Increased Concentrations of Fructose 2,6-Bisphosphate Contribute to the Warburg Effect in Phosphatase and Tensin Homolog (PTEN)-deficient Cells*1

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Background: PTEN deficiency leads to increased glycolytic flux characteristic of cancer cells.

Results: PTEN-deficient cells have high concentrations of fructose 2,6-bisphosphate due to protein stabilization of PFKFB3 that results from impaired APC/C-Cdh1-dependent degradation.

Conclusion: Elevated fructose 2,6-bisphosphate concentrations contribute to the increased rates of glycolysis and proliferation in PTEN-deficient cells.

Significance: Fructose 2,6-bisphosphate might be a crucial mediator of tumorigenesis in PTEN-deficient cells.

Unlike normal differentiated cells, tumor cells metabolize glucose via glycolysis under aerobic conditions, a hallmark of cancer known as the Warburg effect. Cells lacking the commonly mutated tumor suppressor PTEN exhibit a glycolytic phenotype reminiscent of the Warburg effect. This has been traditionally attributed to the hyperactivation of PI3K/Akt signaling that results from PTEN loss. Here, we propose a novel mechanism whereby the loss of PTEN negatively affects the signaling that results from PTEN loss. We discovered that the glycolytic enzyme phosphofructokinase-1 (PFK-1). Reintroduction of either wild-type or phosphatase mutant PTEN in the PTEN KO cells effectively lowers F2,6P2 to the wild-type levels and reduces their lactate production. PTEN KO cells were found to have high protein levels of PFKFB3, which directly contribute to the increased concentrations of F2,6P2. PTEN enhances interaction between PFKFB3 and Cdh1, and overexpression of Cdh1 down-regulates the PFKFB3 protein level in wild-type, but not in PTEN-deficient cells. Importantly, we found that the degradation of endogenous PFKFB3 in PTEN KO cells occurs at a slower rate than in wild-type cells. Our results suggest an important role for F2,6P2 in the metabolic reprogramming of PTEN-deficient cells that has important consequences for cell proliferation.

To meet their bioenergetic requirements, differentiated cells tend to metabolize glucose via oxidative phosphorylation as a way of maximizing ATP production. In contrast, cancer cells are characterized by high rates of glycolysis and metabolize glucose into lactate even in the presence of oxygen, a phenomenon known as the “Warburg effect” or “aerobic glycolysis” (1). Up-regulation of glycolysis is proposed to endow cancer cells with several selective advantages, in particular the incorporation of nutrients into biomass to sustain high rates of proliferation (2, 3). Deregulation of certain cancer-related genes has been linked to the acquisition of the glycolytic phenotype (4). The phosphatase and tensin homolog, PTEN,2 is a tumor suppressor most well known for its ability to oppose the PI3K/Akt signaling pathway through the dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate (5). Deletions in the PTEN gene locus have been reported in multiple cancers (6, 7). Interestingly, PTEN knock-out cells have been used as cellular models of the Warburg effect, given that they exhibit an enhanced glycolytic phenotype and high proliferative rates (8, 9). Excessive PI3K/Akt signaling as a consequence of PTEN loss can account for the enhanced glycolytic rates of these cells (10–13). In our study, however, we are proposing a novel role of PTEN in the regulation of the Warburg effect that is independent of its ability to oppose the PI3K/Akt pathway.

It was recently reported that PTEN, in a phosphatase-independent manner, is capable of enhancing the ubiquitination activity of the APC/C-Cdh1 ligase (14). The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase known to regulate cell cycle progression and to form two complexes with distinct substrate specificity: APC/C-Cdc20 and APC/C-Cdh1 (15). Interestingly, the APC/C-Cdh1 has been demonstrated to mediate the degradation of PFKFB3 (16, 17). PFKFB3 is an isozyme from the family of enzymes 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFK-2/FB-Pase-2) that catalyze the synthesis and degradation of fructose...
2,6-bisphosphate (F2,6P2), a known allosteric activator of the key glycolytic enzyme PFK-1 (18). PFKFB3 is ubiquitously expressed in mammalian tissues and has the highest kinase-to-bisphosphatase ratio; hence it is virtually dedicated to the synthesis of F2,6P2 (19). In light of these studies, we hypothesized that PTEN-deficient cells exhibit enhanced glycolytic rates due to increased concentrations of F2,6P2, which are the result of impaired degradation of PFKFB3 via the APC/C-Cdh1 E3 ligase.

