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6-Phosphogluconate dehydrogenase regulates tumor cell migration in vitro by regulating receptor tyrosine kinase c-Met

Barden Chan [a,*], Paul A. VanderLaan [b], Vikas P. Sukhatme [a]

[a] Division of Interdisciplinary Medicine and Biotechnology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States
[b] Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

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ABSTRACT

6-Phosphogluconate dehydrogenase (6PGD) is the third enzyme in the oxidative pentose phosphate pathway (PPP). Recently, we reported that knockdown of 6PGD inhibited lung tumor growth in vitro and in a xenograft model in mice. In this study, we continued to examine the functional role of 6PGD in cancer. We show that 6PGD expression positively correlates with advancing stage of lung carcinoma. In search of functional signals related to 6PGD, we discovered that knockdown of 6PGD significantly inhibited phosphorylation of c-Met at tyrosine residues known to be critical for activity. This downregulation of c-Met phosphorylation correlated with inhibition of cell migration in vitro. Overexpression of a constitutively active c-Met specifically rescued the migration but not proliferation phenotype of 6PGD knockdown. Therefore, 6PGD appears to be required for efficient c-Met signaling and migration of tumor cells in vitro.

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1. Introduction

Altered cellular metabolism is a hallmark of cancer [1]. Tumors have a tendency to utilize glycolysis to generate ATP even under high oxygen tension, a phenomenon known as aerobic glycolysis or the Warburg effect [2,3]. The importance of the Warburg effect in cancer has been demonstrated in many studies. Inhibition of key enzymes in glycolysis such as pyruvate kinase M2, phosphoglycerate mutase 1, phosphofructokinase 1, lactate dehydrogenase A, and aldolase A results in inhibition of growth of various tumor types [4–10].

In addition to glycolysis, the pentose phosphate pathway (PPP) is also hyperactive in tumors [11,12]. We have previously examined the potential role of the oxidative branch of PPP in lung cancer [13]. Specifically, we found that knockdown of 6-phosphogluconate dehydrogenase (6PGD), the third enzyme in the pathway, inhibited cell growth by a novel mechanism independent of the known functions of PPP such as the production of NADPH, ribose phosphate, and dNTP/NTP [13].

In this study, we sought to identify signaling pathways regulated by 6PGD and discovered that knocking down 6PGD potently blocks c-Met activation. We show that this c-Met inactivation by 6PGD knockdown does not cause growth inhibition but instead lowers the ability of the cancer cells to migrate in vitro in response to human hepatocyte growth factor (HGF). Thus, 6PGD appears to be required for c-Met activation in this lung cancer cell line. To the best of our knowledge, this is the first demonstration that a receptor tyrosine kinase can be inhibited by manipulation of glucose metabolism. This discovery has broad implications potentially extending beyond lung cancer. In particular, targeting 6PGD may inhibit other tumors driven by c-Met activity and cancer metastasis in general.

2. Material and methods

2.1. Materials

A549 was purchased from ATCC. H1975 and MDA231 were gifts from Drs. S. Kobayashi and Z. Husain at BIDMC. Antibodies were commercially available: monoclonal 6PGD (Santa Cruz, SC-100316); polyclonal 6PGD (Sigma, AB1100532); phospho-c-Met (Cell Signaling, 3007); total c-Met (Cell Signal 3148), and actin (Abcam AB3280). Lung cancer arrays were from BioMax (LC242a, LC817, and T045c). Immunostaining reagents Vectastain Elite ABC Peroxidase System (PK-6102), Avidin/Biotin Blocking Kit (SP-2001), and Peroxidase Substrate Kit DAB (SK-4100) were from Vector Laboratories. Recombinant human HGF, NucBlue Fixed Cell Stain ReadyProbes DAPI, pcDNA3.1 Hygro, Lipofectamine® LTX

Abbreviations: 6PGD, 6-phosphogluconate; PPP, pentose phosphate pathway; PKM2, pyruvate kinase M2; LDHA, lactate dehydrogenase A; FBS, fetal bovine serum; HGF, human hepatocyte growth factor.

