Tumor directly invading other organs or structures and/or perforating the visceral peritoneum, N: Regional lymph nodes, Nx: Regional lymph nodes cannot be assessed, N0: No regional lymph node metastasis, N1: Metastasis in 1–3 regional lymph nodes, N2: Metastasis in 4 or more regional lymph nodes, M: Distant metastasis, Mx: Distant metastasis cannot be assessed, M0: No distant metastasis, M1: Distant metastasis

Variable	No. of samples	Normal subject (%)		Cancer patient (%)	
		<60	>60	<60	>60
Patients (Male)	35 cancer patient, 5 normal person	5 (100%)	-	3 (8.57%)	32 (91.42%)
Tumor stage, n (%)					
II	-	-	-	3 (8.57%)	15 (42.85%)
III	-	-	-	-	2 (5.71%)
IV	-	-	-	-	15 (42.85%)
Gleason score					
4–5	-	-	-	1 (2.85%)	4 (11.42%)
6	-	-	-	-	5 (14.28%)
7	-	-	-	-	7 (20.0%)
8–10	-	-	-	2 (5.71%)	16 (45.71%)
Gleason grade					
2	-	-	-	1 (2.85%)	1 (2.85%)
3	-	-	-	-	9 (25.71%)
4	-	-	-	1 (2.85%)	15 (42.85%)
5	-	-	-	1 (2.85%)	7 (20%)
TNM grade					
T2N0M0	-	-	-	3 (8.57%)	12 (34.28%)
T2aN0M0	-	-	-	-	2 (5.71%)
T2N1M1c	-	-	-	-	1 (2.85%)
T3N1M1	-	-	-	-	1 (2.85%)
T3N0M1b	-	-	-	-	2 (5.71%)
T3N0M1	-	-	-	-	1 (2.85%)
T3N1M1b	-	-	-	-	4 (11.42%)
T3N0M1c	-	-	-	-	1 (2.85%)
T4N1M1c	-	-	-	-	1 (2.85%)
T3N0M0	-	-	-	-	2 (5.71%)
T3N1M1c	-	-	-	-	1 (2.85%)
T2bN0M0	-	-	-	-	1 (2.85%)
T2N1M1	-	-	-	-	1 (2.85%)
T4N1M1c	-	-	-	-	1 (2.85%)
T2N1M1b	-	-	-	-	1 (2.85%)

37°C for 1 h to block nonspecific binding sites, followed by washing three times with TBS. The slides were then incubated overnight at 4°C with the anti-PKM2 primary antibody (dilution, 1:1000). Next, the slides were washed three times with TBS, followed by incubation with an HRP-conjugated secondary antibody for 45 min at room temperature. Immunostaining in the slides was visualized using diaminobenzidine tetrahydrochloride, counterstained with hematoxylin and by performing microscopy.

Cell lines and cell culture

Human prostate cancer cell line DU145 was purchased from American Type Culture Collection, USA. The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells in the logarithmic growth phase were collected and used in subsequent experiments.

Small interfering RNA transfection

Human PKM2 was knocked down using specific small interfering RNAs (siRNAs), according to a method described previously [38]. For this, siRNAs specifically targeting PKM2 mRNA (si27, si155, and si156), a siRNA targeting both PKM1 and PKM2 mRNAs (siPK, positive control siRNA), and a scrambled siRNA targeting the firefly luciferase gene without affecting PKM2 expression (siCT, negative control siRNA) were obtained from Bioneer Corporation (Table 2). DU145 cells in the logarithmic growth phase were carefully selected and transfected with the indicated siRNAs after they reached 70% confluency by using Lipofectamine RNAiMAX reagent, according to the manufacturer's protocol. The proficiency of siRNA transfection was examined by performing western blotting analysis.

Cell viability assay

The effect of PKM2 knockdown on the viability of prostate cancer cells was determined using IncuCyte ZOOM™ live cell imaging system (Essen BioScience, MI, USA) that quantitatively detects live cells in real time. The cells were seeded and transfected (forward transfection) with 5, 25, 50 and 100 nM of the indicated siRNAs (si27, si155, si156, siCT, or siPK) for 72 h.

Western blotting analysis

The cells were lysed using the PRO-PREP cell lysis buffer, and the protein content of the lysates was quantified using the BCA protein assay kit. Proteins present in the cell lysates were evaluated by performing western blotting analysis. For this, the total proteins in the cell lysates were resolved by performing SDS-PAGE on 6%–12% polyacrylamide gels. Next, the resolved proteins were transferred onto a PVDF membrane, and the membrane was blocked using 5% skimmed milk for 1 h at room temperature. Next, the membrane was incubated overnight at 4°C with the primary antibodies, followed by incubation with the respective HRP-conjugated secondary antibodies for 1 h at room temperature. Immune complexes were detected using Luminata™ Crescendo Western HRP Substrate. Nuclear and cytoplasmic fractions of the DU145 cells were extracted using the NE-PER subcellular fractionation kit, according to the manufacturer's instructions, and were used for performing subsequent experiments.

Immunofluorescence analysis

DU145 cells were grown in a confocal dish and were transfected with the indicated siRNAs. Next, the cells were fixed in acetone for 10 min at room temperature, followed by washing with ice-cold PBS. Next, the cells were blocked with 10% goat serum for 1 h at room temperature. PKM2 expression was determined by incubating the cells overnight at 4°C with the anti-PKM2 primary antibody, followed by incubation with the respective Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature. Next, the cells were washed three times with PBS, stained with 0.1 µg/mL DAPI in PBS for 1 min, and washed again with PBS. Finally, the cells were examined under a fluorescence microscope (FV10i; Olympus Corp., Tokyo, Japan) at 400× magnification.

Table 2. The sequences of the control and PKM2 siRNAs [38]

siRNAs	Sequences
si27	AGGCAGAGGCUGCCAUCUA
si155	GCCAUAAUCGUCCUCACCA
si156	CCAUAAUCGUCCUCACCAA
siCT (negative control siRNA targeting the firefly luciferase gene)	CUUACGCUGAGUACUUCGA
siPK (positive control siRNA [commercially available] targeting both PKM1 and PKM2	GGACCUGAGAUCCGAACUG

Acridine orange staining

DU145 cells were transfected with the indicated siRNAs after they reached 70% confluency. After the transfection, the cells were treated with 1 μg/mL acridine orange in a serum-free medium for 15 min at 37˚C, followed by washing with PBS. Formation of acidic vesicular organelles (AVOs) was examined using the confocal microscope. The cytoplasm and nuclei showed bright green staining, whereas the AVOs showed incandescent bright red staining.