MATERIALS AND METHODS

Cell Culture and Transfection—Wild-type mouse embryonic fibroblasts (MEF) and PTEN knock-out mouse embryonic fibroblasts (PTEN KO MEF) (a kind gift from Dr. Tak Mak, University of Toronto) were grown at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal bovine serum (HyClone), L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen). The following DNA plasmids were transiently transfected in subconfluent cells with GeneJuice® transfection reagent (Novagen): pcDNA3.1 FLAG-PFKFB3, constructed from pDONR223-PFKFB3 (Addgene plasmid 23668, William Hahn); pcDNA3.1 FLAG-PFKFB3 KENmut, KEN box mutated to AAA; pSG5L HA-PTEN WT (Addgene plasmid 10750, William Sellers); pSG5L HA-PTEN C124S (Addgene plasmid 10744, William Sellers); and pCS2+ MT-Cdh1 (Addgene plasmid 11595, Marc Kirschner). For dsRNA-mediated gene knockdown, the following predesigned dsRNA (Integrated DNA Technologies) at final concentration of 20 nM were transfected using RNAiMAX Lipofectamine (Invitrogen): MMC.RNAI.N001177753.12.1 (PFKFB3 dsRNA oligo 1), MMC.RNAI.N001177753.12.6 (PFKFB3 dsRNA oligo 2), HSC.RNAI.N000314.12.1 (PTEN dsRNA oligo 1), and HSC.RNAI.N000314.12.10 (PTEN dsRNA oligo 2).

Stable Cell Line Generation—The vesicular stomatitis virus G glycoprotein envelope expression plasmid was co-transfected with MaRX IVf Puro HA-PTEN wild-type, MaRX IVf Puro HA-PTEN C124S mutant, or the control MaRX IVf Puro enhanced green fluorescent protein plasmid into 293 GP cells. Selection of successful transductants was carried out by infection of PTEN KO MEF cells. Selection of successful transductants was carried out by treatment with puromycin for several weeks. The stable cell lines PTEN KO MEF + HA-PTEN wild-type PTEN KO MEF + HA-PTEN C124S mutant and PTEN KO MEF + enhanced green fluorescent protein were created.

Fructose 2,6-Bisphosphate Measurement—Fructose 2,6-bisphosphate was extracted and measured using the method described in Ref. 20. Briefly, cells were lysed in 0.1 M NaOH and heated at 80 °C for 5 min. Samples were then centrifuged, and the supernatant was neutralized with ice-cold 1 M acetic acid in the presence of 20 mM Hepes. The mixture was centrifuged again, and F2,6P2 was assayed in the resultant supernatant through its ability to activate potato tuber pyrophosphate phosphofructokinase-1 (PF-PFK). In the assay, the following reagents at the specified final concentrations were used: 50 mM Tris/HCl, pH 8, 5 mM MgCl2, 0.15 mM NADH, 17.5 mM glucose 6-phosphate, 0.5 mM pyrophosphate, 1 mM fructose 6-phosphate, 50 µg/ml aldolase, 1 µg/ml triosephosphate isomerase, 10 µg/ml glycerol-3-phosphate dehydrogenase. Light absorbance of NADH at 340 nm was measured in a spectrophotometer. F2,6P2 was quantified using a standard (a kind gift from Dr. Richard Honzatko, Iowa State University) and expressed as pmol of F2,6P2/µg of cellular protein or as percentage of (or _fold difference when compared with) control cells.

