Gene Regulation:

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Krüppel-like Factor 4 (KLF4) Activates the Transcription of the Gene for the Platelet Isoform of Phosphofructokinase (PFKP) in Breast Cancer*[S]

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Krüppel-like factor 4 (KLF4) is a transcription factor that plays an important role in cell differentiation, proliferation, and survival, especially in the context of cancers. This study revealed that KLF4 activates glycolytic metabolism in breast cancer cells by up-regulating the platelet isoform of phosphofructokinase (PFKP). KLF4 activated the transcription of the *PFKP* gene by directly binding to the *PFKP* promoter. Whereas glucose uptake and lactate production were inhibited by the knockdown of KLF4, they were activated by the overexpression of KLF4. Unlike *PFKP*, the expressions of the other isoforms of phosphofructokinase and glycolytic genes were unaffected by KLF4. The human breast cancer tissues showed a close correlation between KLF4 and PFKP expression. This study also showed that PFKP plays a critical role in cell proliferation in breast cancer cells. In conclusion, it is suggested that KLF4 plays a role in maintenance of high glycolytic metabolism by transcriptional activation of the *PFKP* gene in breast cancer cells.

Breast cancer is the most common type of cancer in women in Western countries (1). Breast cancer shows elevated glycolytic metabolism, which is one of the common characteristics of the malignant cancers (2). The increase in glycolytic rate is a result of the up-regulation of the metabolic transporters and glycolytic enzymes whose expressions are controlled by the transcriptional regulation of genes as well as post-translational modifications of the enzymes (2, 3).

The conversion of fructose 6-phosphate to fructose 1,6-bisphosphate is the first committed step in the glycolytic pathway catalyzed by phosphofructokinase (PFK-1) (4, 5). PFK-1 is a complex tetrameric enzyme and exists in three isoforms: liver (PFKL), muscle (PFKM), and platelet (PFKP). The activity of PFK-1 is regulated by both quantitative changes and isozymic alterations secondary to altered gene expression during neoplastic transformation *in vivo* and *in vitro* (6, 7). The expression of PFK-1 is up-regulated in cancer cells, where glycolysis is enhanced (7, 8). Although an increase in PFKP expression is a characteristic feature of malignant tissues (8), little is known about how PFKP expression is regulated during the development and progression of cancers or the change of cancer phenotypes.

Krüppel-like factor 4 (KLF4) is a transcriptional factor that modulates the expression of several genes that are involved in cell cycle regulation and differentiation (9, 10). The KLF4 levels rise following DNA damage, cell cycle arrest in response to serum withdrawal, and contact inhibition (10). Elevated KLF4 level has also attributed to certain types of cancers. *KLF4* mRNA and protein are overexpressed in up to 70% of breast cancers (11, 12). The increased nuclear expression of KLF4 is considered to be associated with the aggressiveness of breast cancer phenotypes (12). KLF4 has been found to be overexpressed in oral and skin squamous carcinoma cells as well (13). In addition, KLF4 exhibits potent transforming activity when expressed in cultured RK3E epithelial cells (14). Many studies suggest that KLF4 plays an important role in the development and progression of these tumors (15).

This study investigated the role of KLF4 in glycolytic metabolism and proliferation in breast cancer cells and reached a conclusion that elevated KLF4 level in breast cancer cells contributes to the stimulation of glycolytic metabolism by activating *PFKP* transcription. KLF4 activated transcription of *PFKP* by direct binding to the *PFKP* promoter. The knockdown of KLF4 significantly reduced glucose uptake and lactate production by suppressing PFKP expression. On the other hand, the overexpression of KLF4 induced PFKP expression, resulting in increased glucose uptake and lactate production. Therefore, KLF4 is required to maintain high levels of glycolytic metabolism in these cells. In the analyses of breast cancer tissues, there was a statistical positive correlation between KLF4 and PFKP expression. These results strongly suggest that induction of PFKP by KLF4 plays a critical role in regulating the glycolytic metabolism of breast cancer cells.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2.

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EXPERIMENTAL PROCEDURES

Cell Culture—The human breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in a medium containing 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). The prostate cancer cells PC3 (RPMI1640) (Invitrogen), non-tumorigenic epithelial cells MCF10A, and breast cancer cells MCF7, BT-474 (Dulbecco's modified Eagle's medium), MDA-MB-231 (modified Eagle's medium), and SK-BR-3 (McCoy's medium) were maintained in designated media. All of the media were purchased from Invitrogen.

Tissue Procurement from Patients—The tissue samples of conventional breast cancer tissues were prepared from surgical specimens of 31 patients enrolled in the Department of Surgery, College of Medicine, Yonsei University. The study procedures were approved by the Institutional Review Board of the Yonsei Severance Hospital prior to the initiation of the study. The age of the patients ranged from 33 to 71 with a mean age of 40 and a median of 47 at the time of diagnosis. Fresh tumor biopsies from 31 primary sporadic breast carcinomas were collected during surgery and snap-frozen immediately after the histological examination of frozen sections. Tumor biopsies were frozen in liquid nitrogen until being processed. Total RNA was isolated from the tissues using the TRIzol® reagent (Invitrogen) according to the instructions provided by the manufacturer.

Construction of Recombinant Plasmids—For the generation of constructs that express human PFKL, PFKM, PFKP, and KLF4, a full-length cDNA of each gene was amplified from MCF7 cell cDNA using the following primers: *PFKL*, 5′-GGCCGCGGTGGACCTGGAGAA-3′ and 5′-TCAGAAGCCCTTGTCCATGCTCAGG-3′; *PFKM*, 5′GACCCATGAAGAGCACCATGCAGC-3′ and 5′-TTTAGACGGCAGCTTCCCCGGA-3′; *PFKP*, 5′-GGACGCGGACGACTCCCGGGC-3′ and 5′-GTCAGACACTCCAGGGCTGCACATGTTCC-3′; *KLF4*, 5′-CGACGCGCTGCTCCCATCTT-3′ and 5′-GGCAGTGTGGGTCATATCCA-3′. For the generation of the construct that expresses KLF5, the human *KLF5* cDNA was amplified from PC3 prostate cancer cell cDNA using the following primers: 5′-GGCTACAAGGGTGCTGAGCATG-3′ and 5′-TCAGTTCTGGTGCCTCTTCATATGCA-3′. The PCR-amplified cDNA for each gene was cloned into the SmaI site of the pSG5-HA-tagged expression vector. For the generation of promoter-reporter constructs, 5′-flanking regions of the human *PFKL*, *PFKM*, and *PFKP* genes were amplified by PCR from the genomic DNA prepared from MCF7 cells using the following primers: the *PFKL* promoter from −2035 to +21 (each base was numbered, with the first base of the translated ATG being +1), 5′-GCAGTGAGCTGAGATTGCGCCACTG-3′ and 5′-CTCCAGGTCCACCGCGGCAAT-3′; the *PFKM* promoter from −1993 to +5, 5′-GAGACGGTTCAAGGGAATTACCAGG-3′ and 5′-TGAATGGCTGCCTCCTAGAAATAGAGAC-3′; and the *PFKP* promoter from −2008 to +7, 5′-TTCAGTGTGCCCCACCCTCTCCAC-3′ and 5′-CGTCAATGGCGAGGAGGCCGA-3′. The PCR product was cloned into the SmaI site of the pGL3-basic vector (Promega Corp., Madison, WI). The plasmids generated from the process were named pPFKL-Luc, pPFKM-Luc, and pPFKP-Luc for *PFKL*, *PFKM*, and *PFKP*, respectively. The integrity of constructs was confirmed by DNA sequencing.