Monodansylcadaverine (MDC) staining

DU145 cells were transfected with the indicated siRNAs after they reached 70% confluency. After the transfection, the cells were fixed with methanol and stained with 50 μM MDC in a serum-free medium for 15 min at 37˚C, followed by washing with PBS examined by confocal microscopy (600 x, Olympus FV10i).

Quantification of pyruvate and lactate

Liquid chromatography (LC) system used in this study included a LC-321/322/350 pump (Gilson, France), an autosampler (Gilson-234), and a UV/Vis-151 detector (Gilson). Detection and quantification were performed using a Synergi Hydro-RP C18 column (250 × 4.6 mm, 4 μm, 80 Å; Phenomenex, USA) preceded by a pre-column (Phenomenex). The flow rate was maintained at 0.8 and 0.7 mL/min for lactate and pyruvate, respectively. Isocratic mobile phases were composed of water with 0.1% phosphoric acid and water with 20 mM potassium phosphate for lactate and pyruvate, respectively. After extraction, the samples were collected, transferred to a sample tube, and mixed thoroughly with acetonitrile containing thiamine (internal standard). The samples were then centrifuged at 1, 503 X g for 5 min. Finally, supernatants obtained were collected and analyzed by performing HPLC (LC-321/322/350 pump) at 210 and 220 nm for lactate and pyruvate, respectively.

Effect of PKM2 knockdown on the non-metabolic functions of prostate cancer cells

Besides its metabolic role, PKM2 performs other non-metabolic roles. Therefore, we examined PKM2 expression level in both the cytoplasmic and nuclear fractions of DU145 cells by performing western blotting analysis. To examine whether PKM2 knockdown modulated gene transcription, cell proliferation, and cell cycle progression, we performed western blotting analysis of β-catenin, c-MYC, p-STAT3, p-ERK1/2, and MCT4.

Colony formation assay [39, 40]

A total of 700 cells/well were seeded into 6 well plates and then transfected with si156 and siPK (100 nM) for 14 days. Viable colonies were fixed with methanol, stained with 0.05% crystal violet for 20 min, washed with phosphate buffered saline, and air dried. Colonies with more than 50 cells were counted and then normalized to the numbers in the control group. Experiments were performed at least three times.

Statistical analysis

Data are expressed as mean ± SD. Statistically significant differences between the groups were determined using analysis of variance (ANOVA) followed by Tukey multiple comparison tests. For all the tests, a significance level of 5% ($P < 0.05$) was used. Data on survival were analyzed using Kaplan–Meier analysis.

Results

PKM2 is remarkably overexpressed in prostate cancer

PKM2 expression was immunohistochemically examined in the 35 human prostate adenocarcinoma tissue samples obtained from patients aged 55–82 years (duplicate per cases) with stages II, III, and IV tumors and was compared with that in the 5 normal prostate tissue samples obtained from normal subjects aged 28–45 years (duplicate per cases). PKM2 expression was predominantly higher in the prostate adenocarcinoma tissues (depending on the tumor stage) than the normal prostate tissues (Fig. 1A-C), indicating the role of PKM2 in the poor prognosis and outcomes of patients with prostate cancer. Previous studies have reported elevated PKM2 expression in various cancers cells [41-45]. We compared basal PKM2 and PKM1 expression levels in various cancer cell lines by performing western blotting analysis and found that PKM2 protein level was high in the prostate cancer DU145 cells as well as in other cancer cell lines (Fig. 1D).