Lactate Measurement—Cells were seeded at low density in 6-well plates and the next day incubated with fresh medium containing 10% diazoyl fetal bovine serum (FBS) to decrease background levels of lactate. After 72 h, 200 µl of the medium were collected and assayed with a lactate PAP kit (Biomerieux). Lactate readings were normalized to protein amount, which was assayed with BCA Protein Assay Kit (Pierce).

Glycolytic Flux Measurement—Complete medium containing [5-3H]glucose (PerkinElmer) at a concentration of 363.63 µCi/mmol glucose was prepared. Cells seeded in 24-well plates were incubated with 300 µl of the medium at 37 °C for 6 h. In negative control wells, 2-deoxyglucose (Sigma) at final concentration 5 mM was also added. Glass vials with rubber stoppers and hanging wells were set up for each sample, and filter paper (1 × 6 cm) soaked in 200 µl of H2O was placed inside the hanging well. After a 6-h incubation, 250 µl of medium were collected and centrifuged to pellet any cells, and the supernatant was added to the bottom of the glass vial. Vials were sealed and incubated at 37 °C for 48 h to allow evaporation of 3H2O. The filter paper was then transferred to a scintillation tube. In addition, the hanging well was washed once with 200 µl of water, and this was added to the scintillation tube. 3H radioactivity count was measured for each sample. Readings were normalized to protein amount.

Western Blotting—Cells were lysed with Triton X-100 buffer (21). Equal amounts of protein were electrophoresed in SDS-PAGE gels and were transferred overnight onto nitrocellulose membranes. The following antibodies were used to probe the membranes: mouse monoclonal anti-PFKFB3 (Novus Biologicals), mouse monoclonal anti-PTEN (Santa Cruz Biotechnology), mouse monoclonal Cdh1 (Calbiochem), rabbit polyclonal anti-Geminin (Abcam), mouse monoclonal anti-PLK-1 (Invitrogen), mouse monoclonal anti-β-actin (Sigma), mouse monoclonal anti-α-tubulin (Molecular Probes), mouse monoclonal anti-FLAG (Sigma), and mouse monoclonal anti-Myc tag (clone 9B11, Cell Signaling).

Statistical Analysis—Measurements are expressed as means ± S.E. Statistical analysis of the results was performed by unpaired Student’s _t_ test or by one-way analysis of variance followed by Tukey’s honestly significant difference test for pairwise comparison of means. _p_ < 0.05 was considered significant.

RESULTS

PTEN-deficient Cells Have Higher Concentration of F2,6P2 Than Wild-type Cells—PTEN-deficient cells have been reported to exhibit high rates of aerobic glycolysis. To validate this, we measured glycolytic flux through the metabolism of [5-3H]glucose, wherein traceable 3H2O is produced at the enolase reaction and is released into the culture medium (22). To ensure the specificity of the assay, we co-incubated control
wells with the glucose analog 2-deoxyglucose (2-DG), which is converted to 2-DG-P and inhibits hexokinase at the rate-limiting step of glycolysis (23). Results are thus expressed as total [5-3H]glucose-to-3H2O metabolism minus nonspecific metabolism that occurs during inhibition by 2-DG. Wild-type and PTEN KO MEF cells were assayed this way, and we found that, in accordance with previous studies, the PTEN-deficient cells had a significantly higher glycolytic flux than the wild-type cells (Fig. 1A).

Aerobic glycolysis culminates in the production of lactic acid from pyruvate, which in aqueous solutions dissociates almost completely to lactate and H+. We thus assayed the rate of lactate synthesis in wild-type and PTEN KO MEF cells. In line with the [5-3H]glucose results, PTEN KO cells had a higher rate of lactate synthesis.