Transient Transfections and Analyses of the Promoter Activity—MCF7 cells (2×10^5 cells/well) were seeded in 6-well plates and were transfected with promoter-reporter constructs and an expression vector (500 ng of DNA) using Lipofectamine and the Plus reagent (Invitrogen) according to the manufacturer's instructions. The *Renilla* luciferase vector (pRL-SV40, Promega Corp.) was co-transfected for the standardization of transfection efficiency. Following a 48-h transfection, cells were harvested, and then the luciferase activities in 5 μl of cell lysates were measured using the Dual-Luciferase Reporter Assay System® (Promega Corp.) according to the manufacturer's instructions. The luciferase activity was normalized to the *Renilla* luciferase activity. Data shown are the mean ± S.D. of triplicate samples from a representative experiment.

Quantitative RT-PCR—Total RNA was isolated from cultured cells and tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For the quantitative RT-PCR, cDNA was synthesized from 4 μg of total RNA using random hexamers and SuperScript Reverse Transcriptase II® (Invitrogen) according to the manufacturer's instructions. The synthesized cDNA was then diluted with 80 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), 2 μl of which was subsequently used in RT-PCR. A 10-μl mixture containing the cDNA and a set of gene-specific primers was mixed with 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and then subjected to RT-PCR quantification using the ABI PRISM 7500 real-time PCR system (Applied Biosystems). The sequences of the primers used in the RT-PCR were as follows: *PFKL*, 5′-GGCATTTATGTGGGTGCC AAAGTC-3′ and 5′-CAGTTGGCCTGCTTGATGTTCTCA-3′; *PFKM*, 5′-GAGTGACTTGTTGAGTGACCTCCAGAAA-3′ and 5′-CACAATGTTCAGGTAGCTGGACTTCG-3′; *PFKP*, 5′-CGGAAGTTCCTGGAGCACCTCTC-3′ and 5′-AAGTACACCTTGGCCCCCACGTA-3′; *KLF4*, 5′-ATCTTTCTCCACGTTCGCGTCTG-3′ and 5′-AAGCACTGGGGGAAGTCGCTTC-3′; *KLF5* 5′-GCCCTCCCTGAGTTCACCAGTATATTC-3′ and 5′-GCTGGGTAGGGACAGAAAGATGAAGA-3′; *HK2*, 5′-ATGGATGCCTAGATGACTTCCGCA-3′ and 5′-TAAGTGTTGCAGGATGGCTCGGA-3′; *PFKFB3*, 5′-CCGCTCATGAGACGCAATAGT-3′ and 5′-TTGATGCGAGGCTTTTTGGT-3′; *GAPDH*, 5′-CCCCTTCATTGACCTTCAACTA-3′ and 5′-GAGTCCTTCCACGATACCAAAG-3′. All reactions were done in triplicate, and the relative amounts of all mRNAs were calculated by using the comparative C_T method. *GAPDH* mRNA was used as the invariant control.

Western Blot Analysis—Cells were harvested and lysed in 2× SDS loading buffer and then briefly sonicated. Lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were obtained. The protein concentrations of the supernatants were determined using the Bradford assay (Bio-Rad). Aliquots (30 μg) of proteins were subjected to either 6 or 8% SDS-PAGE and transferred to Protran nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were then blocked in Tris-buffered saline, which contained

0.1% Tween 20 (TBST) and 5% skimmed milk solution for 2 h, followed by an incubation for either 3 h or overnight in the TBST containing the primary antibody. After they were washed with the TBST, the membranes were incubated with the ImmunoPure HRP-conjugated goat anti-rabbit or anti-mouse IgG antibody (Pierce) at room temperature for 20 min. The immunoreactive bands were detected by the SuperSignal West Pico Chemiluminescent System (Pierce). The antibodies used in this study were anti-PFK-1 (catalog no. SC-67028), anti-PFKP (catalog no. SC-130227), and anti-HA tag (catalog no. SC-7392) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-KLF4 (catalog no. ab56542) and anti-KLF5 (catalog no. ab24331) from Abcam (Cambridge, UK); and anti-tubulin (catalog no. No.CP-06) from Calbiochem (Brookfield, WI).

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were carried out using the ChIP assay kit (Upstate, Temecula, CA) according to the manufacturer's instructions. Briefly, cells were fixed in formaldehyde for 15 min at room temperature. Cells were lysed in the lysis buffer containing protease inhibitors, and nuclei were isolated. The chromatin was then sheared to an average length of 250–500 bp by sonicating suspended nuclear pellets. The shortened chromatin was precleared with salmon sperm DNA/protein A-Sepharose beads and then immunoprecipitated overnight at 4 °C with an antibody against KLF4. Rabbit IgG control was used to monitor nonspecific interactions. Immune complexes/protein A-Sepharose beads were washed extensively with the immunoprecipitation buffer, and immune complexes were eluted with the elusion buffer. After ethanol precipitation, the DNA was resuspended in water, and then PCR was performed to amplify the KLF4 binding regions of the *PFKP* gene using the following primers: KLF4 region 1, 5′-GACCTGAACATATTTCCATGT-CAGTCGAC-3′ and 5′-CAAAGTCAGAGAGCGCTGCTCT-CAT-3′; KLF4 region 2, 5′-TCCCATAAGCCCGTGCTTC-TATG-3′ and 5′-TCCAGGTGGTGGGAAAAGGTGA-3′; negative control region, 5′-TCACTCACTCCATTCATTC-AGCAGCA-3′ and 5′-TCCTCTGCTGACTTGGGAGTC-ACA-3′.

