Biochemical and Biophysical Research Communications 459 (2015) 327–332

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Secreted pyruvate kinase M2 facilitates cell migration via PI3K/Akt and Wnt/β-catenin pathway in colon cancer cells

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ARTICLE INFO

Article history:
Received 6 February 2015
Available online 28 February 2015

Keywords:
PKM1
PKM2
Secretion
Colon cancer
Migration

ABSTRACT

Pyruvate Kinase M2 (PKM2) is a key glycolytic enzyme, which highly expressed in tumor cells, and plays a pivotal role in the growth, survival and metabolism reprogramming of cancer cells. Besides the location of cytoplasm as a glycolytic enzyme and the location of nucleus as a protein kinase, extracellular PKM2 is present in serum and feces of tumor patients. However, little is known about the secretion of PKM2 and its significance in the progression of colon cancer. Here we demonstrated that PKM2 could be secreted from colon cancer cells, and purified PKM2 protein mimicing the secreted PKM2 was able to promote colon cancer cell migration. Moreover, PI3K/Akt and Wnt/β-catenin signaling were involved in secreted PKM2 induced colon cancer cell migration. The results reveal critical roles of secreted PKM2 in the progression of colon cancer, and indicate that PKM2 may be a therapeutic target for colon cancer.

1. Introduction

One of the important features of metabolism reprogramming of cancer cell is that a shift from oxidative phosphorylation to glycolysis for ATP production [1]. As a rate-limiting step of glycolysis, pyruvate kinase converts phosphoenolpyruvic acid (PEP) and ADP to pyruvate and ATP. Pyruvate kinase M1(PKM1) and Pyruvate kinase M2 (PKM2), which have been known as the most common isoforms in mammalian cells, are encoded by exclusive alternative splicing of the PKM gene and differs at 23 amino acid residues [2]. PKM1 contains exon 9 and is highly expressed in adult tissues, PKM2 includes exon 10 and is predominantly expressed in embryonic tissues and tumors [3]. PKM1 possesses only terameric form, but PKM2 switches between the less active dimer and the active tetramer to endow a selective growth advantage of tumor cells and confer ability to adapt tumor microenvironment [4]. PKM2 tetramer mainly locates in the cytoplasm with a glycolytic activity for ATP production, whereas PKM2 dimer mainly locates in the nucleus with protein kinase activity, which not only provides glycolytic intermediates to divert to biosynthetic processes, but also regulates gene expression to facilitate tumor growth [5]. Replacement of PKM2 by PKM1 leads to reduce glycolytic activity and represses tumorigenesis [6]. PKM2 has been reported to be tightly associated with tumor cell proliferation, survival, migration and angiogenesis [7–9].

PKM2 concentration is higher in plasma of some types of cancer patients, such as colorectal cancer, pancreatic cancer, gastric cancer and biliary tract cancer [9–11]. PKM2 in plasma can be used as a biomarker for tumor diagnosis. In addition, PKM2 is also present in feces and may act as a diagnostic tool for colorectal cancer [12]. Recent reports have been demonstrated that circulating PKM2 facilitates tumor angiogenesis [13]. However, other physiological functions of secreted PKM2 are still poorly known.

In this study, we detected that elevated levels of secreted PKM2 in colon cancer cells. Recombinant PKM2 micmicing secreted PKM2 promoted cell migration, which enhanced expression of N-cadherin, MMP-2 and MMP-9 and downregulated E-cadherin expression. We further found that PI3K/Akt and Wnt/β-catenin signalings are involved in PKM2-mediated migration. These data suggest that a novel function of secreted PKM2 and it may be a suitable target for proventing progression of colon cancer cells.