**FIGURE 1. PTEN KO cells have higher F2,6P2 concentration than wild-type cells. A, cells were incubated with medium containing [5-3H]glucose at concentration 363.63 μCi/mmol of glucose for 6 h at 37 °C. Supernatant was then transferred to glass vials equipped with hanging wells fitted with filter paper. After 48 h of incubation at 37 °C, filter paper was placed in scintillation vials, and 3H radioactive count was measured and normalized to protein concentration. Results are expressed as total [5-3H]glucose converted to 3H2O minus nonspecific metabolism that occurs during inhibition by 2-DG (5 mM). Data are mean ± S.E. of three experiments. B and F, cells were incubated in 10% dialyzed FBS medium for 72 h. 200 μl of medium were collected, centrifuged, and assayed with a lactate PAP kit. Results are normalized to protein concentration. Data are mean ± S.E. of three experiments. C, data are mean ± S.E. of five experiments. E and G, data are mean ± S.E. of two experiments. D, Western blot of PTEN expression in PTEN KO MEF stable cell lines expressing control GFP, PTEN-HA WT, or PTEN-HA C124S. Stable cell lines were created by retroviral infection of PTEN KO MEF cells. G, HEK293T cells were transfected with the indicated dsRNA oligos and were assayed for F2,6P2 concentration. H, effectiveness of PTEN knockdown was determined by Western blotting. ** denotes p < 0.01, * denotes p < 0.05, N.S. denotes nonsignificant, p > 0.05.
F2,6P2 Contributes to Warburg Effect in PTEN KO Cells

lactate production (Fig. 1B). The good correlation between glycolytic flux and lactate production validated the latter as a good read-out of aerobic glycolysis.

According to the proposed hypothesis, cells lacking PTEN are expected to have higher concentrations of F2,6P2 than wild-type cells, which in turn may contribute to their increased glycolytic capacity. To investigate this, an enzymatic assay to measure F2,6P2 was set up and optimized based on the method described in Ref. 20. Cell extracts from wild-type and PTEN KO MEF cells were analyzed in the assay to determine the effects of PTEN deficiency on F2,6P2 concentration. The results were normalized to the protein concentration to control for variable rates of cell proliferation. Even after accounting for higher cell mass, PTEN KO MEF consistently showed increased concentrations of F2,6P2 (Fig. 1C), thus supporting our hypothesis.

Reconstitution of the PTEN Gene Decreases the Concentration of F2,6P2.—The complete loss of a gene, such as PTEN, may trigger compensatory mechanisms to re-establish cellular homeostasis. Hence, we aimed to determine whether the increased concentrations of F2,6P2 in the PTEN KO cells were indeed the result of the absence of PTEN. Furthermore, we wished to elucidate whether the role of PTEN in regulating F2,6P2 is dependent on its phosphatase activity. To this end, PTEN KO MEF cells were infected with retroviruses encoding for control GFP, WT PTEN-HA, and C124S PTEN-HA, a mutant form of PTEN that lacks lipid phosphatase activity. Stable cell lines were generated, and their PTEN expression was assessed by Western blotting (Fig. 1D).

Remarkably, reconstitution of both the wild-type and the mutant PTEN in the PTEN KO cells decreased the concentration of F2,6P2 to a level similar to that of wild-type MEF (Fig. 1E). This result demonstrates a direct role for PTEN in the regulation of F2,6P2 concentrations. Furthermore, the regulation by PTEN appears to be largely independent of its lipid phosphatase activity. This is in agreement with our hypothesis that PTEN regulates F2,6P2 via its ability to promote, in a phosphatase-independent manner, the APC/C-Cdh1-mediated degradation of PFKFB3.

The glycolytic capacity of the retrovirus-infected cells followed a trend similar to the F2,6P2 concentrations. The control GFP-expressing cells maintained a high rate of lactate synthesis, whereas the cells reconstituted with WT and C124S PTEN decreased their lactate production (Fig. 1F). Acidification of the cell culture medium as judged from the red-to-yellow change in color, supported the lactate production results (supplemental Fig. S1A). Of note, the drop in lactate synthesis was statistically more significant in the PTEN WT than the C124S mutant cells. This is to be expected due the role of PTEN phosphatase activity in regulating glycolysis through inhibition of PI3K/Akt signaling.