Overexpression or Knockdown of KLF4 and PFKP—For stable overexpression of the *KLF4* gene, the fragment encoding the full-length cDNA of *KLF4* from the pSG5-KLF4 construct was cloned into the SmaI-XhoI sites of pLL-CMV-puro lentiviral vector. The shRNA technology platform (Sigma-Aldrich MISSION® shRNA) was used to stably knock down gene expression. For the knockdown of KLF4 and PFKP, MISSION® shRNA lentiviral plasmids contained a U6 promoter transcribing non-target shRNA (SHC002), KLF4 shRNAs (shKLF4 number 1, TRCN0000005314; shKLF4 number 2, TRCN0000005315; shKLF4 number 3, TRCN0000005316), or PFKP shRNA (shPFKP, TRCN0000199816) from Sigma-Aldrich. Plasmid DNAs were transfected into HEK-293T cells along with lentiviral packaging mix consisting of an envelope and packaging vector to produce lentivirus packed with *KLF4* cDNA, KLF4 shRNA, or PFKP shRNA cassettes using the manufacturer's instructions. Positive cells harboring *KLF4* cDNA, KLF4 shRNA, or shPFKP cassette were selected by 0.2 μg/ml puromycin (P8833, Sigma-Aldrich) selection after infection of breast cancer cells.

Cell Proliferation Analysis—Cells (2×10^4 cells/well) were seeded in 96-well plates. Cells were allowed to grow for 24–72 h. The number of cells was measured every 24 h using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (catalog no. G3580, Promega Corp.). Briefly, 20 μl of the CellTiter 96® AQ_{ueous} One Solution reagent was added to each well of the 96-well assay plate containing the samples in a 100-μl culture medium. The plate was incubated at 37 °C for 90 min in a humidified, 5% CO_2 atmosphere. Following the incubation, the 96-well assay plate was read at 490 nm using a 96-well plate reader (VERSA max tunable microplate reader, Molecular Devices, Inc.). Data shown are the mean ± S.D. of triplicate samples from a representative experiment.

Glucose Uptake and Lactate Production Assay—For a glucose uptake assay, cells (2×10^5 cells/well) were plated on 6-well plates and treated as indicated in the figure legend (Figs. 2, 4, and 7). Cells were then incubated in a glucose-free DMEM or RPMI1640 medium (Invitrogen) for 6 h. After adding 0.3 MBq (10 μCi) of [^{18}F]fluoro-2-deoxyglucose (16), the cells were incubated at 37 °C for 20 min. The cells were washed twice with PBS and then imaged for 5 min on a PET scanner (Allegro; Philips-ADAC Medical Systems, Cleveland, OH). The images were reconstructed using an interactive reconstruction algorithm (16). Upon completion of the PET imaging, cells were harvested in PBS for radioactivity measurement using the Wallac 148 Wizard 3 γ-counter (PerkinElmer Life Sciences). Data shown are the mean ± S.D. of triplicate samples from a representative experiment. The lactate levels in a culture medium were measured using a lactate assay kit according to the manufacturer's instructions (Biovision, Mountain View, CA).

Microarray Analysis—Each total RNA (200 ng) from KLF4 knockdown or control MCF7 cells was labeled and amplified using the Low Input Quick Amp labeling kit (Agilent Technologies, Santa Clara, CA). The Cy3-labeled aRNAs were resuspended in 50 μl of hybridization solution (Agilent technologies). After labeled aRNAs were placed on Agilent SurePrint G3 Human GE 8 × 60K array (Agilent technologies) and covered by a Gasket 8-plex slide (Agilent Technologies). The slides were hybridized for 17 h at 65 °C. The hybridized slides were washed in 2× SSC, 0.1% SDS for 2 min, 1× SSC for 3 min, and then 0.2× SSC for 2 min at room temperature. The slides were centrifuged at 3000 rpm for 20 s to dry. The arrays were analyzed using an Agilent scanner with associated software. Gene expression levels were calculated with Feature Extraction version 10.7.3.1 (Agilent Technologies). Relative signal intensities for each gene were generated using the robust multiarray average algorithm. The data were processed based on median polish normalization method using GeneSpring GX 7.3.1 (Agilent Technologies). This normalization method aims to make the distribution of intensities for each array in a set of arrays the same. The normalized and log-transformed intensity values were then analyzed using GeneSpring GX 7.3.1 (Agilent Technologies). -Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and less than 50% of controls for down-regulated genes. Hierarchical clustering data were clustered groups that

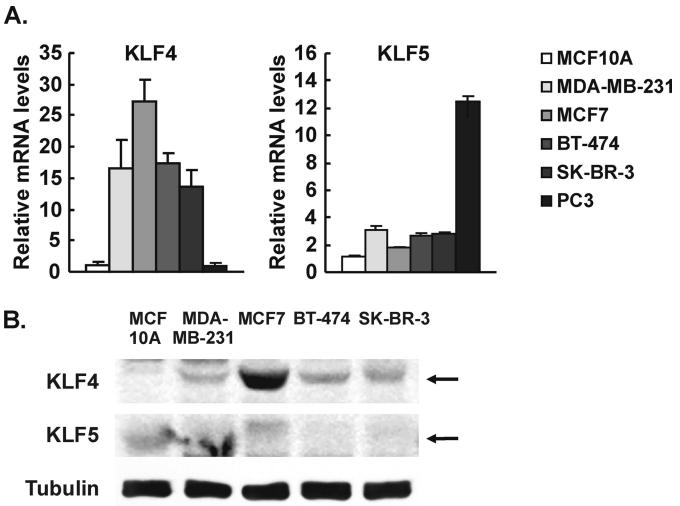

FIGURE 1. **The KLF4 was overexpressed in breast cancer cell lines.** *A*, quantitative RT-PCR analysis was performed to measure the level of mRNAs for *KLF4* and *KLF5* in MCF10A cells, breast cancer cell lines, and prostate cancer cells. Relative amounts of mRNAs are represented as values relative to the level of *GAPDH* mRNA as an invariant control. *B*, the protein levels of KLF4 and KLF5 were measured by Western blot analysis in cell extracts of MCF10A cells and breast cancer cell lines using the antibodies against KLF4 and KLF5. Tubulin was used as an invariant control. *Error bars*, S.D.

