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Original Article

Extracellular PKM2 induces cancer proliferation by activating the EGFR signaling pathway

Ming-Chuan Hsu[1,2], Wen-Chun Hung[2], Hirohito Yamaguchi[1], Seung-Oe Lim[1], Hsin-Wei Liao[1], Chia-Hua Tsai[2], Mien-Chie Hung[1,3,4]

[1]Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; [2]National Institute of Cancer Research, National Health Research Institutes, Tainan, Taiwan; [3]Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan; [4]Department of Biotechnology, Asia University, Taichung, Taiwan

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Abstract: Pyruvate kinase is a key enzyme in the glycolytic pathway that converts phosphoenolpyruvate to pyruvate, and the M2 isoform of pyruvate kinase (PKM2) is associated with cancer. PKM2 has been reported to function independently of its pyruvate kinase activity, which is crucial for cancer cell proliferation. Moreover, there is growing evidence indicating that dimeric PKM2 is released from tumor cells into the circulation of cancer patients. However, the role of secreted PKM2 in cancer is not well understood. Here, we found that the phosphorylation level of epidermal growth factor receptor (EGFR) significantly increased upon the exposure of cells to the recombinant PKM2 protein. In addition, secreted PKM2 induces EGFR phosphorylation and activates the EGFR downstream signaling in triple-negative breast cancer cells. In contrast, knocking down PKM2 decreased EGFR phosphorylation. Moreover, expression of R399E mutant PKM2, which has been reported to preferentially form a dimer, enhanced EGFR phosphorylation, cellular transformation, and cell proliferation more strongly than the wild-type PKM2. Thus, our study revealed a novel function of extracellular PKM2 in the promoting cancer cell proliferation through EGFR activation.

Keywords: PKM2, EGFR, triple-negative breast cancer

Introduction

Pyruvate kinase plays a critical role in aerobic glycolysis, and four pyruvate kinase isoforms exist, including PKM1, PKM2, PKL, and PKR. Pyruvate kinase M1 (PKM1) and M2 (PKM2) are produced by mutually exclusive alternative mRNA splicing of the PKM gene to include either exon 9 (PKM1) or exon 10 (PKM2), respectively [1]. PKM2 catalyzes the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate (ADP), generating one molecule of pyruvate and adenosine triphosphate (ATP) at the final rate-limiting step of glycolysis. PKM2 exists in both tetrameric and dimeric forms. While only the PKM2 tetramer harbors glycolytic activity for ATP production, PKM2 dimer has been reported to function as a nuclear protein kinase to regulate gene transcription and promote tumorigenesis [2, 3]. The conformational conversion between tetrameric and dimeric PKM2 is mediated by allosteric regulation [4, 5]. Posttranslational modifications, e.g., phosphorylation and acetylation, also regulate PKM2 activity in addition to allosteric regulation. For example, oncogenic tyrosine kinases, such as FGFR1, BCR-ABL, JAK2 and FLT3-ITD, phosphorylate PKM2 at tyrosine 105 which enhances its dimer formation [6, 7]. Acetylation of PKM2 at lysine 305 results in the decrease of its pyruvate kinase activity and subsequent autophagic degradation of PKM2 [8], whereas acetylation of PKM2 at lysine 433 switches PKM2 from a cytoplasmic pyruvate kinase to a nuclear protein kinase upon mitogenic and oncogenic stimuli [9]. Oxidation of PKM2 at cysteine 358 has been reported to inhibit its kinase activity [10].

The PKM2 protein is expressed in early embryonic cells, normal proliferating cells, and tumor cells [11-15]. Over the years, there has been increasing evidence pointing to the non-glycolytic functions of PKM2 in tumor cells. For instance, in the cytosol, PKM2 can directly interact with and stabilize mutant epidermal growth

factor receptor (EGFR) protein in lung cancer cells [16]. Activation of EGFR can also induce translocation of PKM2 into the nucleus, where PKM2 promotes the transcriptional activity of β-catenin by binding to c-Src-phosphorylated β-catenin at tyrosine 333, promoting expression of its target genes, such as cyclin D1 and Myc, in tumor cells [17]. They further demonstrated that PKM2 directly binds to histone H3 and phosphorylates it at threonine 11 upon EGFR activation. PKM2-mediated phosphorylation of histone H3 is required for histone H3-K9 acetylation and genes transcription, including cyclin D1 and Myc [18]. More recently, the STAT3 transcription factor was identified as a substrate of PKM2. Specifically, nuclear PKM2 phosphorylates STAT3 at tyrosine 705 and activates its transcriptional activity which then induces the expression of a STAT3 target gene MEK5 and enhances tumor cell growth [2]. Hosios et al., however, later provided evidence to argue against the previously reported protein kinase function for PKM2 [19]. The role of PKM2 as a protein kinase, thus, remains controversial.

Triple-negative breast cancer (TNBC), which lacks ER, PR and ErbB2 expression, exhibits highly proliferative and aggressive characteristics and has poor prognosis. At present, there are no effective targeted therapies to treat TNBC. Interestingly, about 50% of TNBC is reported to have EGFR overexpression that also correlates with poor clinical outcome [20, 21]. PKM2 has also been shown to play a critical role mediating tumor angiogenesis and cancer progression in TNBC cells [22, 23]. Although it has been shown that PKM2 dimers are released from tumor cells into the circulation of cancer patients [24-29], the role of the secreted PKM2 and which molecule(s) it regulates is not well understood. Here, we further investigated the relationship between EGFR and extracellular PKM2 with results indicating that extracellular PKM2 activates the EGFR signaling pathway and enhances cancer cell proliferation.