2. Materials and methods

2.1. Cell culture and conditioned medium collection

The human colorectal cancer cell lines DLD1, HT29, HCT116 and human cervical cancer cell line Hela were maintained in RPMI 1640

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http://dx.doi.org/10.1016/j.bbrc.2015.02.112
0006-291X/

medium supplemented with 10% fetal bovine serum at 37 °C in humidified tissue culture incubator of 5% CO_2. 293T cells were grown in DMEM containing 10% fetal bovine serum at 37 °C in 5% humidified CO_2. Conditioned media from experimental cells were generated as follows: cells with 80% confluency were grown in serum-free DMEM and incubated for 24 h. The conditioned medium containing secreted protein was collected, clarified by centrifugation at 4,000 rpm for 10 min and concentrated by the 30-kDa MW cut-off ultrafiltration membranes (Millipore) and then used to treat cells for following up experiments.

2.2. Protein purification

The cDNA of PKM1 and PKM2 were cloned into pGEX-4T-1 expression vector and purified from *E.coli.* using Glutathione Sepharose 4B (GE Healthcare) as described previously [14]. Briefly, when the *E.coli.* grew to an OD of 0.8, IPTG at final concentration of 1 mM was added to induce protein expression at 16 °C overnight. The cells were harvested and lysed by freeze thaw cycle and sonication. Lysates were passed through Glutathione Sepharose 4B, and purified by GST-affinity purification. The purity of recombinant PKM1 and PKM2 were determined by SDS-PAGE and Commassie blue staining.

2.3. Real-time quantitative PCR

Total RNA from cells were extracted using Trizol regent and 500 μg was used to obtain cDNA by reverse-transcription using PrimeScript RTMasterMix (Takara). Quantitative RT-PCR reaction was carried out using a SYBR Green PCR master mix (Takara) on the Applied Biosystems StepOnePlus™ Real-Time PCR System. PCR amplification was performed with the following specific primer sets: E-cadherin, 5′- GAACGCATTGCCACATACAC-3′ (forward) and 5′- AACTCTCTCGGTCCAGCCCAG-3′ (reverse); N-cadherin, 5′-TTTTG CCCCCAATCCTAAGA-3′ (forward) and 5′-CAGCGTTCCTGTTCCACTC AT-3′ (reverse); MMP-2, 5′-TGGATGATGCCTTTGCTCGTGC (forward) and 5′-ATCGTCATCAAAATGGGAGTCT-3′ (reverse); MMP-9, 5′-CACC CTTGTGCTCTTCCCT-3′ (forward) and 5′-AAGTCTTCCGAGTAGTTTT GG-3′ (reverse); STAT3, 5′-TCTCAACTTCAGACCCGTCAACA-3′ (forward) and 5′-ACAGCTCCACGATTCTCTCCTCC-3′ (reverse); GAPDH, 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGAA CGCCAGTGGA-3′ (reverse). After normalized to GAPDH gene, target gene expression was calculated using the $\triangle\triangle$Ct comparative method. Experiments were performed as triplicate, and the data was described as the mean ± standard deviation.

2.4. Transfection of siRNA

A set of siRNAs against β-catenin (sense 5′-GGGTAGGGTAAATCA GTAATT-3′, antisense 5′-TTACTGATTTACCCTACCCAT-3′) and a scrambled siRNA (sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense, 5′-ACGUGACACGUUCGGAGAATT-3′) were produced by Shanghai GenePharma Co. Ltd. (Shanghai, China). DLD1 cells were seeded in a 6-well plate and transfected with 100 nmol siRNA/well at 70% confluence using Lipofectamine 3000 (Invitrogen, CA, USA). Forty-eight hours after transfection, subsequent experiments were carried out.

PKM2 in Hela cells was depleted by transfection of siRNA as previously described [15].

2.5. SDS-PAGE and western blot

Total proteins were extracted as described previously [7]. Protein concentrations of total proteins were measured using the BCA protein assay and 50 μg of cell lysates were applied to 10% SDS-PAGE, transferred to PVDF membranes, and then incubated with indicated antibodies. The immunoreactive bands were visualized using an ECL system (Amersham Pharmacia Biotech, Piscataway, NJ).