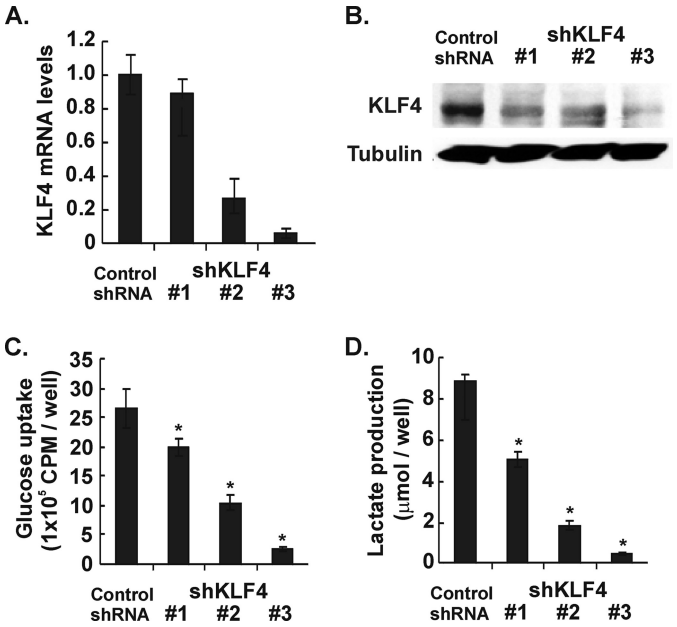

FIGURE 2. **Knockdown of KLF4 suppresses the glycolytic activity in MCF7 cells.** Quantitative RT-PCR analysis (*A*) and Western blot analysis (*B*) were performed to measure the level of mRNAs and protein of KLF4 in MCF7 cells transduced with lentiviruses expressing non-target shRNA or three different shKLF4s (#*1*, #*2*, and #*3*). GAPDH or tubulin expression was used as an invariant control. Glucose uptake (*C*) and lactate production (*D*) were measured in MCF7 cells transduced with lentiviruses expressing non-target shRNA or three different shKLF4s (#*1*, #*2*, and #*3*). Data shown are the mean ± S.D. (*error bars*) of triplicate samples from a representative experiment. *, $p < 0.01$.

behave similarly across experiments using GeneSpring GX 7.3.1 (Agilent Technologies). The clustering algorithm was Euclidean distance, average linkage.

Statistical Analysis—A χ^2 test or Fisher's exact test was used for statistical analyses as appropriates. All statistical tests were two-sided, and p values of less than 0.05 were considered statistically significant. SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

RESULTS

The KLF4 Was Overexpressed in Breast Cancer Cells—In order to confirm the differential expression of KLF4 depending on cancer cell types, the endogenous KLF4 levels were analyzed in breast cancer cell lines (MDA-MB-231, MCF7, BT-474, and SK-BR-3) and prostate cancer PC3 cells in comparison with non-tumorigenic epithelial cells (MCF10A) (Fig. 1). The mRNA levels of *KLF4* were 15–20-fold higher in breast cancer cells than MCF10A cells. As previously reported by other studies (28), the amount of *KLF4* mRNA was significantly down-regulated in prostate cancer PC3 cells. Meanwhile, the mRNA levels of *KLF5*, another KLF family member, were slightly increased in breast cancer cells compared with MCF10A cells. In contrast, the level of *KLF5* was significantly up-regulated in PC3 cells (Fig. 1*A*). The KLF4 protein was exclusively expressed in four breast cancer cell lines compared with MCF10A cells. The protein levels of KLF4 were the highest in MCF7 cells among all four breast cancer cells mentioned above. In contrast, the KLF5 protein was not expressed in four breast cancer cell lines where KLF4 was expressed, while it was expressed in MCF10A cells (Fig. 1*B*). These results indicated that the expression of KLF4 was up-regulated in breast cancer cells compared with non-tumorigenic epithelial cells. It is suggested that KLF4 could have oncogenic potential in breast cancer cells, where the metabolic pathways are reprogrammed to meet their requirement for abnormal proliferation. The focus of this study was placed on the function of highly expressed KLF4 in high glycolytic features of breast cancer cells.

Knockdown of KLF4 Suppresses the Glycolytic Activity and the Expression of PFKP—In order to assess the contribution of KLF4 to the control of glycolysis in breast cancer cells, lentiviruses carrying the three different KLF4 shRNAs were used to knock down KLF4 in MCF7 cells. Three KLF4 shRNAs (#*1*, #*2*, and #*3*) showed different efficacies to knock down *KLF4* mRNA and protein (Fig. 2, *A* and *B*). The glycolytic activity was measured by quantifying the uptake of [^{18}F]fluoro-2-deoxyglucose and lactate production to evaluate the metabolic effects of KLF4 knockdown in MCF7 cells. The uptake of [^{18}F]fluoro-2-deoxyglucose, which is stored as 2-deoxyglucose 6-phosphate in cells, was significantly suppressed over a period of 20 min by knocking down KLF4 compared with control cells (Fig. 2*C*). Lactate production was also significantly suppressed by the knockdown of KLF4 (Fig. 2*D*). The suppression of glucose uptake and lactate production was almost proportionate to the magnitude of KLF4 knockdown by shRNAs. KLF4 shRNA 3 was chosen for future studies based on its potency to decrease the glucose uptake and lactate production as well as *KLF4* mRNA levels. These results strongly suggest that KLF4 is involved in the control of glycolysis in MCF7 cells. To examine which enzymes in the glycolytic pathway are affected at the transcriptional level by KLF4, microarray analysis was performed with RNAs isolated from KLF4 knockdown or control MCF7 cells (supplemental Table S1). The expression of the *PFKP* gene was