We further examined whether the regulation of F2,6P2 concentration by PTEN is a conserved cellular mechanism and not a cell type-dependent effect restricted to the mouse embryonic fibroblasts. Interestingly, silencing of PTEN in HEK293T cells by a dsiRNA oligo that effectively down-regulates PTEN protein levels caused a significant increase in F2,6P2 concentrations (Fig. 1, G and H). In contrast, silencing with an oligo that has no effect on PTEN protein levels failed to alter F2,6P2 concentrations (Fig. 1, G and H).

Abundance of PFKFB3 in PTEN KO Cells Contributes to the Elevated F2,6P2 Concentration.—Having shown that PTEN deficiency results in elevated F2,6P2, we next addressed the premise that PFKFB3 protein abundance is responsible for the increased concentrations of this metabolite. Wild-type and PTEN KO MEF cells were analyzed for PFKFB3 protein abundance by Western blotting in several independent experiments, and the average densitometry values were plotted (Fig. 2A). PTEN KO cells were consistently observed to have higher PFKFB3 protein levels than wild-type MEF cells.

To validate the role of PFKFB3 in contributing to the high F2,6P2 concentrations, we screened two possible dsRNA oligos against PFKFB3 and found oligo 2 to be effective (Fig. 2B). Silencing of PFKFB3 with oligo 2 in the PTEN KO cells resulted in a dramatic decrease in the F2,6P2 concentration so that F2,6P2 levels resembled that of wild-type cells (Fig. 2C). In contrast, knockdown of PFKFB3 in the wild-type MEF cells had only a minor effect on the concentration of F2,6P2 (Fig. 2C). To further substantiate these observations, we performed a complementary experiment wherein human PFKFB3 was overexpressed in wild-type MEF cells. Following overexpression, the levels of PFKFB3 protein in wild-type cells rose to the endogenous levels in the PTEN KO cells (Fig. 2D, lower panel), and this effectively abolished the difference in F2,6P2 concentrations between the wild-type and knock-out conditions (Fig. 2D, upper panel). These data validate the reported regulation of F2,6P2 by PFKFB3 and suggest that the abundance of PFKFB3 protein in PTEN KO cells contributes to their elevated F2,6P2 concentrations. In addition, silencing of PFKFB3 resulted in decreased lactate production, and this effect was more evidently observed in the PTEN KO cells (Fig. 2E).

The enzymatic activity of PFKFB3 is regulated by phosphorylation (24). In view of our hypothesis, we aimed to rule out the involvement of enhanced enzymatic activity of PFKFB3 in PTEN KO cells as opposed to the premise of increased protein abundance. We constructed phospho-mutant and phosphomimic forms of PFKFB3 at sites Ser-461 and Ser-478 and tested their ability to up-regulate F2,6P2 synthesis. Interestingly, the mutants were as capable as wild-type PFKFB3 of up-regulating F2,6P2 (supplemental Fig. S1, B and C), suggesting that phosphorylation of PFKFB3 is unlikely to play a major role in the elevated synthesis of F2,6P2 in PTEN KO cells.

AMP-activated protein kinase, PKC, and PKA have been reported to phosphorylate PFKFB3 at the Ser-461 site (24), whereas the residue Ser-478 is a conserved site for Akt phosphorylation. We therefore treated wild-type and PTEN KO MEF cells with inhibitors of these kinases and examined the levels of F2,6P2 (supplemental Fig. S1C). Blockage of the PI3K/Akt signaling pathway with LY294002 lowered the F2,6P2 concentration in PTEN KO but not in wild-type MEF cells, indicating a PTEN KO-specific effect. However, the degree of inhibition was subject to high variability, and the concentration of F2,6P2 in the PTEN KO cells did not decrease to wild-type levels. Therefore, an elevation in PI3K/Akt signaling activity can only partially account for the high levels of F2,6P2 in PTEN KO cells. In contrast, the inhibition of AMP-activated protein
kinase, mTOR complex I (mTORC1), PKC, and PKA did not result in PTEN KO-specific decreases in F2,6P2 concentrations (supplemental Fig. S1).