2.6. Wound healing assay

Cells were grown to monolayer confluence in 24-wel plates and then a sterile pipette tip was used to form straight-line scratch wounds. Cells were washed twice and then grown in RPMI1640 with 1% serum with indicated concentration of purified PKM2. Cell migration was recorded at the indicated hours. The percentage of wound healing was calculated by comparing the scratch gap among the indicated hours.

2.7. Statistical analysis

Data were presented as mean ± standard error (SE). Differences among groups were tested by one-way analysis of variance (ANOVA). Comparisons between two groups were evaluated using Student's t-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Secreted PKM2 promotes the migratory ability of colon cancer cells

PKM2 has been reported in human plasma that facilitated tumor angiogenesis [13], but the extensive physiological functions of PKM2 remained elusively. To investigate whether colon cancer cells secreted PKM1 or PKM2, the levels of PKM1 and PKM2 in conditioned medium were detected in DLD1, HT29, HCT116 cells and 293T cells. Secreted PKM1 levels were relative high in HCT116 and 293T cells, and were very low in DLD1 and HT29 cells (Fig. 1A). PKM2 was secreted by DLD1, HT29 and 293T cells, but not in HCT116 cells (Fig. 1A). In order to explore the physiological function of secreted PKM1 and PKM2, the full length PKM1 and PKM2 gene were cloned, expressed and purified in *E. coli* (Fig. 1B). The purified PKM1 and PKM2 were used to treat DLD1 cells to determine their effects on migration of DLD1 cells. As shown in Fig. 1C and D, purified PKM2 significantly promoted DLD1 cell migration, whereas purified PKM1 had on effect on the migration of DLD1 cells. These results indicated that secreted PKM2 has a stimulative effect on DLD1 cell migration.

3.2. Silencing PKM2 inhibits the migration of colon cancer cells

Cell migration, which involves the secretion of various factors to facilitate motility, is a key parameter in tumor metastasis [16,17]. Several reports have shown that tumor cells secrete proteins to extracellular, some can be used as a biomarker, and some have physiological functions on affecting of cell migration [18]. To further determine whether PKM2 in secretions also promoted the migration of DLD1 cells, we knocked down the expression of PKM2 in Hela cells (named siPKM2) and compared with the negative control siRNA (named sicont). Conditioned media were collected from the siPKM2 cells and sicont cells, respectively. The results from western blot confirmed that PKM2 knockdown significantly decreased PKM2 expression and secretion in DLD1 cells (Fig. 2A). Furthermore, the effect of PKM2 knockdown on cell migration was determined by wound healing assay. The quantification of wound closure showed that DLD1 cells, were treated with the conditioned media collected from siPKM2 cells and sicont cells, were at 8.56% and 31.69%, respectively (Fig. 2B and C). These results confirmed