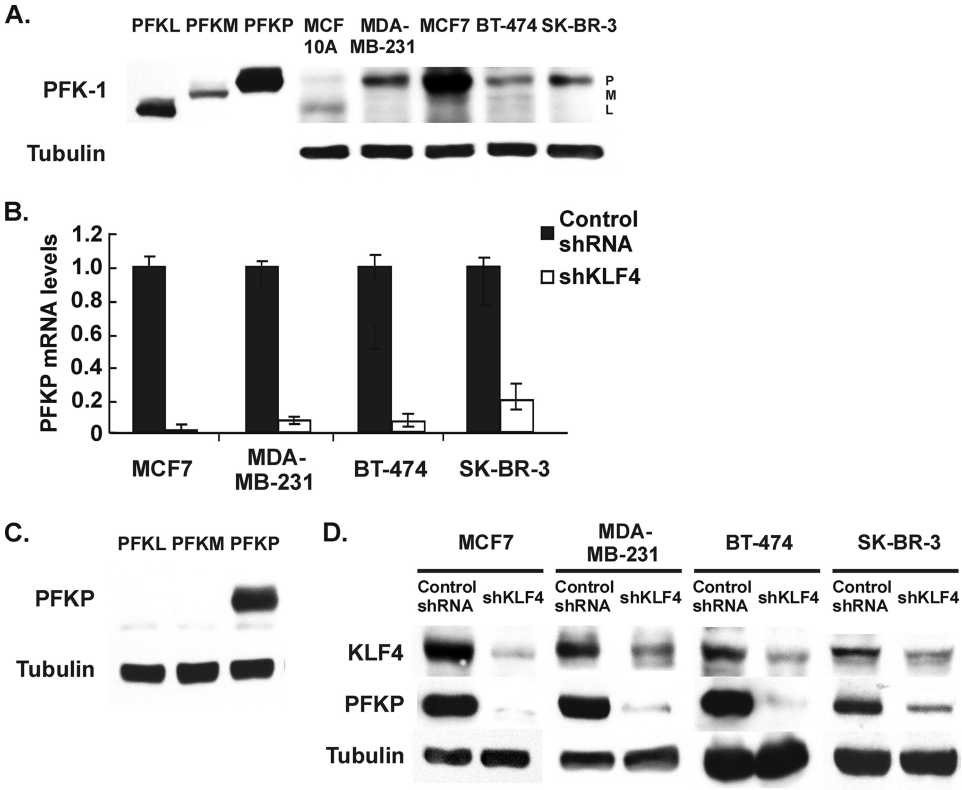

FIGURE 3. **Knockdown of KLF4 suppresses the expression of PFKP in breast cancer cells.** *A*, the levels of PFK-1 isoforms were measured by Western blot analysis in protein extracts of MCF10A cells and breast cancer cell lines as indicated in the figure, using anti-PFK-1 antibody, which can bind all three PFK-1 isoforms. We measured the size of each PFK-1 isoform in HEK-293 cells transfected with the pSG5-PFKL, pSG5-PFKM, or pSG5-PFKP expression constructs (*lanes 1–3*). *B*, quantitative RT-PCR analysis was performed to measure the level of mRNAs for *PFKP* in breast cancer cell lines (MCF7, MDA-MB-231, BT-474, and SK-BR-3) transduced with lentiviruses expressing non-target shRNA or shKLF4. *C*, Western blot analysis, using an antibody against PFKP, was performed with the extracts from HEK-293 cells transfected with pSG5-PFKL, pSG5-PFKM, and pSG5-PFKP expression vector. Anti-PFKP antibody binds only to PFKP and not to PFKL and PFKM. *D*, Western blot analysis was performed to measure the levels of KLF4 and PFKP in breast cancer cells transduced with lentiviruses expressing non-target shRNA or shKLF4. GAPDH or tubulin expression was used as an invariant control. *Error bars*, S.D.

markedly decreased by ~4-fold by KLF4 knockdown (supplemental Table S1), whereas the expressions of other glycolytic enzymes, such as glucose transporters, hexokinase isoforms, and other phosphofructokinase isoforms, remained unchanged (data not shown). To determine the major isoform of PFK-1 in breast cancer cells, Western blot analyses were performed. The molecular size of each isoform of PFK-1 was discriminated using an antibody that reacts with all three isoforms (Fig. 3*A*) that were overexpressed in HEK293 cells exogenously. PFKP was the major isoform in breast cancer cells, including MCF7, MDA-MB-231, BT-474, and SK-BR-3 cells, whereas PFKL was the major isoform in MCF10A cells (Fig. 3*A*). Consistent with the result in microarray analysis, the *PFKP* mRNA was drastically suppressed by KLF4 knockdown in all breast cancer cell lines (Fig. 3*B*). To assess the amount of PFKP protein, the PFKP isoform-specific antibody was used for Western blot analysis after verification of the specificity using the recombinant PFK-1 isoforms (Fig. 3*C*). The reduction of PFKP protein by KLF4 knockdown was clearly confirmed in four breast cancer cell lines (Fig. 3*D*). These results indicate that PFKP is the primary isoform of PFK-1 expressed in breast cancer cells and is the major target of KLF4 leading to a high glycolytic state in breast cancer cells.

KLF4 Enhances the Glycolytic Activity in Breast Cancer Cells—To confirm the role of KLF4 in glycolytic metabolism, the overexpression of KLF4 was performed using the lentivirus expressing KLF4. First, this experiment was conducted in non-tumorigenic epithelial cells, MCF10A, in which the expression level of KLF4 was extremely low. However, the KLF4 expression in MCF10A induced severe cytostatic effects, and the analysis of metabolic effects could not be performed as a result (data not shown). Next, the KLF4 protein was overexpressed in MCF7 and MDA-MB-231 cells (Fig. 4*A*), where the expression of KLF4 is relatively high. In contrast to the knockdown of KLF4, the overexpression of KLF4 significantly increased glucose uptake and lactate production (Fig. 4, *B* and *C*), although the changes were not marked. Because the basal expression of KLF4 in breast cancer cells was already high, the additional expression of KLF4 was supposed to be relatively less effective than the changes induced by the knockdown of KLF4.