PFKFB3 Degradation via the APC/C-Cdh1 Ligase Is Impaired in PTEN KO Cells—According to the proposed hypothesis, up-regulation of PFKFB3 in PTEN KO cells may be attributed to decreased PFKFB3 protein degradation via the APC/C-Cdh1 ligase. Hence, we first confirmed the previous work by Song et al. (14) reporting the impaired function of APC/C-Cdh1 in PTEN-deficient cells. To this end, PTEN KO and wild-type cells were treated with cycloheximide, a protein synthesis inhibitor, and the half-life of two known APC/C ligase substrates, Geminin and PLK-1, was followed in a time course lysis experiment. These substrates were chosen because they are specifically recognized by the substrate receptor Cdh1 and not by Cdc20. Analysis by Western blotting showed that both Geminin and PLK-1 appear to be degraded at a slower rate in the PTEN KO cells (Fig. 3A), confirming that the function of the APC/C-Cdh1 ligase is impaired in PTEN-deficient cells.

Next, as proof of principle, we examined the relationship of PTEN, PFKFB3, and Cdh1 at the level of protein interaction. As observed in Fig. 3B, immunoprecipitation of Cdh1 resulted in specific pulldown of co-transfected PFKFB3, suggesting that PFKFB3 and Cdh1 interact directly and supporting the notion of PFKFB3 as an APC/C-Cdh1 substrate. Importantly, co-overexpression of PTEN was observed to increase the binding of PFKFB3 to Cdh1 (Fig. 3B).

To test more directly the physiological role of PTEN in enhancing the degradation of PFKFB3 by the APC/C-Cdh1 ligase, HEK293 cells were treated with PTEN dsiRNA or negative control dsiRNA and then transfected with wild-type PFKFB3 or a KEN box mutant form of PFKFB3 (KEN to AAA). The KEN box is a protein motif recognized by the APC/C-Cdh1 ligase for ubiquitination of its substrates (25). In cells treated with negative control dsiRNA, co-overexpression of Cdh1 was capable of decreasing the protein levels of PFKFB3 WT but had no effect on the KEN box mutant (Fig. 3C). This is in agreement with the reported role of the APC/C-Cdh1 ligase in mediating PFKFB3 degradation (16, 17). In contrast, Cdh1 overexpression failed to affect the levels of PFKFB3 WT in cells where PTEN had been knocked down (Fig. 3C), supporting the premise that PTEN is required for the efficient degradation of PFKFB3 by the

FIGURE 2. Abundance of PFKFB3 in PTEN KO cells contributes to elevated F2,6P2. A, steady-state PFKFB3 protein abundance in PTEN KO and wild-type MEF cells was analyzed by Western blotting and quantified by densitometry. Data are mean ± S.E. of four experiments. PFKFB3 protein level in PTEN KO MEF is expressed as fold change when compared with wild-type MEF cells. B and D (lower panel), Western blotting analysis with PFKFB3 and β-actin antibodies of cells transfected with negative control dsiRNA, PFKFB3 dsiRNA oligo 1, or PFKFB3 dsiRNA oligo 2 (B) or with human PFKFB3-FLAG pcDNA3.1 or pcDNA3.1 empty vector (D, upper panel). C and D (upper panel), F2,6P2 concentrations were assayed in wild-type and PTEN KO MEF cells transfected with negative control dsiRNA or PFKFB3 dsiRNA oligo 2 (C) or with human PFKFB3-FLAG pcDNA3.1 or pcDNA3.1 empty vector (D, upper panel). Data are mean ± S.E. of three experiments, respectively. E, cells were treated as in C and assayed for lactate following 72 h of incubation in 10% FBS medium. Results are normalized to protein concentration. Data are mean ± S.E. of three experiments. ** denotes p < 0.01, * denotes p < 0.05, NS denotes nonsignificant, p > 0.05.
APC/C-Cdh1 ligase. Although the overall lower protein levels of PFKFB3 in the PTEN-silenced cells seem contradictory, we found that this is due to lower transfection efficiency in these cells when compared with those treated with control dsiRNA (supplemental Fig. S1D).