* Corresponding author. Address: CLS728 E8, 330 Brookline Ave, Beth Israel Deaconess Medical Center, Boston, MA 02215, United States.
E-mail address: cchan@bidmc.harvard.edu (B. Chan).

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& Plus Reagent, RPMI medium, and fetal bovine serum were from Life Technologies (PHG0254; R37606; V870-20; 15338030; 11875-119; 10437-028). c-Met siRNAs and Silentfect were from Origene (SR302873) and Bio-Rad (170-3361), respectively. 24-well Transwell Systems (8.0 μm, 6.5 mm inserts) were from Coster (3422). pBABE-puro TPR-cMet [14] was from Addgene (10902). Hematoxylin was from Santa Cruz (SC-24973). Doxycycline hyclate was from Sigma (D9891). Hygromycin, puromycin, and Primocin were from Invivogen (ant-hg-1, ant-pr-1, ant-pm-1).

2.2. Cell culture

Doxycycline-inducible shRNA knockdown of 6PGD in H1975 cells has been described [13]. H1975, A549, and MDA231 cells were maintained in RPMI/10%FBS/100 μg/ml Primocin. Production of stable A549 and MDA231 lines with inducible 6PGD shRNAs and siRNA transfection was performed as described [13]. Stable A549 and MDA231 cells were selected and maintained in 2 and 1 μg/ml puromycin, respectively. The coding sequence of TPR-cMet was excised from pBABE-puro and subcloned into pcDNA3.1 Hygro. The resulting plasmid was linearized with FspI and transfected into H1975 cells using Lipofectamine® LTX & Plus Reagent. Stable lines were selected and maintained with 100 μg/ml hygromycin and 0.5 μg/ml puromycin.

2.3. Immunohistochemistry

Specificity of the antibody for 6PGD detection was validated as follows. Expression of 6PGD was assessed in mock treated cells using a control shRNA or 6PGD-specific shRNAs. Cells were harvested. A portion was analyzed by western blot. 2×10^6 cells were resuspended in 1% agarose and fixed in 10% formalin. The samples were paraffin-embedded and sectioned by Dana Farber/Harvard Cancer Center Research Pathology Cores (Boston, MA), followed by immunostaining.

Immunostaining was done using the Vectastain Elite ABC kit, Avidin/Biotin Blocking Kit, and Peroxidase Substrate Kit DAB according to the manufacturer's protocols. 6PGD antibody treatment was for 1 h at room temperature at 1:100 dilution.

Staining for 6PGD protein was semi-quantitatively scored using a 0–3 scale based on previously used methodologies by Fisher et al. and Adams et al. [15,16]. Briefly, 0 is for negative (no cytoplasmic staining), 1+ represents weak staining (focal moderate staining in <50% of cells, or pale cytoplasmic staining in any proportion of cells not easily appreciated under low power), 2+ designates moderate staining (focal darkly staining areas, in <50% of cells or moderate cytoplasmic staining of >50% of cells), and 3+ denotes strong (dark cytoplasmic staining that is easily visible with a low power objective and involves >50% of cells). When a tumor sample was given in duplicate; an average of the scores was recorded for that sample.

2.4. In vitro assays

Cell proliferation assay was done as described [13]. Migration was tested using a 24-well Transwell system (8.0 μm). H1975 cells were grown in the presence of 2 μg/ml doxycycline for 5 days. On day 6, cells were trypsinized and reseeded at 2×10^6 per 10-cm plate in full medium and 2 μg/ml doxycycline. On the next day, cells were trypsinized, washed in PBS once, and resuspended in 1%FBS/RPMI/2 μg/ml doxycycline at 200,000 cells/ml. This cell suspension (100 μl) was transferred to the Transwell insert and incubated at 37 °C overnight. On the next day, 1%FBS/RPMI/10 ng/ml HGF without doxycycline (650 μl) was added to the bottom chamber. After a 5-h incubation at 37 °C, cells not migrated were removed carefully by a cotton-swab. Migrated cells were fixed with 10% formalin at room temperature for 15 min, washed once in PBS, and stained with DAPI in 0.5% Triton/PBS for 10 min. After a brief rinse in PBS, migrated cells were visualized by DAPI staining, recorded using a Zeiss fluorescence microscope, and counted in three random fields. Migration was reported as percent migration of control cells.