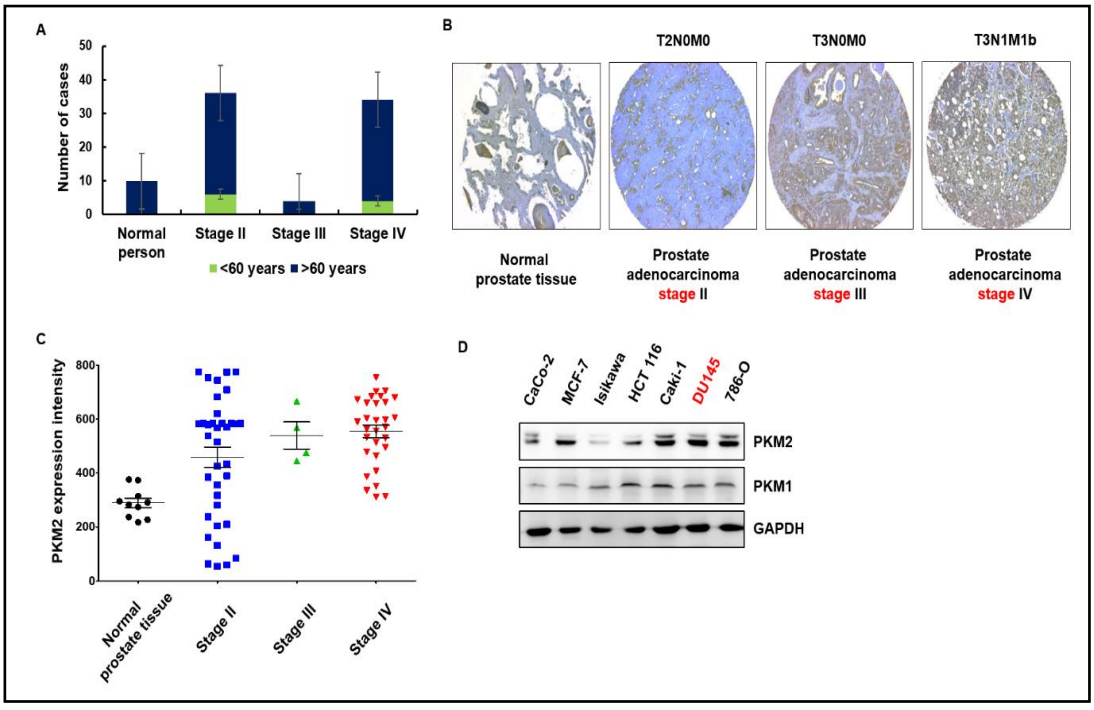

Fig. 1. Expression patterns of pyruvate kinases. (A) Number of cases of human normal prostate tissue samples and prostate adenocarcinoma tissue samples (belonging to three different tumor stages). (B) Normal prostate tissue and prostate adenocarcinoma tissue samples were immunostained for PKM2 by using the specific antibody. (C) PKM2 immunoreactive scoring between the normal prostate tissue samples and prostate adenocarcinoma tissue samples belonging to the three different tumor stages. (D) Comparison of PKM1 and PKM2 expression levels in seven cancer cell lines using GAPDH as an internal loading control. (CaCo: human epithelial colorectal adenocarcinoma cells; MCF-7: human breast adenocarcinoma cells; Ishikawa: human Asian endometrial adenocarcinoma cells; HCT 116: human colon carcinoma cells; Caki-1: human clear cell renal *cell* carcinoma; DU145: human prostate cancer cells; 786-O: human renal carcinoma cells from the kidney).

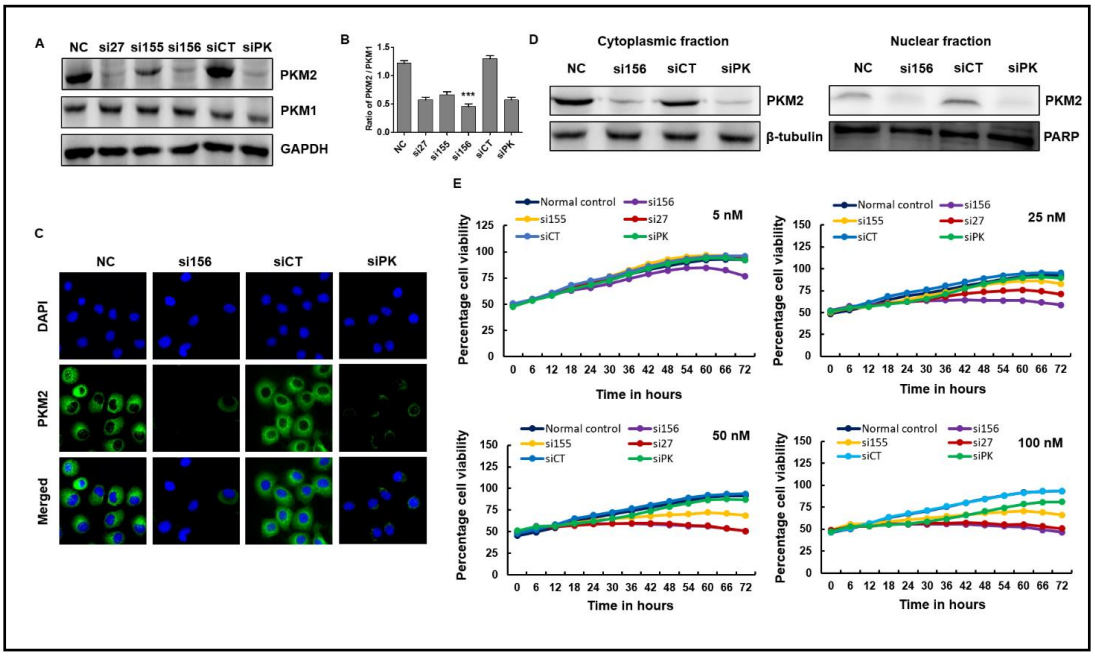

Fig. 2. Effect of siRNA transfection on PKM2 expression and cancer cell survival. (A) Expression level of PKM1 and PKM2 after transfected with indicated PKM2 siRNAs (100 nM concentration for 72 h) using GAPDH was used as an internal loading control. (B) The statistical quantification of the western blot bands of PKM2/PKM1. One-way ANOVA was used to compare the means of different groups. Differences between the means were considered to be significant at $P<0.05$ by using Tukey multiple comparison tests; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with the normal control cells. (C) Immunofluorescence analysis of PKM2 expression after siRNA transfection. DAPI was used for nuclear staining, and Alexa Fluor 488-conjugated goat anti-rabbit antibody was used to detect PKM2. (D) PKM2 expression level in the cytoplasmic and nuclear fractions of DU145 cells. Respective blots are shown. (E) Assessment of the effect of the transfection of different concentration (5, 25, 50, and 100 nM siRNAs upto 72 h) of the indicated siRNAs on the viability of prostate cancer DU145 cells using IncuCyte ZOOM™ live cell imaging system. siCT (siControl): siRNA targeting the firefly luciferase gene (negative control); siPK: commercially available siRNA targeting both PKM1 and PKM2 mRNAs (positive control); and si27, si155, and si156: experimental siRNAs targeting PKM2 mRNA.

Effect of PKM2 knockdown on it's expression in prostate cancer cells

In order to determine the most effective siRNA concentration that could completely silence PKM2 protein expression, DU145 cells were transfected with various concentrations ranging from 5 to 100 nM (Only highest doses shown here) of the indicated PKM2 siRNAs for 48 (data not shown) and 72 h. Results of the western blotting analysis of whole-cell lysates showed a robust reduction in PKM2 protein expression in the DU145 cells transfected with 100 nM si27 or si156 for 72 h without affecting PKM1 protein expression (Fig. 2A and 2B) compared with that in the normal control and negative control cells. As expected, the transfection of DU145 cells with siPK inhibited both PKM1 and PKM2 expression. Immunofluorescence staining detected PKM2 protein in the cytoplasm (green fluorescence) and nucleus (blue fluorescence with DAPI staining) of DU145 cells. However, these immunofluorescence signals were not detected in the DU145 cells transfected with the PKM2 siRNAs (Fig. 2C). Results of western blotting analysis showed markedly decreased PKM2 protein levels in both the cytoplasm and nucleus of si156-transfected DU145 cells (Fig. 2D), thus confirming the results of immunofluorescence analysis.