Materials and methods

Cell cultures and reagents

Breast cancer cell lines MDA-MB-231, MDA-MB-468, and MCF7, and human embryonic kidney cell line HEK 293T cells were cultured in DMEM medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. MDA-MB-231 and MDA-MB-468 cells with PKM2 knockdown and stable expression of wild-type PKM2 or R399E mutant PKM2 (R399E-PKM2) were grown in the same medium supplemented with 1 μg/ml puromycin. MCF7 cells stably expressing wild-type EGFR were maintained in the same medium with 1 mg/ml G418. Gefitinib was purchased from ChemieTek. Phospho-Erk (Cat No. 9101S), phospho-Akt (Cat No. 4060S), Akt (Cat No. 9272), PKM2 (Cat No. 4053S), phospho-ErbB3 (Cat No. 2842P), ErbB3 (Cat No. 12708P), phospho-Met (Cat No. 3077S) antibodies were purchased from Cell Signaling Technology; phospho-Y1068 (Cat No. ab32430) from Abcam; EGFR (Cat No. 04-338), Erk (Cat No. 06-182), phosphotyrosine, clone 4G10 (Cat No. 05-321) from Millipore; and Met (Cat No. SC-161) from Santa Cruz.

Plasmids

Human PKM2 ORF clone and pGIPZ-shPKM2 were obtained from the shRNA/ORF Core Facility (UT MD Anderson Cancer Center, Houston, TX, USA). To knockdown endogenous PKM2 and re-express flag-PKM2, we created the pGIPZ-shPKM2/flag-PKM2 dual expression vector [30]. First, we replaced turboGFP gene with flag-PKM2 gene in pGIPZ-shPKM2 vector which targets the 3'-UTR of endogenous PKM2 gene. We then generated the pGIPZ-shPKM2/R399E-flag-PKM2 mutant by site-directed mutagenesis using the following primers: (forward) 5'-TTATTTGAGGAACTCGAACGCCTGGCGCCC-3' and (reverse) 5'-GGGCGCCAGGCGTTCGAGTTCCTCAAATAA-3'. Human PKM2 ORF was cloned into pET-28a vector to express recombinant protein PKM2 in bacteria. pET-28a-R399E-PKM2 expression vector was generated by site-directed mutagenesis with the same primers shown above.

Western blotting and immunoprecipitation

Cells were harvested by using RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, and 50 mM NaF). Lysates were incubated on ice for 20 minutes and centrifuged at 13,000 *g* for 10 minutes to remove the cell debris. Protein concentrations were measured by using BCA protein assay kit (Pierce). Equal amounts of total protein were subjected to polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes. The blots were incubated for 1 hour at room tem-