To determine whether or not KLF4 could specifically increase the expression of PFKP, KLF4 was overexpressed in MCF7 and MDA-MB-231 cells, and then the levels of mRNAs for glycolytic enzymes were measured (Fig. 5*A*). The amount of *PFKP* mRNA was increased significantly by the overexpression of KLF4 in both MCF7 and MDA-MB-231 cells, whereas *PFKL* and *PFKM* mRNA levels remained unchanged. Similarly, the mRNA levels of *HK2* and *PFKFB3*, which were known to be highly expressed in cancer cells (2, 17), were not affected by the

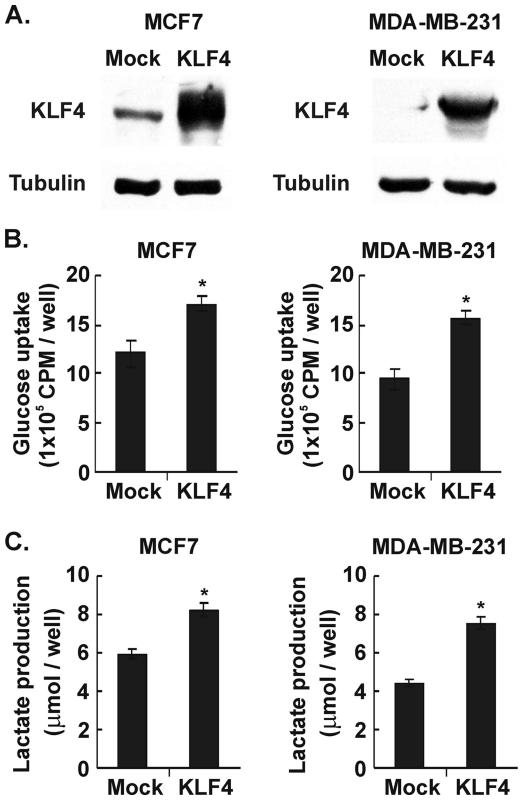

FIGURE 4. **KLF4 enhances the glycolytic activity in breast cancer cells.** *A*, the protein levels of KLF4 were measured by Western blot analysis in the protein extracts of the MCF7 and MDA-MB-231 cells transduced with lentiviruses expressing control or KLF4. Tubulin was used as an invariant control. Glucose uptake (*B*) and lactate production (*C*) were measured in MCF7 and MDA-MB-231 cells transduced with lentiviruses expressing control or KLF4. Data shown are the mean ± S.D. (*error bars*) of triplicate samples from a representative experiment. *, $p < 0.05$.

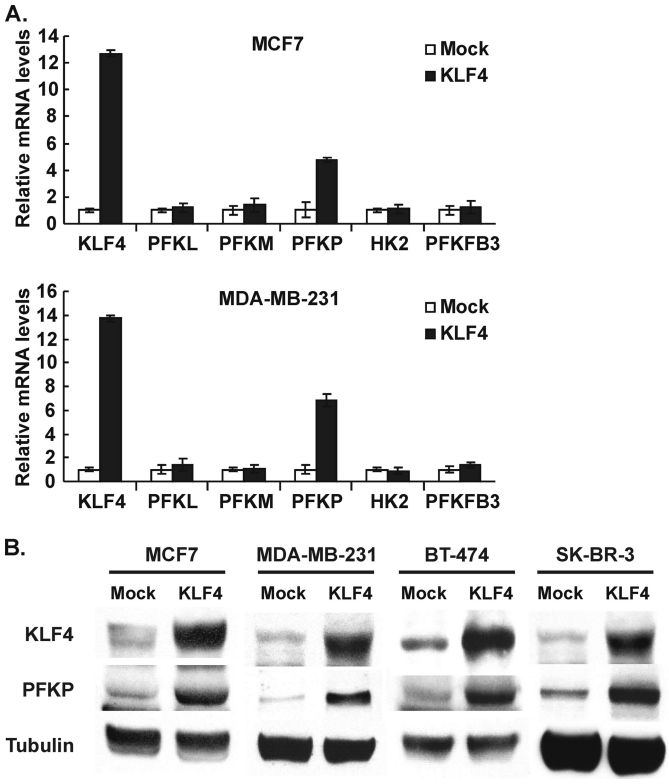

FIGURE 5. **The KLF4 induces the expression of PFKP in breast cancer cells.** *A*, quantitative RT-PCR analysis was performed to measure the level of mRNAs for *KLF4*, *PFKP*, and other glycolytic genes in MCF7 and MDA-MB-231 cells transduced with lentiviruses expressing control or KLF4. The amounts of mRNA were represented as values relative to the levels in the cells transduced with control lentivirus. *B*, the protein levels of KLF4 and PFKP were measured by Western blot analysis in the protein extracts of breast cancer cell lines (MCF7, MDA-MB-231, BT-474, and SK-BR-3) transduced with lentiviruses expressing control or KLF4. Tubulin was used as an invariant control. *Error bars*, S.E.

overexpression of KLF4 (Fig. 5*A*). The induction of PFKP protein levels by KLF4 was checked in MCF7, MDA-MB-231, BT-474, and SK-BR-3 cells. Consistent with mRNA levels, the protein levels of PFKP were increased significantly by KLF4 overexpression in all breast cancer cell lines (Fig. 5*B*). These results suggest that KLF4 induces the expression of PFKP in breast cancer cells.

The effect of KLF4 overexpression on PFKP expression was also studied in human prostate cancer PC3 cells, which were known to have the minimal expression of KLF4. The overexpression of KLF4 in PC3 cells led to an increase in glucose uptake and lactate production to a much greater extent than in MCF7 cells with high KLF4 expression. The PFKP protein was also markedly increased by KLF4 expression (supplemental Fig. S1, *A–C*). These findings indicate that KLF4 increases the expression of PFKP as well as the glycolytic activity in cancer cells.

The Relationship between KLF4 and PFKP Expression in Human Breast Cancer Tissues—To determine the major isoform of PFK-1 in breast cancer cells, the mRNA levels of *PFKL*, *PFKM*, and *PFKP* genes were measured in breast cancer cells and MCF10A cells using RT-PCR (Fig. 6*A*). The breast cancer cells showed the higher levels of mRNAs for all of the *PFK-1* isoforms compared with MCF10A cells. The magnitude of increase in *PFKP* mRNA was especially higher than those of the other isoforms in all cancer cells compared with MCF10A cells.

To assess whether there is correlation between KLF4 and PFKP expressions in primary breast cancers, the mRNA levels of *KLF4* and *PFKP* were compared in 31 human breast cancer tissues. We obtained a statistically positive correlation between these two variables in primary breast cancers (Pearson $R^2 = 0.437$; $p < 0.001$) (Fig. 6*B*).