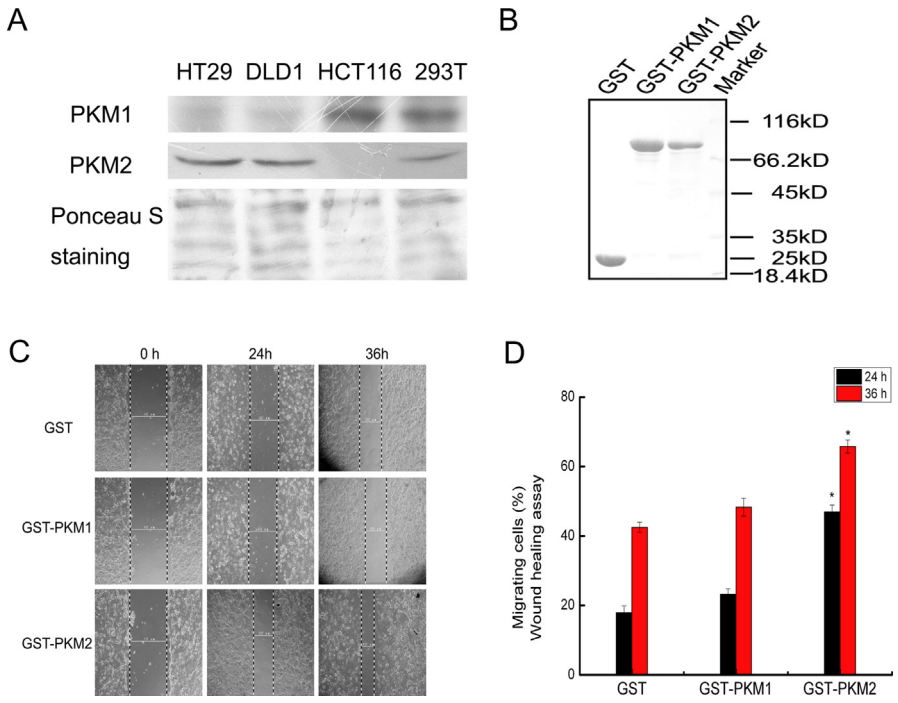

Fig. 1. Secreted PKM2 facilitates cell migration in DLD1 cells. (A) Detection of the secreted PKM1 and PKM2 protein levels in three colon cancer cell lines and 293T cells by western blotting. (B) SDS-PAGE was used to analyze the purified GST, GST-PKM1 and GST-PKM2 recombinant proteins. (C) Representative results of the wound healing assays examined the effect of GST, GST-PKM1 and GST-PKM2 proteins with 0.5 μM on the migration of DLD1 cells. (D) Showed the statistical graph of wound closure rate of result C. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus GST group.

that knockdown of PKM2 suppressed secreted PKM2 and cell migration.

3.3. *The β-catenin signaling pathway is involved in secreted PKM2-mediated cell migration*

E-cadherin and N-cadherin are calcium-dependent cell adhesion proteins, which can form adherens junction-like structures and play critical roles in the regulation of cell adhesion and migration [16,19]. To further elucidate the mechanism by which secreted PKM2 induced DLD1 cell migration, we found that secreted PKM2 upregulated N-cadherin expression and down-regulated E-cadherin expression in mRNA levels (Fig. 3A). By contrast, secreted PKM1 had no effect on expression of E-cadherin and N-cadherin (Fig. 3A). Results of western blot analysis of E-cadherin and N-cadherin were also identical with that of qPCR assay (Fig. 3B). Among the matrix metalloproteinases (MMP) family members, MMP-2 and MMP-9 are of great interest because of their roles in tumor invasion and migration [20]. As critical transcription factors, signal transducers and activators of transcription 3 (STAT3) and β-catenin have an important role in the regulation of migration, invasion and motility of tumor cells [21,22]. As shown in Fig. 3C, secreted PKM2 increased expression of MMP-2 and MMP-9, and did not change STAT3 mRNA levels. Moreover, protein levels of β-catenin were increased in secreted PKM2 induced DLD1 cells (Fig. 3D). Whereas, expression levels of MMP-2, MMP-9, STAT3 and β-catenin did not show alterations in secreted PKM1 induced DLD1 cells. Collectively, these data indicate that β-catenin signaling has an important role in secreted PKM2 induced colon cancer cell migration.