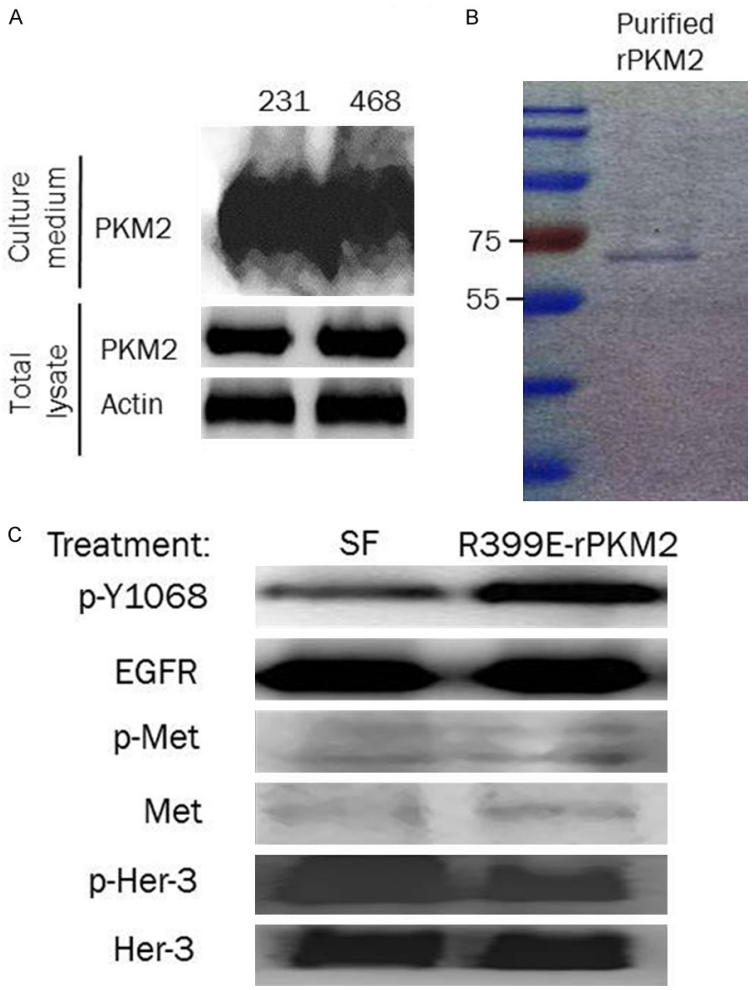

Figure 1. Recombinant PKM2 induces phosphorylation of receptor tyrosine kinases. A. The triple negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 were grown in serum-free DMEM medium for 24 hours. The conditioned medium was collected and centrifuged at 4000 rpm for 5 min to remove cell debris. The supernatant was concentrated approximately 20-fold by a 30-kDa MW cut-off ultrafiltration membrane. The concentrated medium was subjected to western blot assay for the detection of secreted PKM2. B. The recombinant protein R399E-PKM2 was expressed in bacteria and purified from bacterial lysates. Purification of recombinant protein R399E-PKM2 was validated by Coomassie blue staining. C. MDA-MB-468 cells were grown in serum-free medium for 24 hours and then treated with 2 μM of recombinant R399E-PKM2 protein for 30 min. Cell lysates were collected and subjected to western blot analysis to detect the phosphorylation level of RTKs.

perature with 5% non-fat milk in 0.05% TBST and then overnight at 4°C with primary antibodies followed by horseradish peroxidase-labeled secondary antibodies. Immunoreactive proteins were detected by using enhanced chemiluminescence reagent. For immunoprecipitation, cells were lysed in lysis buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) and centrifuged at 13,000 *g* for 10 minutes to remove the cell debris. Cell lysates were then subjected to immunoprecipitation with antibodies.

Soft agar colony formation assay

Cells were suspended in DMEM medium containing 0.3% agarose and plated onto 0.6% solidified agarose containing DMEM medium in six-well culture plates at a density of 2,000 cells per well. After incubation at 37°C with 5% CO_2 for 2-3 weeks, the number of colonies was counted and photographed. The results were expressed as the means ± SD of triplicate counts within the same experiment.

Non-contacting co-culture transwell cell culture system

MDA-MB-231 stable cells were seeded onto the membrane of transwell cell culture inserts (upper chamber), and MDA-MB-231 cells were simultaneously plated onto the bottom of the 24-well transwell (lower chamber). Serum-free medium was added to both chambers, and the cells were incubated in serum-free medium for 48 hours. The co-culture transwell was purchased from Corning Incorporated (Cat. No. 3450).

MTT assay

Cells were seeded onto 96-well plates at a density of 5,000 cells per well and incubated with different concentrations of gefitinib for 72 hours. After incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well at a final concentration of 0.5 mg/ml, and the plates were incubated at 37°C for another 4 hours. The supernatant was discarded and 100 μl of DMSO was added to each well. The absorbance at 570 nm was recorded using a microplate reader.

