Upregulation of Cytosolic Phosphoenolpyruvate Carboxykinase Is a Critical Metabolic Event in Melanoma Cells That Repopulate Tumors

Yong Li, Shunqun Luo, Ruihua Ma, Jing Liu, Pingwei Xu, Huafeng Zhang, Ke Tang, Jingwei Ma, Yi Zhang, Xiaoyu Liang, Yanling Sun, Tiantian Ji, Ning Wang, and Bo Huang

Abstract

Although metabolic defects have been investigated extensively in differentiated tumor cells, much less attention has been directed to the metabolic properties of stem-like cells that repopulate tumors [tumor-repopulating cells (TRC)]. Here, we show that melanoma TRCs cultured in three-dimensional soft fibrin gels reprogram glucose metabolism by hijacking the cytosolic enzyme phosphoenolpyruvate carboxykinase (PCK1), a key player in gluconeogenesis. Surprisingly, upregulated PCK1 in TRCs did not mediate gluconeogenesis but promoted glucose side-branch metabolism, including in the serine and glycerol-3-phosphate pathways. Moreover, this retrograde glucose carbon flow strengthened rather than antagonized glycolysis and glucose consumption. Silencing PCK1 or inhibiting its enzymatic activity slowed the growth of TRCs in vitro and impeded tumorigenesis in vivo. Overall, our work unveiled metabolic features of TRCs in melanoma that have implications for targeting a unique aspect of this disease. Cancer Res; 75(7); 1–6. ©2015 AACR.

Introduction

Stem cell–like cancer cells (SCLCC) are a self-renewing, highly tumorigenic subpopulation of cancer cells, playing crucial roles in initiation, promotion, and progression of tumorigenesis. In vivo imaging of unperturbed tumors has visualized SCLCCs in mouse and zebrafish models (1, 2). Further experimental evidences demonstrate the existence of SCLCCs in murine brain, skin, and intestinal tumors (3–5). These tumorigenic SCLCCs are of considerable clinical importance, because they are resistant to cytotoxic therapies and most likely responsible for treatment failure and cancer recurrence. Meanwhile, a poor prognosis in cancer patients has been reported to link to SCLCCs (6), explaining the importance of targeting SCLCCs in tumor treatment. To achieve the goal of better targeting SCLCCs, their intrinsic, especially metabolic features, need to be better elucidated and explained. This is because that reprogrammed energy metabolism is fundamental for cancer cell growth, survival, differentiation, and migration (7). Currently, the identification of SCLCCs through conventional methods that depend on cell surface markers often lacks specificity and is thus unreliable (8). Recently, we developed a mechanical method to select and grow SCLCCs from the general population of tumor cells by culturing single tumor cells in three-dimensional (3D) soft fibrin gels, and found that as few as 10 selected cells are sufficient to grow tumors in immunocompetent mice (9). We thus functionally define these soft fibrin gel–selected cells as tumor-repopulating cells (TRC; refs. 9, 10). Using this method to generate TRCs, here we identify that cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), also known as PCK1, is a novel metabolic feature of melanoma TRCs.

Materials and Methods

Mice and cell lines

Female C57BL/6 mice, 6–to 8-week-old, were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College (Wuhan, China). Murine cell lines B16 (melanoma), H22 (hepatocarcinoma), and EL4 (lymphoma) were purchased from China Center for Type Culture Collection. Murine embryonic stem cell (mESC) line (W4) was used as described before (11). Murine mesenchymal stem cells (mMSC) were isolated and cultured from mouse bone marrow as described previously (12).

Three-dimensional soft fibrin gel preparation

Salmon fibrinogen and thrombin were purchased from Searle Holdings. Three-dimensional soft fibrin gels were prepared as described previously (9).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Y. Li and S. Luo contributed equally to this article.

Corresponding Author: Bo Huang, Chinese Academy of Medical Sciences, 5 DongDan SanTao, Beijing 100730, China. Phone: 86-10-6522-9258; Fax: 86-10-6522-9258; E-mail: tjhuangbo@hotmail.com

doi: 10.1158/0008-5472.CAN-14-2615
©2015 American Association for Cancer Research.

www.aacrjournals.org

Published OnlineFirst February 24, 2015; DOI: 10.1158/0008-5472.CAN-14-2615
RT-PCR and real-time PCR

Total RNA extracted from tissues and cell lines with TRIzol reagent (Invitrogen) were used for RT-PCR and real-time PCR analysis. Total RNA (1 µg) was reverse transcribed into cDNA using the Reverse Transcription System (Promega). Real-time PCR was performed with a FastStart Universal SYBR Green Master Kit (Roche) on an ABI 7900 System. mRNA levels were normalized to GAPDH. The sequences for all the primers were provided in Supplementary Table S1.