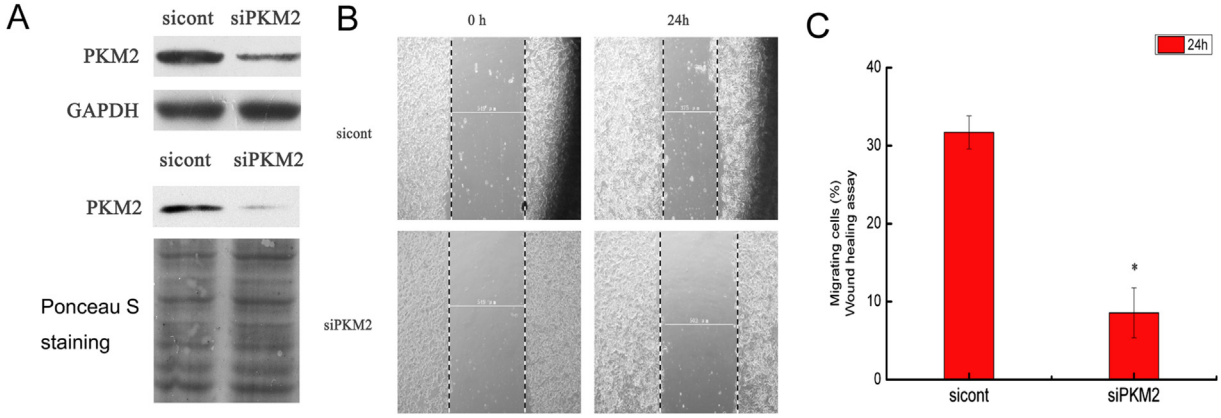

Fig. 2. Knockdown of PKM2 decreases cell migration in DLD1 cells. (A) Hela cells were transfected with siRNA specific blocked PKM2 for 48 h, and then western blot was used to detect PKM2 expression levels and secreted PKM2 in siPKM2 and sicont cells. (B) Representative results of the wound healing assays examined the effect of conditioned medium from siPKM2 and sicont cells on the *in vitro* migration of DLD1 cells. (C) Showed the statistical graph of wound closure rate of result B. $^{*}p < 0.05$ versus sicont group.

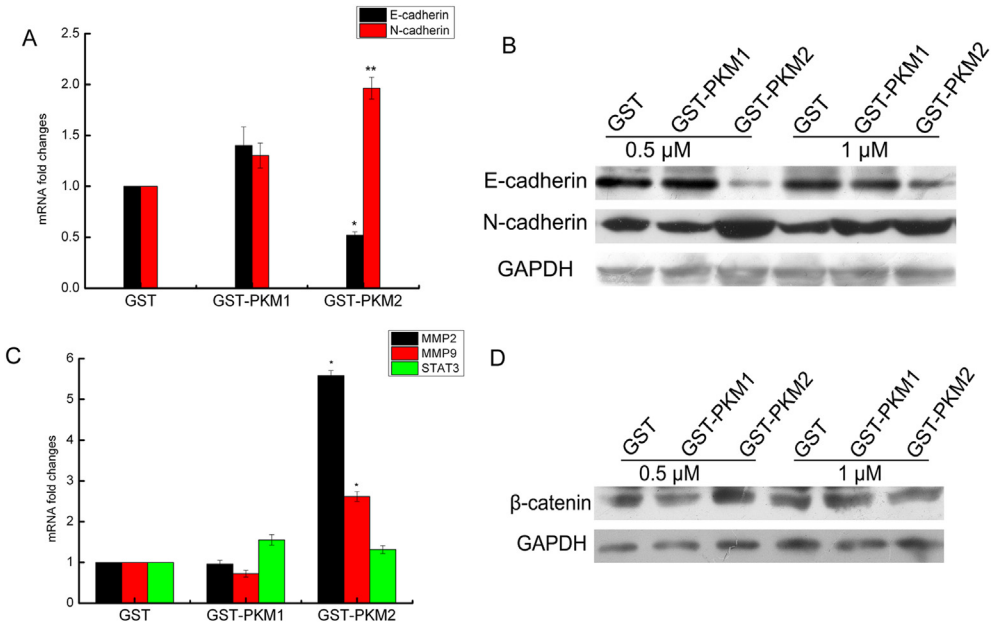

Fig. 3. The β-catenin signaling pathway is involved in secreted PKM2-mediated cell migration. (A) Relative mRNA levels of E-cadherin and N-cadherin in DLD1 cells incubated with 0.5 μM of GST, GST-PKM1 and GST-PKM2 recombinant proteins, respectively. (B) Western blotting analysis of E-cadherin and N-cadherin expression in DLD1 cells by treated with GST-PKM2 with different concentrations at 24 h. (C) Relative mRNA levels of MMP-2, MMP-9 and STAT3 in DLD1 cells incubated with 0.5 μM of GST, GST-PKM1 and GST-PKM2 recombinant proteins, respectively. (D) Western blotting analysis of β-catenin expression in DLD1 cells by treated with GST-PKM2 for different concentrations at 24 h.