Subsequently, we addressed whether the protein stability of endogenous PFKFB3 is affected in PTEN KO cells when compared with wild-type cells. We thus treated cells with cycloheximide and monitored the protein level of PFKFB3 over time. In wild-type MEF cells, PFKFB3 was practically undetectable after 10 h of treatment. In contrast, PFKFB3 remained relatively constant in the PTEN KO cells throughout the 10 h (Fig. 3D), suggesting that the degradation of PFKFB3 is compromised in cells lacking PTEN.

In virtue of the known cell cycle-dependent regulation of PFKFB3 by the APC/C-Cdh1 ligase (17, 26, 27), we deemed it relevant to investigate any possible cell cycle-specific differences in PFKFB3 stabilization between the PTEN KO and wild-type cells. Cells were synchronized and released at the G2/M boundary with nocodazole. Cdh1 protein levels were observed to drop progressively, denoting transition through mid G1-phase, whereas appearance of the APC/C-Cdh1 substrate Geminin at the later time points indicated entry into S-phase (Fig. 3E). In the wild-type cells, there was a nice correlation between Cdh1 protein level drop and rise of PFKFB3 protein, as has been previously reported (25). On the other hand, PFKFB3 levels remained fairly stable in the PTEN KO cells and did not appear to be affected by the fluctuation of Cdh1 (Fig. 3E). Taken together, all these results provide evidence to support the hypothesis that PFKFB3 degradation via the APC/C-Cdh1 ligase is impaired in PTEN-deficient cells.

**PFKFB3 Contributes to the High Proliferative Rate of PTEN KO Cells**—We showed that PFKFB3 down-regulation in PTEN-silenced cells translates into decreased cellular lactate production, thus suggesting a role for F2,6P2 as a mediator of aerobic glycolysis. One of the proposed advantages that the Warburg effect confers to cancer cells is the ability to sustain high proliferative rates (2). Accordingly, we questioned
F2,6P₂ Contributes to Warburg Effect in PTEN KO Cells

FIGURE 4. PFKFB3 contributes to the high proliferative rate of PTEN KO cells. A and B, PTEN KO and wild-type MEF cells were transfected with PFKFB3 dsRNA and negative control dsRNA, and their growth was monitored with a Nomarski microscope (A) or quantified by Trypan blue cell counting (B). Data are mean ± S.E. of two experiments.

whether metabolic reprogramming of PTEN KO cells via PFKFB3-mediated synthesis of F2,6P₂ may contribute to the high proliferative capacity of these cells. To address this, we silenced PFKFB3 in both wild-type and PTEN KO MEF cells and assessed their proliferative capacity over a 3-day period. Monitoring of the cells by microscopy showed that PTEN KO cells transfected with negative control dsRNA proliferated significantly faster than equally treated wild-type cells (Fig. 4A), an observation that has been reported extensively in the literature for wild-type and PTEN KO cells (28). Remarkably, transfection of cells with PFKFB3-targeting dsRNA caused a dramatic decrease in cell proliferation, particularly in the PTEN KO cells (Fig. 4A). These results were confirmed by Trypan blue exclusion counting of similarly treated PTEN KO and wild-type cells at days 0, 1, and 2 (Fig. 4B).

The fact that cell proliferation was more evidently affected in the PTEN KO cells is consistent with our previous results regarding the up-regulated levels of PFKFB3 in these cells. Furthermore, these data suggest that the contribution of F2,6P₂ to the glycolytic phenotype of PTEN KO cells has a significant impact on cell proliferation, conforming to prevalent ideas about the physiological relevance of the Warburg effect.

DISCUSSION

PTEN deficiency is known to result in a glycolytic phenotype and an increased cellular proliferation rate. In this study, we demonstrate that elevated F2,6P₂ concentrations are important in mediating the increased rates of glycolysis and proliferation in PTEN KO MEF cells. Furthermore, we show that the phosphofructokinase-2 isoform PFKFB3 is responsible for the F2,6P₂ increase in PTEN-deficient cells. Finally, we provide mechanistic evidence that PFKFB3 protein stabilization as a result of impaired APC/C-Cdh1-dependent degradation is a major contributing factor to the increase in F2,6P₂ concentration. These findings are of great significance given that elevations in glycolytic flux play an important role in tumorigenesis and PTEN is one of the most frequently mutated tumor suppressor genes in cancer.