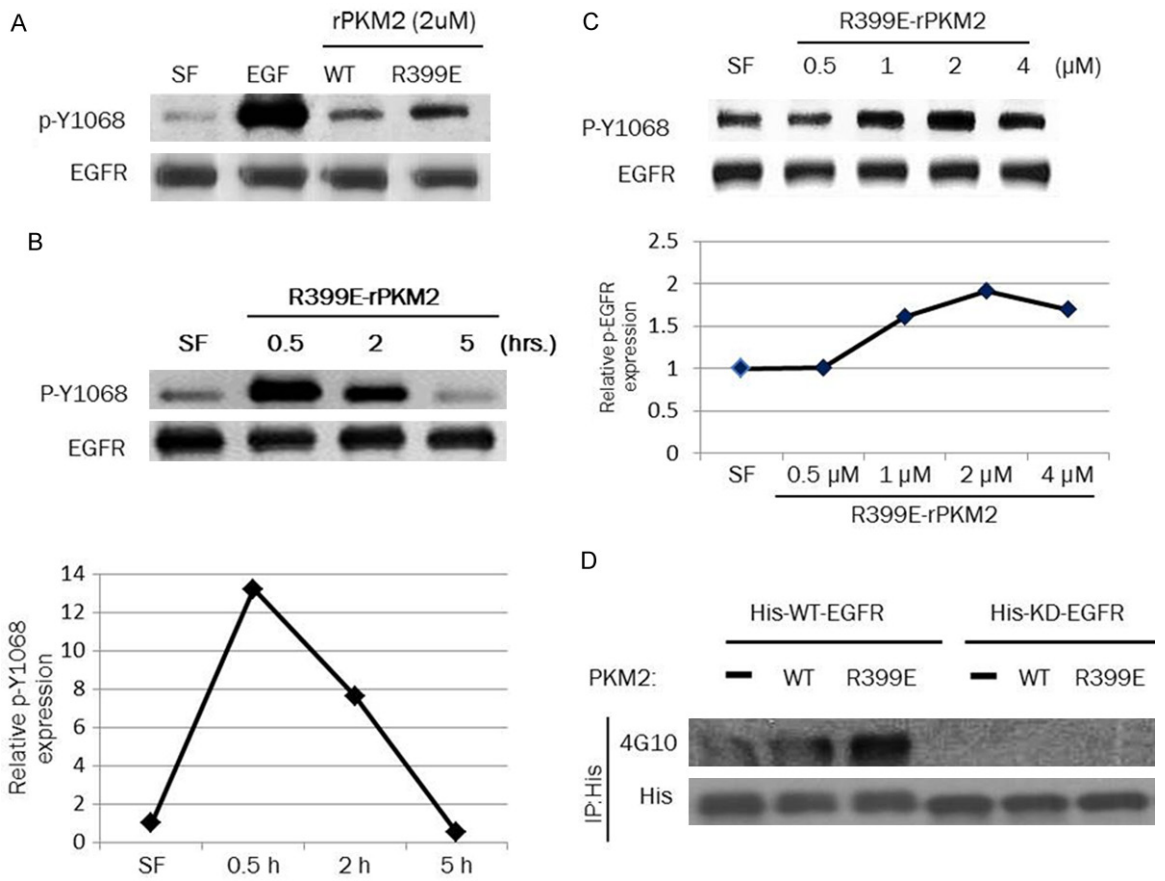

Figure 2. Recombinant protein PKM2 induces EGFR phosphorylation. A. Cells were serum starved for 24 hours and then treated with 2 μM of recombinant wild-type PKM2 and R399E-PKM2 proteins respectively for 30 min. Cell lysates were harvested and subjected to western blotting to detect phospho-EGFR (Y1068) and total EGFR. EGF treatment was added as positive control for EGFR phosphorylation. B. Cells were treated with 2 μM of recombinant R399E-PKM2 proteins for different time points, and the lysates were collected and subjected to western blotting to detect phospho-EGFR (Y1068) and total EGFR. Protein band intensity was quantified by Image J software. C. Cells were serum starved for 24 hours and then treated with different doses of recombinant R399E-PKM2 proteins for 30 min. Cell lysates were harvested and subjected to western blotting to detect phospho-EGFR (Y1068) and total EGFR. Protein band intensity was quantified by Image J software. D. HEK 293 cells were co-transfected the indicated expression vector. Cell lysates were collected and subjected to immunoprecipitation using the His antibody, followed by western blotting to detect phosphotyrosine (4G10) and His.

Results

Extracellular PKM2 induces EGFR phosphorylation

PKM2 exists as a dimer in the blood circulation of cancer patients, and PKM2 dimers have been shown to facilitate tumor angiogenesis [25]. However, the physiological functions of extracellular PKM2 in TNBC remain elusive. We first determined whether TNBC cells secret PKM2 by examining the levels of PKM2 in conditioned medium from MDA-MB-231 and MDA-MB-468 triple-negative breast cancer cells. Secreted PKM2 was detected in the culture medium of both cell lines (**Figure 1A**). To explore the physiological function of secreted PKM2, the full-length R399E mutant PKM2 recombinant protein, which has been reported to preferentially form dimers [2], was purified from *E. coli* (**Figure 1B**). Since multiple RTKs, including EGFR, are overexpressed in TNBC [31-35], we then asked whether extracellular PKM2 mediates the activities of RTKs in TNBC. Previously, recombinant PKM2 proteins at concentration of 2-3 μM was shown to promote tumor growth and angiogenesis *in vitro* and *in vivo* [25], and therefore we used this dose for all subsequent experiments. Serum starved MDA-MB-468 cells were treated with recombinant R399E-PKM2 protein. Treatment of recombinant R399E-PKM2 protein induced EGFR but not c-Met or ErbB3 phosphorylation (**Figure 1C**).