3.4. Secreted PKM2 activates the PI3K/Akt signaling pathway

It has been reported that β-catenin expression is mediated by upstream pathways, including PI3K/Akt signaling, which plays critical roles in cell migration and proliferation [14,23]. DLD1 cells were treated with 30 μM LY294002 (a PI3K inhibitor) for 1 h prior to secreted PKM2 treatment. As shown in Fig. 4A and B, LY294002 significantly inhibited secreted PKM2 induced cell migration.

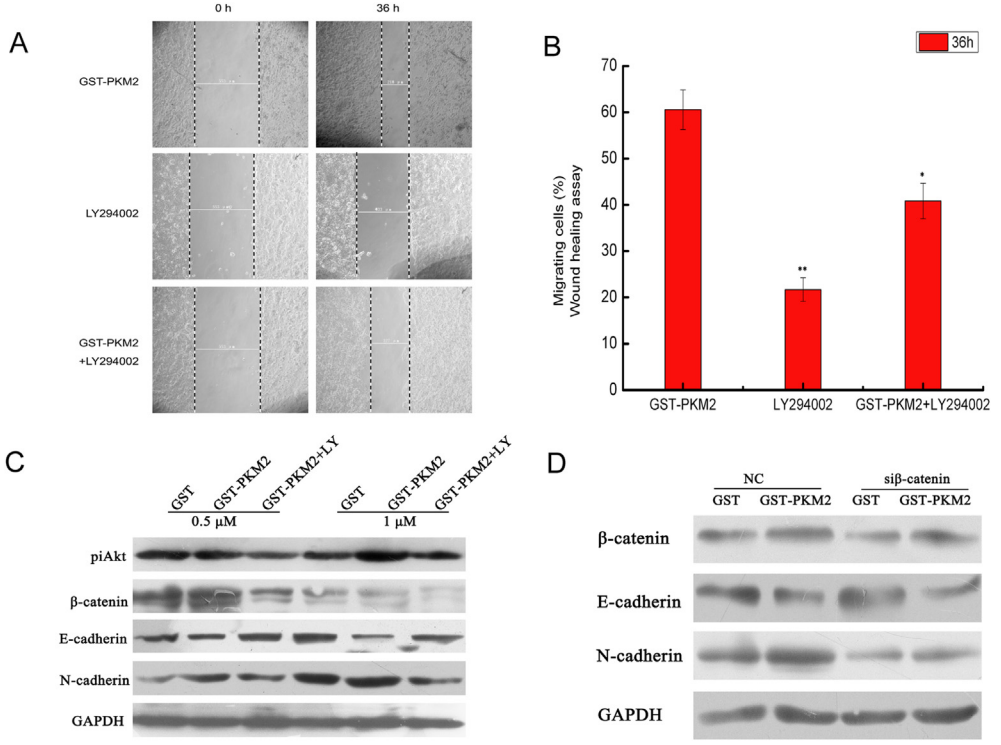

Fig. 4. Secreted PKM2 mediates cell migration via PI3K/Akt signaling pathway. (A) DLD1 cells were pretreated with 30 μM LY294002 for 1 h followed by incubation for 36 h with 0.5 μM of GST-PKM2. Wound healing assay was measured at 0 and 36 h after incubation. (B) Showed the statistical graph of wound closure rate of result A. *$p < 0.05$ and **$p < 0.01$ versus GST group. (C) Western blotting analysis of piAkt, β-catenin, E-cadherin and N-cadherin in DLD1 cells treated with GST-PKM2 in the presence of LY294002. (D) DLD1 cells were transfected with indicated siRNAs for 48 h, and treated with GST-PKM2 for 24 h. Then, expression levels of β-catenin, E-cadherin and N-cadherin were analyzed by western blotting.