Enhanced glycolytic rates in PTEN-deficient cells have been previously attributed to increased PI3K/Akt-dependent signaling and activation of the mTORC1. In their study, Tandon et al. (29) proposed the mTORC1 substrate ribosomal protein S6 kinase as a major activator of glucose metabolism. However, they showed that silencing of S6 kinase affected glycolytic rate and viability in both control and PTEN knockdown cells. In contrast, we provide evidence that regulation of glycolysis and cell proliferation via PFKFB3 is largely specific for PTEN-deficient cells. We also observed that treatment of cells with a PI3K inhibitor partially reversed the F2,6P₂ increase in PTEN knock-out cells, whereas inhibition of other candidate protein kinases was without significant effect. This is consistent with studies that implicate the PI3K/Akt signaling pathway in the regulation of cellular F2,6P₂ synthesis (11, 30). Notwithstanding, a lipid phosphatase-deficient mutant of PTEN was able to reduce the F2,6P₂ concentration and glycolytic rate in PTEN-deficient cells to a similar degree as wild-type PTEN. This strongly argues for a role of PTEN in regulating cellular F2,6P₂ synthesis via a mechanism that is independent of PI3K/Akt signaling.

Here, we show that elevated F2,6P₂ concentrations in PTEN KO cells are a consequence of PFKFB3 protein stabilization due to impaired APC/C-Cdh1 ligase function. PTEN was recently reported to accelerate the degradation of various APC/C-Cdh1 substrates in a phosphatase-independent manner (14). We confirmed such a role of PTEN upon observing a slower rate of degradation of APC/C-Cdh1 substrates, Geminin and PLK-1, in the PTEN KO MEF cells. Importantly, PFKFB3 was observed to follow a similar pattern of decreased protein degradation in the PTEN-deficient cells. At the level of protein interaction, we showed that PTEN augments the binding between Cdh1 and PFKFB3. In the study by Song et al. (14), PTEN was demonstrated to enhance the association of Cdh1 with the APC ligase complex, resulting in more efficient assembly of the ligase and ubiquitination of protein substrates. Consequently, the increased binding of PFKFB3 to Cdh1 in the presence of PTEN is likely to result from enhanced assembly of the APC/C-Cdh1 complex and concomitant recruitment of PFKFB3 to the functional ligase. Finally, we showed that only in the presence of PTEN can overexpression of Cdh1 effectively down-regulate PFKFB3 protein levels. This is dependent on the presence of the KEN box motif on PFKFB3, consistent with previous studies regarding the importance of the KEN box for Cdh1-mediated degradation of PFKFB3 (16, 17). Systemic overexpression of PTEN in transgenic mice was recently shown to reduce
PKFB3 protein levels (31). This demonstrates that PTEN can regulate PKFB3 protein stability under in vivo conditions. However, our results highlight the up-regulation of PKFB3 and the resultant change in glycolytic flux as a consequence of endogenous PTEN loss, which is commonly observed in cancer (6, 7).

The Warburg effect has been proposed to support the proliferative demands of cancer cells (2). In accordance, we showed that PKFB3 directly contributes to the proliferation rate of ME3 cells, particularly in the absence of PTEN. Tudzarova et al. (27) showed that PKFB3 silencing prevents G1/S transition of the cell cycle by down-regulating glycolytic flux at a nutrient-sensitive restriction point. Interestingly, PTEN KO embryonic stem cells have been reported to have accelerated G1/S transition (28). It would thus be interesting to investigate whether PTEN-deficient cells exploit their elevated PKFB3 protein levels to bypass the G1/S restriction point and accelerate their progression through the cell cycle. Furthermore, the therapeutic potential of targeting PKFB3 and F2,6P2 as metabolic mediators of proliferation in PTEN-deficient cancers is worthy of consideration.

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