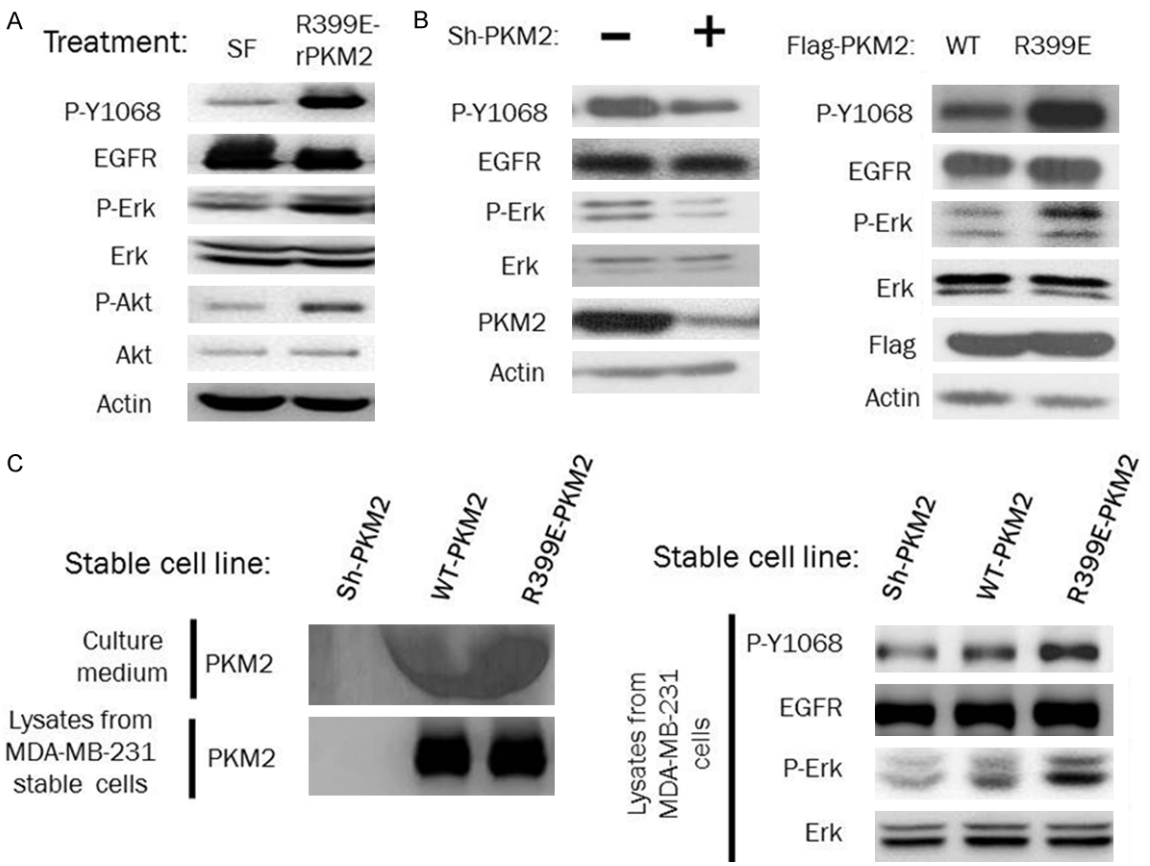

Figure 3. Extracellular PKM2 induces EGFR phosphorylation and downstream signaling. A. Cells were cultured in serum-free medium for 24 hours and then incubated with recombinant R399E-PKM2 protein for 30 min. Cell lysates were subjected to western blotting with the indicated antibodies. B. PKM2-knockdown, wild-type PKM2 and R399E mutant PKM2-expressing stable cells were harvested and lysates were subjected to western blotting with the indicated antibodies. C. PKM2-knockdown, WT-PKM2- and R399E-PKM2-expressing stable cells were seeded onto the membrane of transwell cell culture inserts (upper chamber), and MDA-MB-231 cells were simultaneously plated onto the bottom of the transwell (lower chamber). Serum-free medium was added to both chambers, and cells were incubated in serum-free medium for 48 hours. The conditioned medium was collected and concentrated to detect secreted PKM2 in the culture media. MDA-MB-231 cell lysates were subjected to western blotting with the indicated antibodies.

To further characterize recombinant PKM2-induced EGFR phosphorylation in TNBC cell lines, MDA-MB-468 cells were treated with recombinant wild-type (WT) PKM2 or R399E mutant PKM2 proteins for 30 minutes. Compared with WT PKM2, R399E mutant PKM2 induced higher EGFR phosphorylation in the absence of EGF (**Figure 2A**). We next determined the phosphorylation levels of EGFR stimulated by recombinant R399E-PKM2 protein at different time points or using different doses. A substantial increase in EGFR phosphorylation was observed at 30 minutes after recombinant R399E-PKM2 protein treatment for 5 hours and remained elevated for at least two hours with the 2-μM dose (**Figure 2B**). In cells treated with different doses of recombinant PKM2, EGFR phosphorylation was increased following the addition of 1 to 4 μM of recombinant R399E-PKM2 protein (**Figure 2C**). In addition, HEK 293 cells co-transfected with wild-type EGFR and R399E mutant PKM2 had higher EGFR tyrosine phosphorylation than in cells co-transfected with kinase-dead EGFR and wild-type or R399E mutant PKM2. These data indicated that the dimeric R399E mutant PKM2 may not directly phosphorylate EGFR but instead may induce EGFR auto-phosphorylation (**Figure 2D**).

Extracellular PKM2 activates EGFR downstream signaling

Activation of EGFR stimulates its downstream signaling, leading to genes expression and cellular transformation such as anchorage-inde-