3. Results and discussion

We previously found that different human lung carcinoma samples overexpress 6PGD as compared to normal lung tissues by western blot [13]. To further characterize this observation, we performed immunohistochemical staining of 6PGD. We first validated the specificity of an anti-human 6PGD antibody in IHC application. This antibody specifically detected endogenous 6PGD in A549 cells (Fig. 1A), as no staining of cells was observed when 6PGD was knocked down (Fig. 1B and C).

Using this optimized procedure, we stained for 6PGD expression on human tissue arrays containing a range of lung carcinoma specimens with associated clinical stage data. A summary of the tumor characteristics for the arrays is presented in Suppl. Table 1. Normal lung parenchymal uniformly had very low levels of 6PGD staining (Fig. 1E), whereas 6PGD expression levels widely varied in lung carcinoma specimens (Fig. 1F–I). Semi-quantitative scoring of 6PGD staining indicates that 6PGD expression positively correlates with advancing lung carcinoma stage, as assessed by Spearman Correlation analysis (Fig. 1J). Therefore, 6PGD expression appears to correlate with lung tumor progression. These results are similar to a separate study documenting 6PGD activity as a prognostic marker for breast cancer [17].

Thus far, we have limited our statistical analysis to correlation between 6PGD expression and tumor staging. An expanded study with a larger sample size will be needed to more carefully define how 6PGD expression relates to other lung tumor characteristics such as carcinoma types, metastasis status, and tumor grade.

Next, we sought to identify the effect of 6PGD knockdown in intracellular signaling. Using cells expressing 6PGD and cells with 6PGD knocked down, we performed a simple phosphotyrosine blot of total cytoplasmic proteins. Knockdown of endogenous 6PGD in H1975 cells significantly blocked tyrosine phosphorylation of unknown proteins with high molecular weight (Fig. 2A, lane 3). This loss of tyrosine phosphorylation could be completely recovered by overexpression of mouse 6PGD even when endogenous 6PGD was knocked down (Fig. 2A, lane 4).

Based on the large protein size and the fact that they were supposed to be tyrosine phosphorylated, we speculated that these proteins were receptor tyrosine kinases. Indeed, we found that knocking down 6PGD with shRNA potently blocked c-Met phosphorylation at tyrosine 1234/1235 (Fig. 2B). As tyrosine phosphorylation of these sites is required for kinase activity of c-Met [18], knockdown of 6PGD was expected to have blocked c-Met activity as well. To rule out the possibility of shRNA side effects, we rescued c-Met phosphorylation in cells with endogenous 6PGD knocked down using mouse 6PGD (Fig. 2C). Overexpression of mouse 6PGD alone enhanced c-Met phosphorylation (Fig. 2C, lane 2). Knockdown of 6PGD once again potently inhibited c-Met phosphorylation (Fig. 2C, lane 3). Tyrosine phosphorylation of c-Met in 6PGD knockdown was completely rescued by mouse 6PGD (Fig. 2C, lane 4). Thus, knockdown of 6PGD inhibits c-Met activation. We also expanded this analysis to additional cell lines and found inhibition of c-Met phosphorylation by 6PGD knockdown in A549 and MDA231 cells (Suppl. Fig. 1).