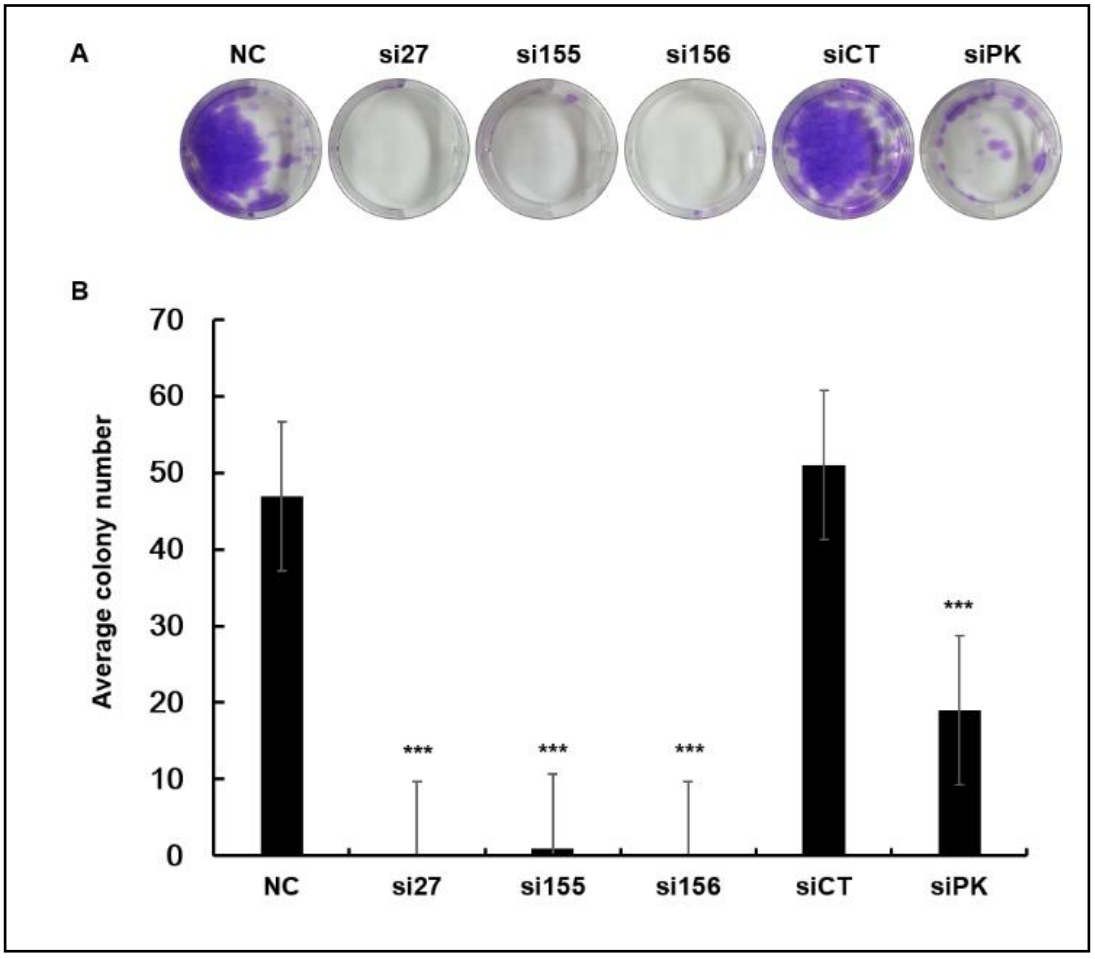

Fig. 3. PKM2 knockdown decreased the colony formation ability of prostate cancer cells. (A) Representative photographs of the colony formation assay showing the proliferation of DU145 cells transfected with the PKM2 siRNAs in six-well plates. The cell colonies were scored visually and were counted using a light microscope. (B) Quantitative analysis of the colony number. Data are representative of three independent experiments. One-way ANOVA was used to compare the means of different groups. Differences between the means were considered to be significant at $P<0.05$ by using Tukey multiple comparison tests; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with the normal control cells.

Effect of PKM2 knockdown on the proliferation of prostate cancer cells

Unrestrained growth is the fundamental property of all malignant tumors. To investigate the effects of PKM2 knockdown on the proliferation of prostate cancer cells and inhibition of cancer cell metabolism, DU145 cells were transfected with the indicated PKM2 siRNAs (5, 25, 50 or 100 nM) for 72 h. Among all three siRNAs, si27 and si156 noticeably reduced the viability of DU145 cells in a time-dependent manner than siPK (Fig. 2E). PKM1 and PKM2 knockdown by siPK significantly reduced the viability of DU145 cells in a time-dependent manner, with the maximum decrease in cell viability being observed at 72 h (Fig. 2E). However, no noteworthy reduction in viability was observed in the normal control (untransfected) and negative control (siCT-transfected) cells, suggesting that PKM2 knockdown inhibited the proliferation of DU145 prostate cancer cells. The reduction in the viability of PKM2 siRNA-transfected DU145 cells indicates the importance of PKM2 in prostate cancer cell survival.