Knockdown of PFKP Severely Suppresses the Glycolytic Activity and Cell Growth in MCF7 Cells—To determine whether PFKP was required to maintain a high glycolytic activity and proliferation in breast cancer cells, PFKP shRNA was used to knock down PFKP in MCF7 cells. Knockdown of PFKP using lentivirus carrying shRNA led to the disappearance of almost all of the *PFKP* mRNA and protein in MCF7 cells (Fig. 7, *A* and *B*). The knockdown of PFKP significantly decreased the glucose uptake and subsequently inhibited the lactate production (Fig. 7, *C* and *D*). PFKP knockdown also markedly inhibited the cell proliferation (Fig. 7*E*). FACS analyses showed that PFKP knockdown suppressed cell cycle progression by decreasing the numbers of cells in S phase and increasing the numbers of cells in G_1 or G_2/M phases (supplemental Fig. S2, *A–D*). However, apoptosis was not significantly affected by PFKP knockdown, whereas KLF4 knockdown increased the percentage of cells in

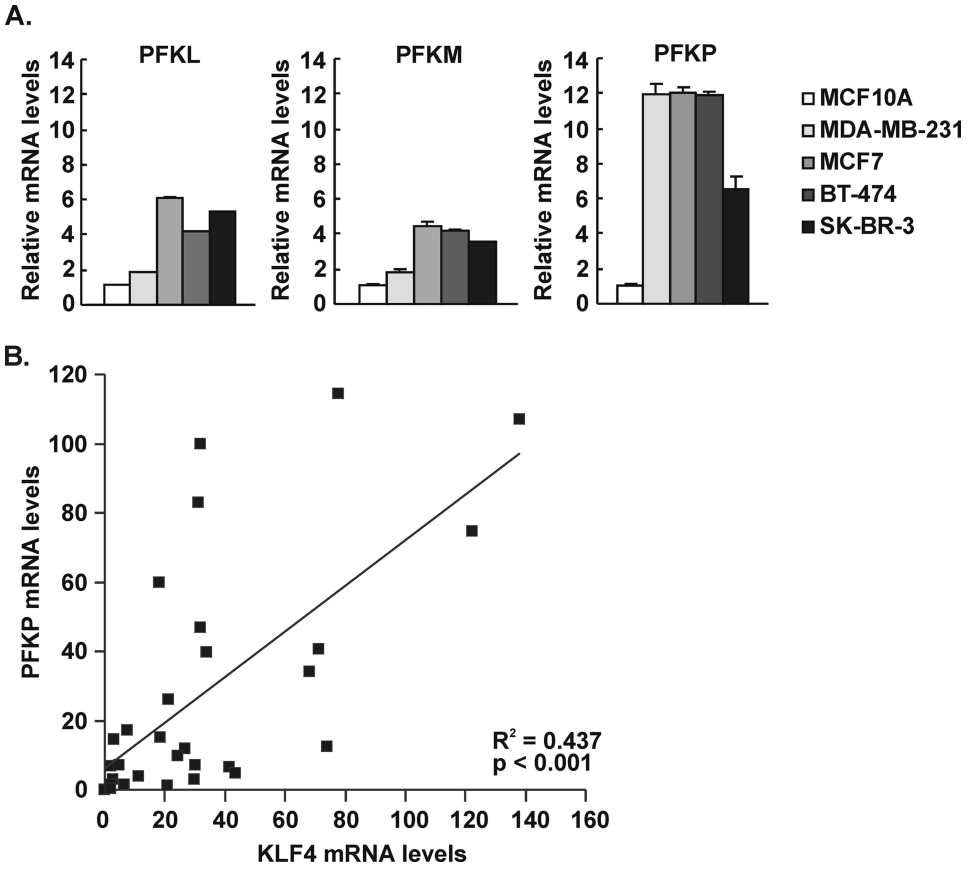

FIGURE 6. **The relationship between KLF4 and PFKP expression in human breast cancer cell lines and primary cancer tissues.** *A*, quantitative RT-PCR analysis was performed to measure the level of mRNAs for *PFKL*, *PFKM*, and *PFKP* in MCF10A cells and four breast cancer cell lines. Relative amounts of mRNAs were represented, using *GAPDH* mRNA as an invariant control. *B*, quantitative RT-PCR analysis was performed to measure the level of mRNAs for *KLF4* and *PFKP* in each breast cancer tissue of 31 cases. The relative amounts of *PFKP* and *KLF4* mRNA in each tissue are expressed by the ratio of each mRNA per *GAPDH* mRNA. The *p* value indicated statistical significance in all of the 31 cases. *Error bars*, S.E.

apoptosis. These results suggest that PFKP plays a critical role in vigorous glycolytic activity in MCF7 cells for their rapid proliferation.

KLF4 Binds to the PFKP Promoter and Transactivates PFKP Expression—To elucidate how KLF4 specifically controls *PFKP* transcription, the promoter activities were assayed using the promoter-reporter constructs containing the sequences ~2 kb upstream of *PFKL*, *PFKM*, or *PFKP* genes in MCF7 cells. KLF4 increased the activity of the *PFKP* promoter by 5-fold, whereas it had no effects on the *PFKL* or *PFKM* promoter activities. The overexpression of KLF5 did not activate the *PFKP* promoter activity (Fig. 8*A*). Within about 1 kb upstream from the ATG start codon of *PFKP*, the GC contents were noticeably high, and more than 20 putative binding sites of KLF4 were detected by the analysis using the MatInspector program of Genomatix software (available from the Genomatix Web site). To assess whether KLF4 actually occupies the *PFKP* promoter, ChIP assays were performed in MCF7 cells. The proximal region from −825 to −1277 was amplified from chromatin immunoprecipitates by anti-KLF4 antibody, but the distal region from −2247 to −1768 was not amplified (Fig. 8*B*). The primer for PCR in more proximal regions could not be designed because of the extremely high GC contents. These results suggest that KLF4 transactivates *PFKP* by directly binding to the proximal promoter region within 1 kb upstream from the ATG start codon.