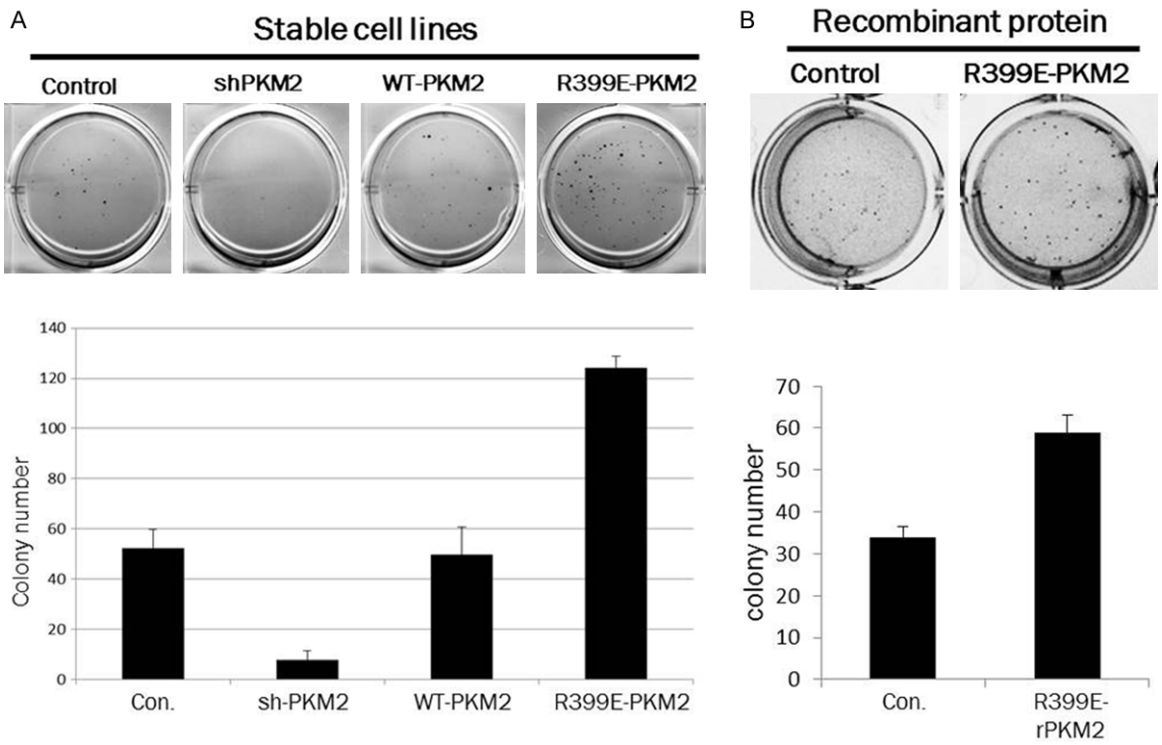

Figure 4. Extracellular PKM2 enhances the cellular transformation of cancer cells. A. Control, PKM2-knockdown, WT-PKM2- and R399E-PKM2-expressing stable MDA-MB-231 cells were subjected to soft agar assay. Quantitative analysis of colony numbers is shown in the lower panel. Values are the means ± SD of three independent experiments. B. MDA-MB-231 cells were subjected to soft agar assay. Cells were treated with 2 μM recombinant protein R399E-PKM2, and culture medium was replaced every 4 days. After incubation for three weeks, the number of colonies was counted and photographed. Quantitative analysis of colony numbers is shown in the lower panel. Values are the means ± SD of three independent experiments.

pendent growth, and aberrant activation of EGFR is associated with tumor progression and poor patient prognosis [36-39]. To investigate whether extracellular PKM2 activates EGFR downstream signaling, MDA-MB-231 cells were incubated with recombinant R399E-PKM2 protein for 30 minutes and the lysates were subjected to Western blot analysis with the antibodies against phosphorylated Erk and Akt. Recombinant R399E-PKM2 protein treatment induced EGFR phosphorylation and activated its downstream signaling, including the Erk and Akt pathways (**Figure 3A**). To further validate that PKM2 regulates the EGFR signaling pathway, we knocked down endogenous PKM2 in MDA-MB-231 cells and then re-expressed WT or R399E mutant PKM2 in these PKM2-knockdown cells. Knocking down PKM2 decreased the phosphorylation levels of EGFR and Erk (**Figure 3B**, left). Re-expression of R399E-PKM2 in PKM2 knockdown cells resulted in higher EGFR and Erk phosphorylation than those re-expressing the wild-type enzyme (**Figure 3B**, right).

Because PKM2 is secreted into the culture medium and activates EGFR signaling, we hypothesized that PKM2 mediates EGFR signaling pathway in an autocrine/paracrine manner. To test this hypothesis, PKM2 knockdown stable cells or knockdown cells with stable expression of WT-PKM2 or R399E-PKM2 were seeded in the upper chamber of a non-contacting co-culture transwell cell culture system. Parental MDA-MB-231 cells were seeded simultaneously in the lower chamber. Cells in both the upper and lower chambers were incubated in serum-free medium for 48 hours. Cells in the lower chamber were then harvested to detect phosphorylated EGFR and Erk. We also collected the culture medium to validate the expression of secreted PKM2. PKM2 was detected in culture medium from WT-PKM2- and R399E-PKM2-expressing stable cells but not from the culture medium of PKM2 knockdown stable cells (**Figure 3C**). Furthermore, we found more PKM2 in the culture medium of R399E-PKM2-expressing stable cells that was accompanied by higher EGFR and Erk phosphorylation than with