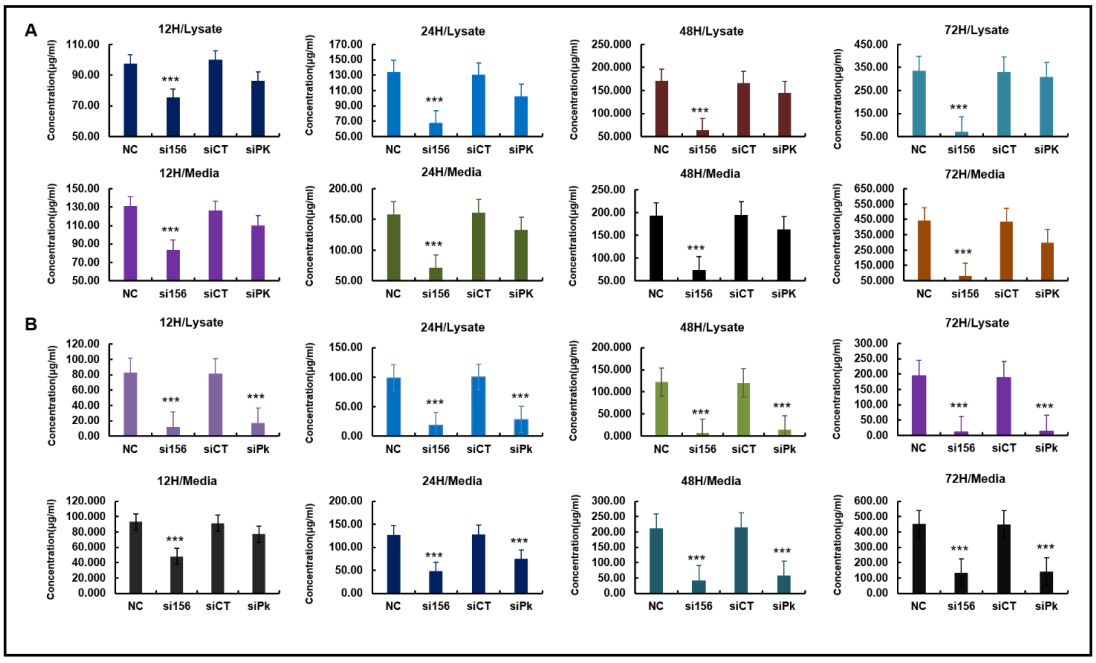

Fig. 4. Effect of PKM2 siRNA transfection on cellular metabolite levels in DU145 cells. (A) Quantitative level of pyruvate in the lysates and media of the PKM2 siRNA-transfected cells (100nM for 72 h). (B) Effect of lactate level in the PKM2 knockdown cells (100 nM of siRNAs for 72 h). One-way ANOVA was used to compare the means of the different groups. Differences between the means were considered to be significant at $P<0.05$ by using the Tukey multiple comparison tests; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with the normal control cells.

PKM2 knockdown inhibits colony formation by DU145 cells

We performed a long-term colony formation assay, which more diligently simulates the *in vivo* environment, to determine whether PKM2 knockdown exerted antitumor effects in prostate cancer cells. *PKM2* knockdown by si156 affected in colony formation by DU145 cells than the positive control siPK (Fig. 3). Therefore, cell reproductive of ability of DU145 was affected after PKM2 knockdown.

PKM2 silencing alters metabolism in prostate cancer cells

PKM2 plays a critical role in glucose metabolism and catalyzes the dephosphorylation of PEP to pyruvate, which is then converted to lactate by LDHA. To determine the effect of PKM2 knockdown on the generation of cellular metabolites, we quantitatively analyzed the pyruvate and lactate level in lysates and media of prostate cancer cells by performing HPLC. PKM2 knockdown by si156 inhibited pyruvate production than the positive control siPK in a time-dependent manner (Fig. 4A). Moreover, PKM2 knockdown induced LDHA inhibition, which in turn reduced lactate production (Fig. 4B, 5A-B). However, these changes in the cellular metabolites were not observed in the normal control and negative control cells. Further evaluation of the lysates of the PKM2 siRNA-transfected DU145 cells by performing western blotting analysis showed that PKM2 knockdown considerably inhibited the expression of the glucose transporter GLUT1 and lactate exporter MCT4 (Fig. 5), which may have decreased glucose uptake and lactate efflux, respectively. Because tumor cells highly depend on glycolysis for energy production, PKM2 knockdown-associated significant reduction in glycolysis in prostate cancer cells may have reduced ATP generation and the Warburg effect.

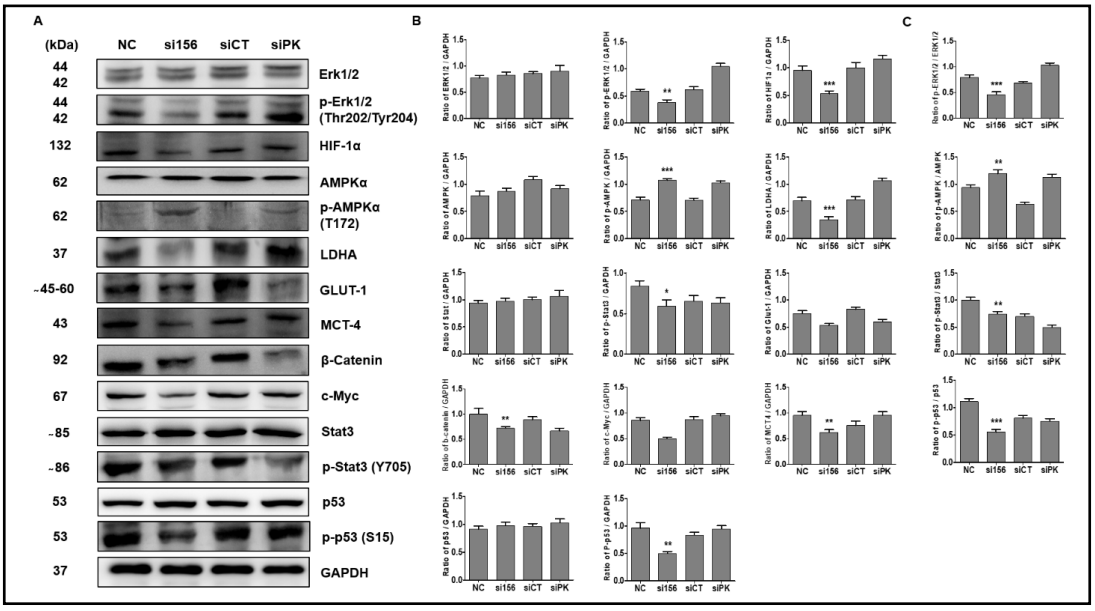

Fig. 5. (A) Effect of PKM2 knockdown on the expression levels of different glycolytic signaling proteins. DU145 cells were transfected with the indicated siRNAs (100 nM for 72 h), and western blotting was performed to determine the expression levels of the glycolytic proteins. Representative blots are shown. (B) The intensities of the bands were measured and depicted in the bar graph by the ratio of GAPDH. (C) The ratio of p-ERK1/2/ ERK1/2, p-AMPK/AMPK, p-Stat3/Stat3 and p-p53/ p53 were measured and depicted in the bar graph. One-way ANOVA was used to compare the means of different groups. Differences between the means were considered to be significant at $P<0.05$ by using Tukey multiple comparison tests; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with the normal control cells.