DISCUSSION

KLF4 is a transcription factor that activates or represses genes involved in cell cycle regulation and differentiation. KLF4 functions as a cytostatic factor by activating the expression of $p21^{WAF1/CIP1}$ (18) and by repressing the genes of cyclin D (19), cyclin B1 (20), and cyclin E (21). In contrast, KLF4 converts the senescence-like cell cycle arrest by RAS^{V12} into oncogenic transformation (22). KLF4 directly suppresses the expression of p53, which plays a critical role in RAS^{V12}-induced senescence. The neutralizing action of activated RAS^{V12} against KLF4 cytostatic action as well as the suppression of RAS^{V12}-induced senescence by KLF4 contributes to oncogenic transformation. KLF4 has known to be one of the four transcription factors (OCT3/4, SOX2, KLF4, and c-MYC) that are essential to conversion of differentiated cells into induced pluripotent stem cells (23). A recent study suggested that KLF4 was a key factor in activating and maintaining the expression of the catalytic subunit of human telomerase in induced pluripotent stem cells, embryonic stem cells, and even several cancer cells (24). It has been reported that KLF4 expression is strongly correlated with cancer progression in head and neck squamous carcinomas (14, 25) and contributes to human telomerase expression in these cancers (24). These mechanism-oriented explanations describe the context-dependent tumor suppressor or oncogenic function of KLF4. The level of KLF4 is extremely low in MCF10A

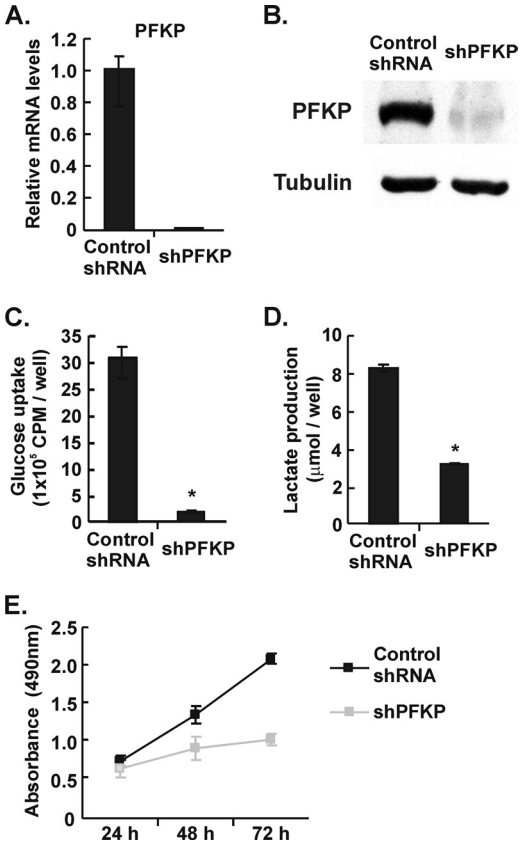

FIGURE 7. **Knockdown of PFKP severely suppresses the glycolytic activity and cell growth in MCF7 cells.** The levels of mRNA (*A*) and protein (*B*) of PFKP were measured in MCF7 cells transduced with lentiviruses expressing non-target shRNA or shPFKP. *GAPDH* or tubulin expression was used as an invariant control. Glucose uptake (*C*), lactate production (*D*), and cell proliferation (*E*) were measured in the cells described above. Data shown are the mean ± S.D. (*error bars*) of triplicate samples from a representative experiment. *, $p < 0.01$.

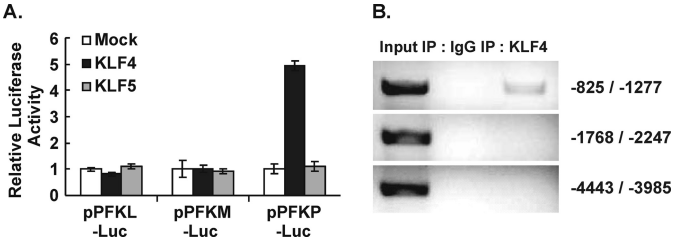

FIGURE 8. **KLF4 binds to PFKP promoter and transactivates PFKP expression.** *A*, the activities of three PFK-1 isoform promoters were assayed in MCF7 cells, exogenously overexpressing KLF4 or KLF5. Cells were transfected with the pPFKL-Luc, pPFKM-Luc, or pPFKP-Luc constructs in combination with the empty vector, pSG5-KLF4, or pSG5-KLF5. The luciferase activities were analyzed 48 h after transfection. Luciferase activities were normalized to the activities of SV40 promoter-driven *Renilla* luciferase. Data shown are the mean ± S.D. of triplicate samples from a representative experiment. *B*, chromatin immunoprecipitation (*IP*) analyses were performed on MCF7 cells using KLF4-specific antibody or nonspecific IgG. The indicated chromosomal regions, denoted as nucleotide numbers from the transcription start site of the *PFKP* gene, were amplified by PCR after precipitation with KLF4-specific antibody or nonspecific IgG and analyzed by 1.2% agarose gel electrophoresis.

cells, which are non-tumorigenic epithelial cells, and the deliberate expression of KLF4 strongly inhibits cell proliferation and survival (data not shown). In contrast, the majority of breast cancers express a large amount of KLF4 (11), and the preferential nuclear localization of KLF4 is known to be a poor prognostic marker showing the early stage infiltrating phenotype (12). The depletion of KLF4 from breast cancer cells reportedly restores p53 levels and causes p53-dependent apoptosis (22). These findings imply that the oncogenic action of KLF4 involves other factors that neutralize the cytostatic activity of KLF4.

In this study, we showed another mechanism of oncogenic potential of KLF4 to activate the glycolytic pathway in breast cancer cells. The knockdown of KLF4 in breast cancer cells markedly suppresses the glucose utilization, which is caused by the suppression of PFKP expression. Glucose transporter 1 (GLUT1) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3/4 (PFKFB3/4) have been reported to be a cause of the high glycolytic phenotype of breast cancers (17, 26, 27), but this study shows that PFKP plays a crucial role in glycolytic activities and cell proliferation in breast cancer cells. The microarray analysis indicates that *PFKP* is a unique gene under the control of KLF4 among the glycolytic enzyme genes. PFK-1, constituted by PFKM, is inhibited by such allosteric inhibitors as citrate and ATP, whereas PFK-1 containing PFKL and PFKP isotypes is less sensitive to the inhibitory effect of these allosteric effectors and more sensitive to fructose 2,6-bisphosphate, a potent activator (3). In breast cancer, the up-regulation of PFKP may contribute to maintaining high glycolytic status even at an increased citrate level and sufficient ATP concentration, which are necessary for the anabolic metabolic pathway supporting cell proliferation and growth. PFKP is the most prominent isotype of PFK-1 in breast cancer cells and determines the rate of glycolysis and lactate production. The proximal region of the PFKP promoter is a highly GC-rich region, where multiple putative KLF-binding regions exist. KLF4 specifically activates PFKP expression by direct binding to its proximal promoter, whereas it has no effects on the activities of PFKL and PFKM promoters.

This study shows that KLF4 contributes to the stimulation of glycolytic metabolism in breast cancer cells by activating *PFKP* transcription. These results suggest that KLF4 and PFKP may be sensitive targets in designing selective breast cancer chemotherapy.

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