Next, we examined the reason for this c-Met inhibition. Our western blots indicated that 6PGD knockdown did not regulate c-Met protein level (Fig. 2B–D). We then tested whether this c-Met inhibition could be compensated by exogenous HGF. Adding

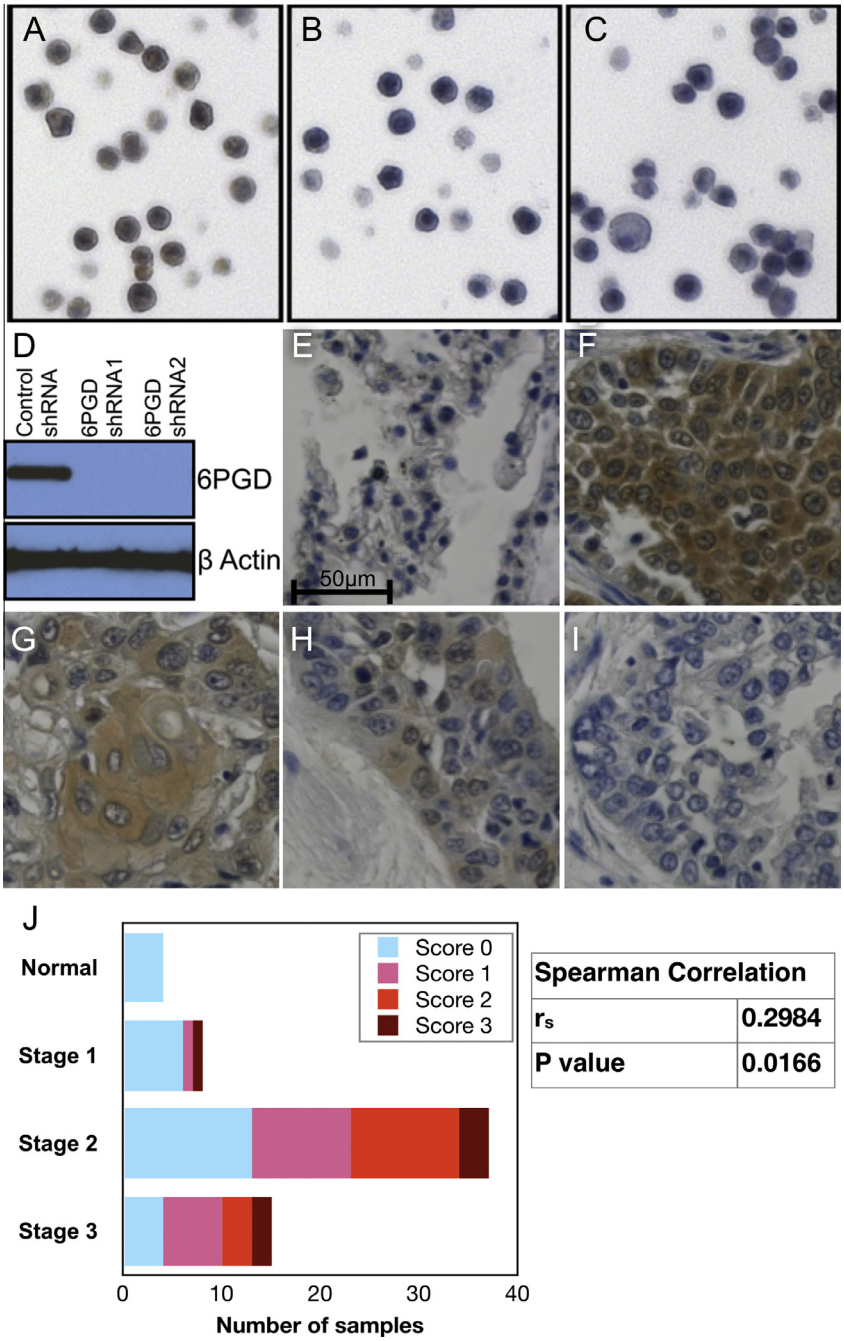

Fig. 1. 6PGD is overexpressed in human lung cancer. An anti-6PGD antibody was validated for specific 6PGD detection in IHC application. After 6PGD was knocked down, A549 cells were formalin-fixed, paraffin-embedded, sectioned, and stained for 6PGD expression using an antibody from Santa Cruz (SC100316) and the VECTASTAIN elite ABC peroxidase system (Vector labs). 6PGD was visualized using DAB as a substrate. Samples were counter-stained by hematoxylin. Note that 6PGD was only detected in control cells (A), and there was no DAB staining in 6PGD knockdown samples (B, C). (D) A western blot confirming complete 6PGD knockdown in A549. (E) 6PGD expression is very low in normal lung tissue. (F–I) 6PGD showed a range of expression levels in lung carcinoma. Examples of squamous cell carcinoma are shown: (F) 3+, (G) 2+, (H) 1+, and (I) 0. (J) 6PGD expression overall positively correlates with advancing stage of disease in human lung cancer. In total, 60 lung carcinomas and 4 normal lung samples were stained for 6PGD expression.

recombinant human HGF up to 50 ng/ml failed to restore c-Met phosphorylation (Fig. 2D). Therefore, 6PGD knockdown interferes with c-Met activation independent of endogenous HGF production.

We previously reported that 6PGD knockdown induced senescence and blocked cell growth in H1975 [13]. We next tested whether low c-Met activity caused by 6PGD knockdown was the reason for the growth phenotype. Knocking down c-Met directly by siRNA (Fig. 3A) only marginally affected cell growth in H1975 (Fig. 3B), in contrast to the significant proliferation inhibition observed when 6PGD was knocked down [13]. Therefore, consistent with a published report, c-Met is not needed for optimal growth in H1975 cells [19]. Diminished c-Met phosphorylation in 6PGD knockdown cells is unlikely to be the reason for the reduced proliferation.

To directly test whether low c-Met activity in 6PGD knockdown cells was the reason for growth inhibition, we restored c-Met activity in these 6PGD knockdown cells by expression of TPR-cMet. This protein is a fusion protein with the cytoplasmic kinase domain of