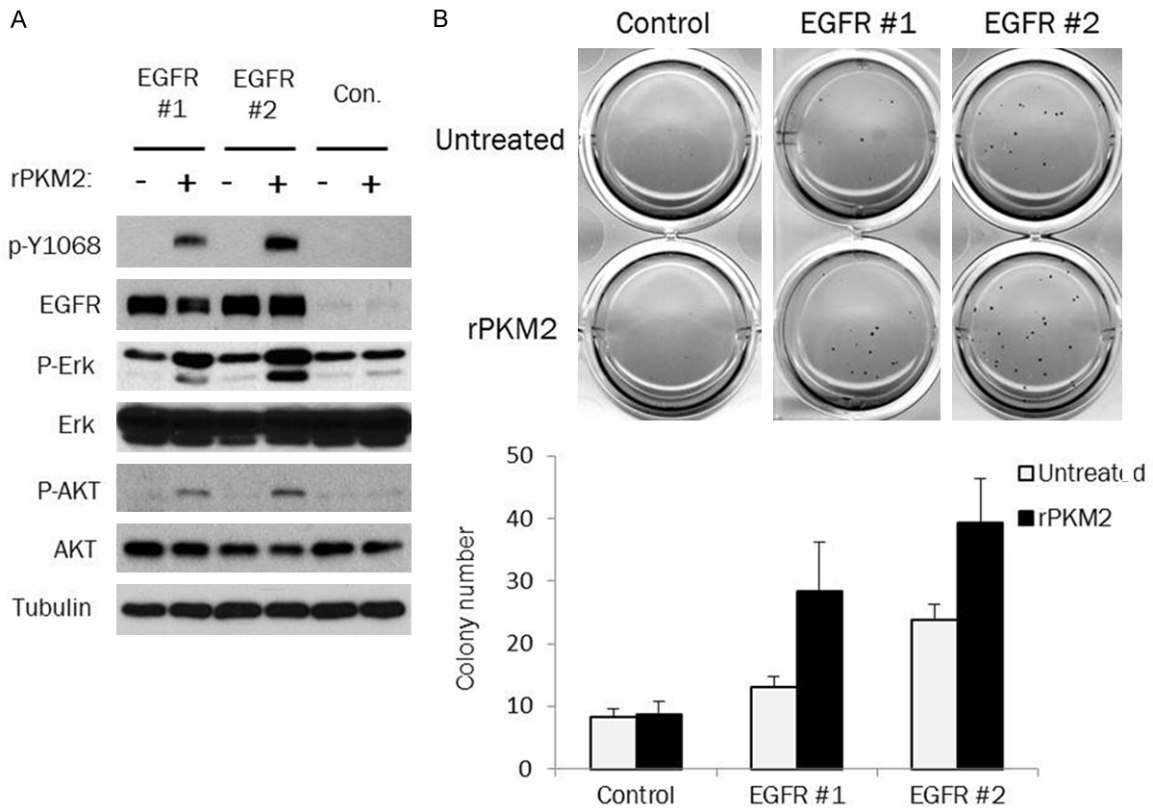

Figure 5. Recombinant PKM2-mediated cellular transformation is EGFR dependent. A. Two independent stable clones of MCF7 with exogenous EGFR expression were cultured in serum-free medium for 24 hours and then treated with 2 μM recombinant R399E-PKM2 proteins for 30 min. Cell lysates were subjected to western blotting with the indicated antibodies. B. Two independent stable clones of MCF7 with exogenous EGFR expression were subjected to soft agar assay. Cells were cultured in DMEM medium containing 2 μM recombinant R399E-PKM2 proteins, and the culture medium was changed every 4 days. After incubation for three weeks, the number of colonies was counted and photographed. Quantitative analysis of colony numbers is shown in the lower panel. Values are the means ± SD of at least three independent experiments.

those expressing from WT-PKM2. Parental cells co-cultured with PKM2-expressing stable cells also had higher EGFR and Erk phosphorylation than those with PKM2-knockdown cells. Together, our results support the notion that secreted PKM2 activates EGFR signaling.

Extracellular PKM2 enhances cellular transformation of TNBC cells

Our data above indicated that extracellular PKM2 induced EGFR phosphorylation and activated EGFR downstream signaling. Because EGFR promotes cancer cell proliferation and tumorigenesis, we further investigated the functional relevance of extracellular PKM2 in TNBC. Anchorage-independent cell growth is an important hallmark of cellular transformation and has been shown to be promoted by EGFR signaling. Thus, we next assessed anchorage-independent growth of PKM2-knockdown cells and PKM2-knockdown cells re-expressing WT or R399E mutant PKM2 by soft agar assay. PKM2-knockdown cells formed significantly less number of colonies compared to the control cells. In contrast, restoring the expression of PKM2, particularly the R399E mutant, greatly enhanced anchorage-independent growth compared to those cells expressing the WT-PKM2 (**Figure 4A**). We treated MDA-MB-231 cells with recombinant R399E-PKM2 protein and found that it also promoted anchorage-independent growth compared with cells without protein treatment (**Figure 4B**). These results suggested that PKM2 enhances cellular transformation, by promoting anchorage-independent growth of cells.

The effects of extracellular PKM2 on anchorage-independent growth are EGFR dependent

To further validate that PKM2-enhanced EGFR signaling and anchorage-independent cell gr-