Purified PKM2 treatment also upregulated the phosphorylation level of Akt, while LY294002 significantly reversed GST-PKM2 induced the expression of E-cadherin, N-cadherin and β-catenin (Fig. 4C). To further examine the role of β-catenin in PKM2 induced expressions of E-cadherin and N-cadherin, DLD1 cells were transfected with siRNA against of β-catenin. As shown in Fig. 4D, GST-PKM2 induced the expression of E-cadherin and N-cadherin was reversed by knockdown of β-catenin. These results indicated that PI3K/Akt signaling played critical roles in secreted PKM2 induced colon cancer cell migration.

4. Discussion

PKM2 is elevated in the serum and feces in cancer patients of different types, which can be used as a marker for cancer prognosis [9,11]. Recent studies have shown that circulating PKM2 accelerates tumor growth by promoting tumor angiogenesis [13]. In the present study, we demonstrated that extracellular PKM2 secreted from colon cancer cells to promote cell migration of DLD1 cells in an autocrine manner. More importantly, PI3K/Akt and Wnt/β-catenin signaling were critcial in secreted PKM2 induced colon cancer cell migration.

Why secreted PKM2 but not secreted PKM1 facilitated colon cancer cell migration? PKM1 and PKM2 differ at 23 of 531 amino acids through alternative splicing of the PKM gene. PKM1, which has high affinity for PEP and does not affect by allosteric regulation, presents only as a highly active tetrameric form. However, PKM2 exists in both dimeric form with protein kinase activity and tetrameric form with glycolytic activity [7]. The different amino acid sequence between PKM1 and PKM2 is mainly localized at the dimer–dimer interface [13,24]. During cancer progression, various environment signalings stimulate the conversion of PKM2 from tetrameric form to dimeric form, thus provide a growth advantage of the tumor cells [6]. It is conceivable that secreted PKM2 but not secreted PKM1 plays critical roles in cancer cell migration dependent on its protein kinase activity. This opinion is consistent with the study that dimer PKM2 in blood circulation is benefit for promoting angiogenesis, while tetramer PKM2 is not required [13].

The most puzzling is that secreted PKM2 binds to what cell surface receptors to stimulate PI3K/Akt and subsequently activate Wnt/β-catenin signaling to mediate colon cancer cell migration. Our previous study demonstrated that cell surface GRP78 bound with secreted GRP78 to activate PI3K/Akt and Wnt/β-catenin signaling to promote tumor cell proliferation [14]. In order to investigate if secreted PKM2 could bind to cell surface GRP78 to activate migration-related signaling pathways, chromatin immunoprecipitation assay was used to analyze the potential interacton between PKM2 and GRP78. However, the interaction between PKM2 and GRP78 was not detected in our experiment (data not shown). Therefore, cell surface GRP78 was unlike the receptor of secreted PKM2. Moreover, finding out the cell surface receptor that interacted with secreted PKM2 was important for understanding the mechanisms of extracellular PKM2 induced colon cancer cell migration. Interestingly, knockdown of PKM2 led to decrease the levels of secreted PKM2 and tumor cell migration.

In summary, our study demonstrates that PKM2 secreted from tumor cells activates PI3K/Akt and then stimulates Wnt/β-catenin signalings to promote cell migration of colon cancer cells. It indicates that secreted PKM2 may be as a therapeutic target for preventing tumor metastasis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 31271516, No. 31201072), Shanxi Province Science Foundation for Youths (2012021028-4) and Research Fund for the Doctoral Program of Higher Education of China (20111401110011).

We wish to thank Prof. Xun Hu, who works in the cancer institute a key laboratory for cancer prevention and intervention at Zhejiang Univerisity School of Medicine, for providing the plasmids of PKM1-pQE-30.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.112.

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