PKM2 knockdown inhibits the Akt/mTOR signaling pathway

PKM2 overexpression inhibits autophagy by activating mTORC1 in various cancers [37, 46, 47]. Therefore, to determine whether PKM2 knockdown induced autophagy in prostate cancer cells, we examined the expression levels of major autophagy signaling proteins in the PKM2 siRNA-transfected DU145 cells by performing western blotting analysis. PKM2 knockdown significantly downregulated the expression of p-Akt, which in turn inhibited the phosphorylation of mTOR and promoted autophagy induction (Fig. 6). Total Akt and mTOR expression levels were unchanged in the normal and negative control cells. Moreover, PKM2 knockdown increased AMPK expression, which in turn decreased mTOR phosphorylation (Fig. 5 and 6). This reduction in mTOR phosphorylation decreased p-P70S6K and p-GSK3β levels compared with those in the normal control and negative control cells (Fig. 6). These data suggest that PKM2 knockdown exerts anticancer effects in DU145 cells by inhibiting the Akt/mTOR signaling pathway.

PKM2 knockdown induces autophagic cell death

To investigate the effect of PKM2 knockdown on autophagic cell death, we performed acridine orange and MDC staining and western blotting analysis to determine the rate of autophagy in the PKM2 siRNA-transfected DU145 cells. PKM2 knockdown significantly increased autophagosome formation in DU145 cells after 72-h incubation (Fig. 7A & B). Autophagy induction was further confirmed based on the upregulation of autophagy proteins by performing western blotting analysis (Fig. 7C & D). Moreover, PKM2 knockdown by using si156 blocked the nuclear availability of PKM2 (Fig. 2C and 2D), which in turn reduced the phosphorylation of ERK1/2 and STAT3 (Fig. 6). Moreover, PKM2 knockdown downregulated β-catenin and c-MYC expression, which in turn decreased the expression of glycolytic enzymes LDHA, GLUT1, and of HIF1α (Fig. 5 and 6).

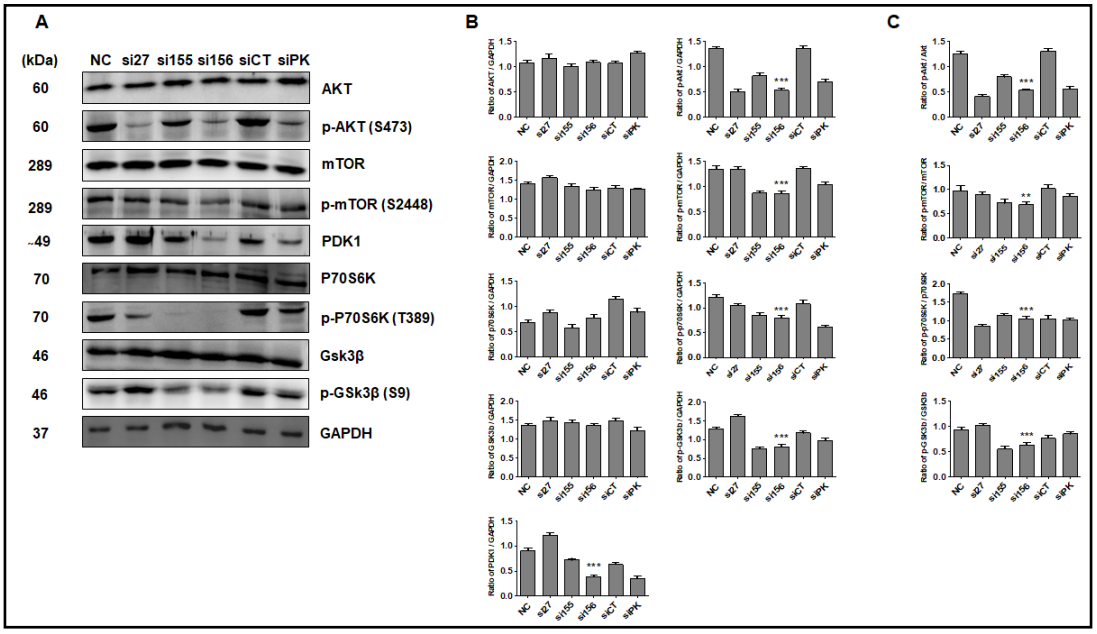

Fig. 6. Expression patterns of different signaling pathway proteins after PKM2 knockdown by siRNAs (100 nM for 72 h). (A) The effect of PKM2 knockdown on anticancer signaling pathways in DU145 cells. The cells were transfected with the indicated siRNAs, and western blotting was performed to determine the expression levels of the different signaling pathway proteins. Representative blots are shown. (B) The intensities of the bands were measured and depicted in the bar graph by the ratio of GAPDH. (C) The ratio of p-Akt/Akt, p-mTOR/mTOR, p-p70S6K/ p70S6K and p-GSK3β/ GSK3β were measured and depicted in the bar graph. One-way ANOVA was used to compare the means of different groups. Differences between the means were considered to be significant at $P<0.05$ by using Tukey multiple comparison tests; *$P<0.05$, **$P<0.01$, and ***$P<0.001$ compared with the normal control cells.

Discussion

Prostate cancer is one of the most common malignant cancers, with high incidence and mortality rates [48-51]. Identification of prostate cancer markers is important for determining its progression and for developing selective and effective therapeutic regimens. To maintain anabolic processes, prostate cancer cells require a large and nonstop supply of glucose [52]. Therefore, high glucose uptake and lactate production are important to heat-seal of prostate cancer metabolism, and increased PKM2 expression plays a significant role in maintaining aerobic glycolysis in prostate cancer cells [53]. In the present study, we examined the effect of PKM2 knockdown on the metabolic and non-metabolic functions in prostate cancer cells and determined the mechanism underlying prostate cancer cell death.

PKM2 overexpression is associated with the tumorigenesis of various cancers and with the poor outcomes of patients with these cancers. In the present study, we detected high PKM2 expression in prostate cancer DU145 cells. Moreover, intense PKM2 immunostaining was observed in stage III and stage IV prostate adenocarcinoma tissue samples compared with that in normal prostate tissue samples, thus confirming that increased PKM2 expression is associated with prostate tumorigenesis. Moreover, we observed that the transfection of DU145 cells with the PKM2 siRNAs drastically decreased their proliferation in a time-dependent manner, suggesting a relationship between PKM2 overexpression and prostate cancer cell proliferation and survival.