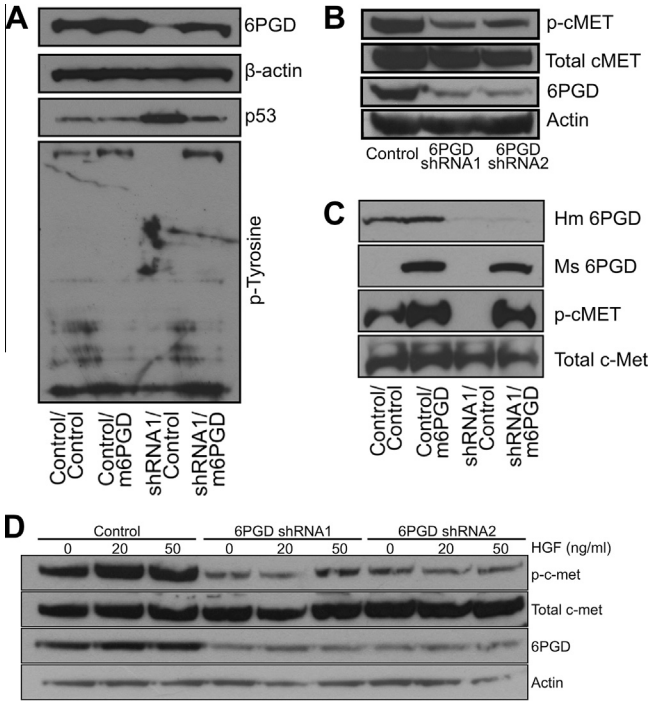

Fig. 2. 6PGD is required for efficient c-Met phosphorylation. (A) A phosphotyrosine blot of total lysates from cells with and without 6PGD. Top gel shows 6PGD detection. At 1:200 dilution, this anti 6PGD antibody (Santa Cruz, SC-100316) simultaneously detected endogenous human and exogenous mouse 6PGD. Knockdown of endogenous human 6PGD (bottom gel, lane 3) blocked tyrosine phosphorylation of some high molecular weight proteins, which was restored by expression of mouse 6PGD (bottom gel, lane 4). (B) Inhibition of c-Met phosphorylation caused by 6PGD knockdown. (C) Inhibition of c-Met phosphorylation by 6PGD knockdown was restored by expression of mouse 6PGD. Note that monoclonal 6PGD antibody (Santa Cruz, SC-100316) at 1:5000 dilution detected only the endogenous human 6PGD (top gel), whereas the polyclonal anti-6PGD antibody (Sigma, AB1100532) preferentially detected overexpressed mouse 6PGD (second gel from the top) [13]. (D) Western blot showing exogenously added HGF did not rescue c-Met phosphorylation when 6PGD was knocked down. Experiments were done at least two times. Representative data are shown.

c-Met fused with the leucine zipper domain of a transcription factor [14,20]. Forced dimerization confers constitutive activity to this protein.

Overexpressed TPR-cMet was phosphorylated at tyrosines corresponding to residues 1234/1235 in full-length c-Met critical for its activity [18] (Fig. 3C, lane 2). 6PGD knockdown alone again inhibited endogenous c-Met phosphorylation (Fig. 3C, lane 3). Importantly, knockdown of 6PGD could only inhibit tyrosine phosphorylation of endogenous c-Met but not TPR-cMet (Fig. 3C, lane 4), suggesting that in these cells, c-Met signaling was restored.

Overexpression of TPR-cMet alone only marginally affected cell growth (Fig. 3D, bar 3), consistent with the notion that c-Met activity played a minor role in growth in these cells. Knocking down 6PGD, as we reported previously [13], potently inhibited proliferation (Fig. 3D, bar 4). Despite the restoration of c-Met signals by TPR-cMet in 6PGD knockdown, cells lacking 6PGD but with TPR-cMet expression still failed to grow efficiently (Fig. 3D, bar 5). Therefore, we concluded that c-Met inhibition by 6PGD knockdown is not the cause of growth inhibition.

As c-Met is known to be critical in tumor metastasis [21], we tested the effect of TPR-cMet in migration of these cells. Overexpression of TPR-cMet alone enhanced cell migration of H1975 (Fig. 4A and B). Knockdown of 6PGD inhibited the migration of H1975 cells in vitro in response to HGF (Fig. 4C). In contrast to proliferation, the migratory phenotype of cells with 6PGD knockdown was significantly restored by TPR-cMet (Fig. 4D). Therefore, 6PGD knockdown inhibits cell migration by blocking c-Met activation.

It was particularly interesting that rescuing c-Met signaling by TPR-cMet could not restore cell growth but significantly rescued migration. Our results, therefore, suggest that cell proliferation and migration of H1975 cells are controlled by two independent signaling pathways. Receptor c-Met appears to play a crucial role in cell migration in these cells. At this time, the altered cell signals in 6PGD knockdown that lead to inhibition of cell growth remain to be identified. Additional experiments will be needed to complete the full picture of 6PGD-regulated signals, especially those that are crucial to senescence suppression and optimal growth.

Receptor tyrosine kinases can affect the activity of genes involved in glucose metabolism. For example, FGFR1 has been shown to tyrosine phosphorylate pyruvate kinase M2 (PKM2) and lactate dehydrogenase-A (LDHA) thereby modulating their activity and