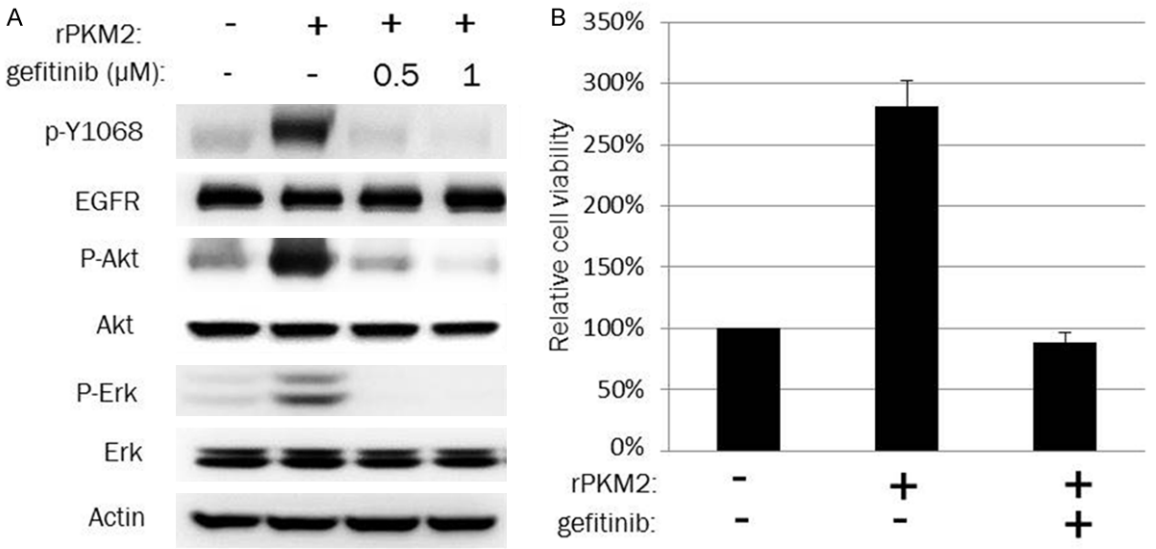

Figure 6. EGFR inhibitor gefitinib blocks recombinant PKM2-mediated cell proliferation. A. Cells were cultured in serum-free medium for 24 hours and then treated with recombinant R399E-PKM2 proteins (2 μM) and with or without gefitinib for 30 min. Cell lysates were collected and subjected to western blotting with the indicated antibodies. B. Cells were incubated with 2 μM recombinant R399E-PKM2 proteins with or without 1 μM gefitinib and subjected to MTT assay to measure cell viability after 72 hours. Values are the means ± SD of at least three independent experiments.

owth require EGFR, we generated two independent MCF-7 stable clones that ectopically express EGFR (MCF-7/EGFR). Serum-starved MCF-7/EGFR stable cells were treated with recombinant R399E-PKM2 protein; both MCF-7/EGFR clones exhibited increased phosphorylation levels of EGFR, Erk, and Akt upon the treatment. In contrast, MCF-7 control cells, which expressed virtually no EGFR, did not exhibit Erk or Akt activation upon R399E-PKM2 protein treatment (**Figure 5A**). Next, to determine whether the PKM2/EGFR axis is involved in cellular transformation, we evaluated anchorage-independent growth of these stable cells with or without recombinant R399E-PKM2 protein treatment by soft agar colony formation assay. R399E-PKM2 protein treatment enhanced the ability of anchorage-independent growth in these two MCF-7/EGFR stable cells but not in MCF-7 control cells (**Figure 5B**). Similarly, treatment with EGFR kinase inhibitor gefitinib inhibited recombinant PKM2-induced activation of downstream signaling (**Figure 6A**) and proliferation in MDA-MB-231 cells as measured by MTT assay (**Figure 6B**). Together, our data suggested that extracellular PKM2 promotes breast cancer cell proliferation and anchorage-independent growth through activation of EGFR.

Discussion

In this study, we investigated the functional role of extracellular PKM2 in TNBC and found the treatment of recombinant R399E-PKM2 protein induced the phosphorylation of EGFR, which is highly expressed and correlated with poor prognosis in TNBC. Previous studies revealed that EGFR activation induces the non-glycolytic function of nuclear PKM2 in transcriptional regulation including its function as a protein kinase via a molecular switch [9, 17, 18]. In contrast to earlier findings, however, a more recently study could not provide evidence to support the role of PKM2 as a protein kinase [19]. Our results indicated that extracellular PKM2 activated the EGFR pathway in an autocrine manner to promote tumor cell growth and induced EGFR phosphorylation. It is not yet clear whether PKM2 may function as an EGFR ligand or may activate EGFR through other mechanisms, such as activation of other receptor tyrosine kinases, which in turn activate EGFR. Our current study reveals a novel function of PKM2 and provides new insights into our understanding of PKM2 functions. Further studies will be required to dissect the mechanism underlying extracellular PKM2-mediated activation of EGFR.

A recent study reported that cytosolic PKM2 can prolong the half-life of mutant EGFR protein by direct interaction to maintain cell survival signaling in lung cancer cells [16]. While their results identified a specific role of cytosolic PKM2 in regulating the protein stability of mutant EGFR, the effects of cytosolic PKM2 on wild-type EGFR and its downstream signaling were less apparent. Dimeric PKM2 has been shown to localize not only in the cytosol and nucleus of cells but also in the blood circulation of cancer patients. In contrast to the study by Yang *et al.* [16], our results indicated that extracellular PKM2 can induce the phosphorylation of wild-type EGFR and its downstream signaling in TNBC cells. Thus, both studies focused on the PKM2-mediated EGFR regulation, but the underlying mechanisms are different, which may be due to cancer types, and/or EGFR mutation status.

In addition to the PKM2-mediated EGFR regulation, we recently demonstrated that EGFR phosphorylates PKM2 and attenuates its pyruvate kinase activity, resulting in promotion of glycolysis, tumor growth, and immune escape in TNBC cells [30]. Our prior data also indicated that EGFR-induced PKM2 phosphorylation reduces PKM2 tetramers [30]. However, it is uncertain whether phosphorylated PKM2 by EGFR preferentially forms a dimer. If so, phosphorylated PKM2 by EGFR may promote EGFR activation through a feed-forward mechanism. More studies will be required to dissect the intricate interplay between EGFR and PKM2 in cancers.

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Address correspondence to: Mien-Chie Hung, Department of Biotechnology, Asia University, Taichung, Taiwan. E-mail: mhung@mdanderson.org

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