Compared with normal cells that metabolize glucose into carbon dioxide, tumor cells metabolize large amounts of glucose for lactate and energy production in the presence of large amounts of oxygen through a process called as the Warburg effect, which increases

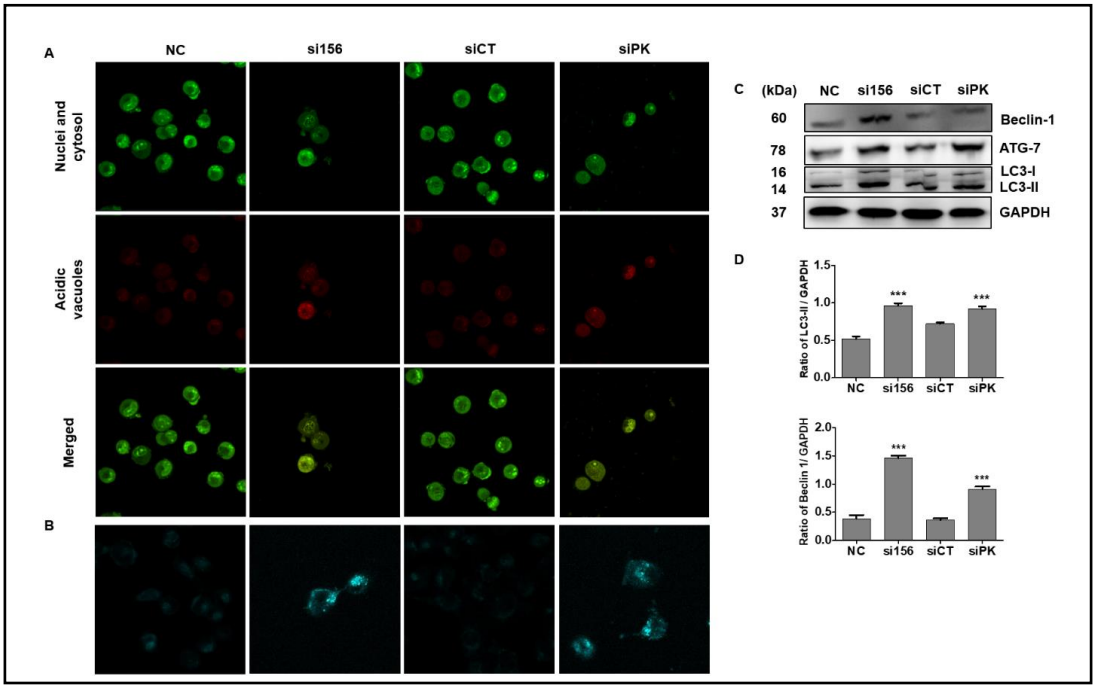

Fig. 7. Assessment of autophagy induction after PKM2 knockdown. (A) DU145 cells were transfected with the indicated siRNAs (100 nM for 72 h) and were stained with acridine orange. Formation of autophagic vacuoles was observed under a confocal microscope. Acridine orange staining resulted in the fluorescent green staining of both the cytoplasm and nucleus and fluorescent bright red or orange-red staining of the autophagic vacuoles. (B) MDC staining showing the induction of autophagy in DU145 cells after PKM2 knockdown as compared with normal and negative control. Cells was examined using confocal microscopy. Scale bars indicate 50 μm. (C) Western blotting of autophagic proteins in cells transfected with the indicated siRNAs. Western blotting was performed using the whole-cell lysates, with GAPDH as the internal loading control. Representative blots are shown. (D) The ratio of LC3-II/GAPDH, Beclin 1/GAPDH were measured and depicted in the bar graph. One-way ANOVA was used to compare the means of different groups. Differences between the means were considered to be significant at $P<0.05$ by using Tukey multiple comparison tests; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with the normal control cells.

glucose consumption and makes tumor cells immortal [8]. One study confirmed that cancer cell proliferation was guided by PKM2-mediated high aerobic glycolysis. In the present study, PKM2 knockdown reduced pyruvate level and subsequently lactate level.

PKM2 regulation in prostate cancer is still unclear. Various studies have suggested that PKM2 is converted from a tetrameric state to a dimeric state in cancer cells to promote aerobic glycolysis and glycolytic intermediate production for synthesizing cellular building blocks [53]. Moreover, nuclear PKM2 plays an important role in regulating the transcription of various genes [25, 29, 54], suggesting its important role in prostate cancer tumorigenesis. Results of western blotting and immunofluorescence analyses performed in the present study support the presence of PKM2 in the nucleus of prostate cancer cells.

PKM2 is a glycolytic enzyme that act as a transcriptional co-activator of HIF1α to activate the transcription of several genes involved in cancer angiogenesis, metastasis, and invasion [55-57]. PKM2 knockdown downregulates HIF1α expression, which may affect the transcription of genes encoding the glucose transporter GLUT1 and glycolytic enzymes LDHA and PDK1, thus affecting glucose uptake, lactate production, and mitochondrial pyruvate utilization. Moreover, PKM2 knockdown decreases the nuclear abundance of PKM2, which in turn decreases p-STAT3 level and affects the transcriptional activation of various STAT3-dependent genes [29]. Nuclear PKM2 binds to and activates c-Src-phosphorylated β-catenin (Y333), thus acting as a protein kinase [58, 59]. In the present study, PKM2 siRNA transfection