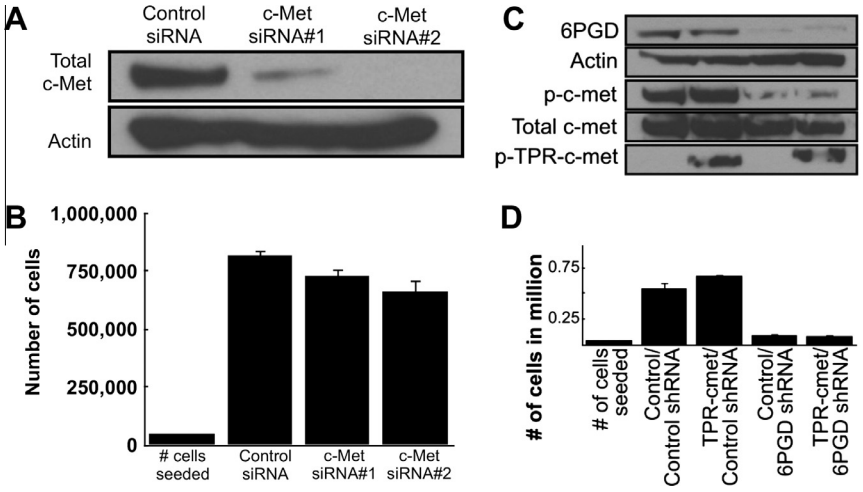

Fig. 3. c-Met played a minor role in growth in H1975 cells. (A) Endogenous c-Met was knocked down by siRNAs. (B) Proliferation assay showing c-Met knockdown marginally affected the growth of H1975 cells. (C) Expression of a constitutively active c-Met construct. Note that knockdown of 6PGD again suppressed phosphorylation of endogenous c-Met (lane 3). However, tyrosine phosphorylation of TPR-cMet was not affected by 6PGD knockdown (lane 4). Therefore, in lane 4, c-Met signaling was expected to be restored by TPR-cMet even when 6PGD was knocked down. (D) Results of a proliferation assay showing the effects of TPR-cMet expression. TPR-cMet alone marginally affected cell growth (bar 3). 6PGD knockdown blocked cell growth (bar 4), which could not be restored by TPR-cMet expression (bar 5). Therefore, inhibition of cell growth caused by 6PGD knockdown was not a result of c-Met inhibition. Experiments were done twice. Representative data are shown.

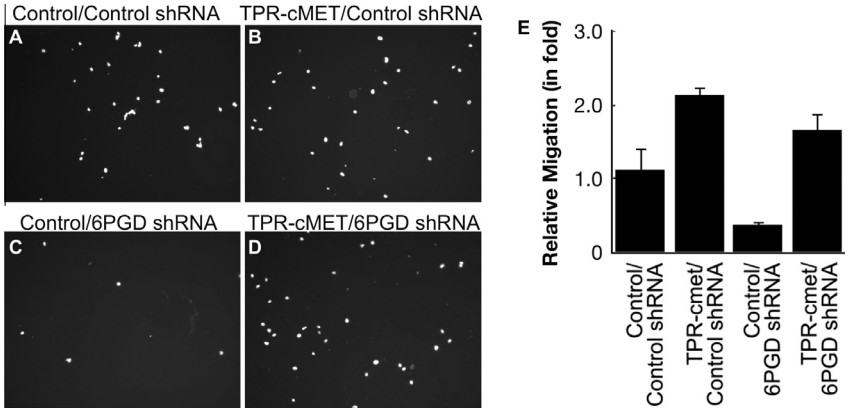

Fig. 4. Knockdown of 6PGD inhibited cell migration by suppressing c-Met activity in response to HGF. After overnight serum starvation on one side of a porous membrane, cells were stimulated with 10 ng/ml HGF on the opposite side to induce migration. Five hours later, migrated cells were fixed, permeabilized, and visualized by DAPI staining. (A) Basal level of H1975 cells migration in a Transwell system. (B) Expression of TPR-cMet enhanced cell migration. (C) 6PGD knockdown inhibited H1975 ability to migrate. (D) In contrast to proliferation, migration of H1975 with 6PGD knocked down was restored by TPR-cMet expression. (E) Quantification of migration results. Experiments were done three times. Representative data are shown.

function [22,23]. These outside-to-inside signals presumably exist to ensure rapid metabolic responses in cancer cells to external stimuli for optimal growth. However, to the best of our knowledge, 6PGD is the first metabolic enzyme that regulates this signaling axis in the opposite direction by regulating a receptor tyrosine kinase activity and function from the inside of a cell. The underlying mechanism of this novel 6PGD function is currently under investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.048.

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