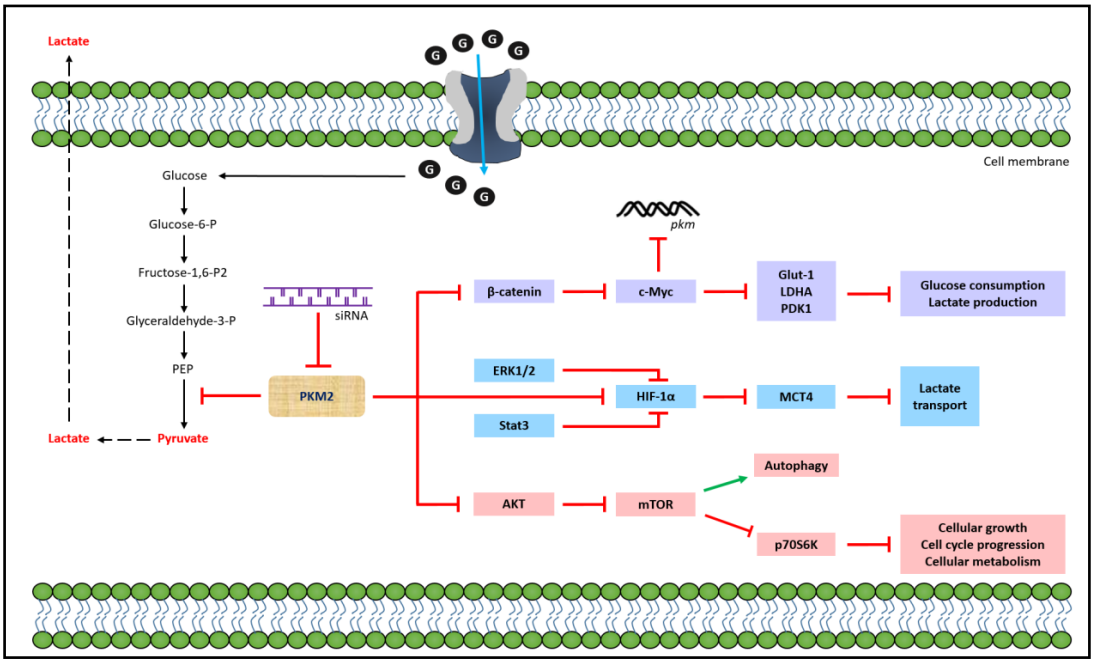

Fig. 8. Schematic representation of the effect of PKM2 knockdown on prostate cancer cell survival and related pathways. PKM2 knockdown inhibits the Akt/mTOR signalling pathway, which activates autophagy and reduces the survival of human prostate cancer cells.

inhibited the nuclear abundance of PKM2, thus inhibiting β-catenin phosphorylation. This subsequently affected c-MYC expression, which upregulates the expression of glycolytic enzymes, thus affecting cell proliferation [54]. PKM2 promotes the phosphorylation and activation of ERK1/2 [30], which was blocked after PKM2 siRNA transfection, thus affecting prostate cancer cell proliferation.

Autophagy is a self-degradative housekeeping process that is important for maintaining a balance of energy sources in response to nutrient stress during tumor development [60]. Autophagic cell death is an alternative cell death pathway to apoptosis [61, 62]. Activation of the Akt/mTOR signaling pathway inhibits autophagy, indicating that this pathway is crucial for controlling cell growth and survival [63]. Akt, a serine/threonine-specific protein kinase, is the master regulator of cell survival under stress condition (such as nutrient stress, oxygen deprivation, and low pH) [64]. On the other hand, mTOR, also a serine/threonine kinase, regulates the intake of nutrients and expression of growth factors and induces the synthesis of important proteins [65]. P70S6K, a downstream effector of mTOR, regulates cell growth, cell cycle progression, and cell metabolism by initiating the translation of mRNAs into necessary proteins [66]. PKM2 activates mTOR signaling by phosphorylating the mTORC1 inhibitor AKT1S1, which ultimately inhibits autophagy [37]. In the present study, the accretion of autophagosomes in the PKM2 siRNA-transfected cells suggests that PKM2 knockdown inhibits Akt expression and initiates autophagic cell death in DU145 cells, which may be mediated by the Akt/mTOR signaling pathway. The inhibition of the Akt/mTOR signaling pathway suppresses the expression of downstream proteins such as P70S6K and GSK3β, which in turn inhibits ribosomal protein synthesis, cell cycle progression, and glycogen synthesis. These findings suggest that PKM2 knockdown is a beneficial therapeutic strategy for treating prostate cancer by inhibiting the key resistance factor of autophagy, i.e., the Akt/mTOR pathway; glucose uptake; and metabolic remodeling.

Clonogenic cell survival assay is a basic tool for determining the ability of a cell to multiply indefinitely and to form a large colony or a clone; a cell with these properties is referred to as a clonogenic cell [67]. In the present study, PKM2 knockdown inhibited the ability of DU145 cells to form colonies (a single DU145 cell can produce a colony containing 50 or more cells) compared with that of normal and negative control cells.

Conclusion

Tumor cells attain growth advantages over normal cells by reprogramming their metabolism. PKM2, the rate-limiting glycolytic enzyme, plays a significant role in metabolic reprogramming in cancer cells. The high expression and enzymatic activity of PKM2 allows improved regulation of glycolysis and stable synthesis of macromolecular building blocks in cancer cells. Moreover, PKM2 regulates non-metabolic functions in cancer cells, thus highlighting its ability to regulate gene transcription, cell proliferation, cell cycle progression, and feedback-regulated cellular metabolism (Fig. 8). Thus, PKM2 promotes the survival of cancer cells by regulating both the metabolic and non-metabolic functions in these cells. Therefore, determination of the cellular functions of PKM2 will help in successfully developing a therapeutic strategy for treating prostate cancer in the near future. Our results indicate that targeting the metabolic and non-metabolic functions of cancer cells by targeting PKM2 is a potentially attractive strategy for prostate cancer therapy. To the best of our knowledge, the present study is first to report that DU145 prostate cancer cells show increased proliferation because of PKM2 overexpression and that PKM2 knockdown induces autophagy in DU145 cells by inhibiting the Akt/mTOR pathway, glycolysis, and glutaminolysis.

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HSK and PD conceived and designed the experiments. PD, AK, JHP, and RS performed the experiments. ISK, MYA, and KY analyzed the data. PD and HSK wrote the manuscript. BML critically revised the manuscript. This work is supported by grants from the National Research Foundation (NRF) of Korea (NRF-2019R1A4A2001451, NRF-2018M3A9C8021792, and NRF-2019R1A2C2002923), which is funded by the Korean Government. We would like to thank Karen Nelson for critically reading the manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

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