RNA interference

TRCs were harvested from fibrin gel by digestion with dispase II (1 mg/mL, Roche) and seeded onto dishes precoated with fibrin gels. After attachment, cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. After 6 hours, the transfected cells were harvested and seeded back to 3D fibrin gels. siRNAs for three genes PCK1 (5’-GCCGCAACAUUGUAUGUCAUG-3’ or 5’-CGCCGCAACAUUGUAUGUCAUG-3’), G9a (5’-GGUGACUUCAGAUGGUGGCC-3’), and SUV39h1 (5’-GGUGACUUCAGAUGGUGGCC-3’) and the corresponding scramble control oligonucleotides were purchased from RiboBio. The silence efficiency was confirmed by real-time PCR (Supplementary Fig. S1A and S1B).

Recombinant plasmids

PCK1-overexpressing vectors were constructed by inserting murine PCK1 cDNA into pcDNA3.1 plasmids. To construct PCK1 promoter-controlled EGFP-expressing vectors, 1.2-kb mouse gene fragments containing the PCK1 promoter sequence were, instead of the original CMV promoter, inserted into upstream of the EGFP cDNA in pEGFP-C1. Lipofectamine 2000 (Invitrogen) was used to transfer the plasmids into cells. In some cases, 800 µg/mL G418 was used to select stably expressing clones. All the constructs were identified here (Supplementary Fig. S1C and S1D).

Intracellular serine and glycine measurement

The intracellular i-serine and glycine were measured by high performance liquid chromatography analysis, which was performed as described previously with some modifications (13).

Statistical analysis

Results were expressed as mean values ± SEM and interpreted by repeated-measure ANOVA. Differences were considered to be statistically significant when the P value was <0.05. Other detailed experimental procedures are described in Supplementary Materials and Methods.

Results and Discussion

Hepatocytes abundantly express phosphoenolpyruvate carboxykinase cytosolic forms (PCK1), to produce glucose via the gluconeogenic pathway. However, malignant hepatocytes lose gluconeogenesis by striking downregulation of PCK1 in favor of tumor glycolysis (14). Stem cell–like cancer cells have been shown to rely on glycolysis for glucose metabolism (15, 16), which prompted us to speculate a further downregulation of PCK1 in TRCs. Surprisingly, hepatic TRCs were found to upregulate PCK1 expression when compared with control H22 cells cultured in rigid plate (Fig. 1A and B). In addition to H22 tumor cells, B16 melanoma cells and EL4 lymphoma cells were examined and both B16 and EL4 TRCs upregulated PCK1 (Fig. 1A and B). CD133 is commonly used to mark SCLCC including melanoma (17). We found that PCK1 expression was much higher in CD133+ B16 cells, compared with CD133− B16 cells (Supplementary Fig. S2A). PCK1 was also found to be highly expressed in mouse embryonic stem cells (mESC) as well as mouse mesenchymal stem cells (mMSC; Fig. 1A), indicating that the upregulation of PCK1 expression might be a unique feature of undifferentiated cells. In addition, using PCK1 promoter-EGFP constructs further confirmed the expression of PCK1 in cultured B16 TRC spheroids.

Figure 1.
The expression of PCK1 is upregulated in TRCs. A, the expression of PCK1 in H22 hepatocarcinoma, B16 melanoma, and EL4 lymphoma TRCs as well as mESCs and mMSCs was analyzed by RT-PCR. Tumor cells cultured in 2D rigid dish were used as control. Data shown are representative of three independent experiments. B, the expression of PCK1 in H22, B16, and EL4 TRCs was analyzed by real-time PCR. Tumor cells cultured in 2D rigid dish were used as control. Results represent means ± SEM from three independent experiments; *P < 0.05; **P < 0.001. C, PCK1 promoter-EGFP-expressing B16 cells were cultured in 3D soft fibrin gels for 5 days to form TRC spheroids and the fluorescence of spheroids was measured. Then, these cultured TRCs were seeded in conventional rigid dish for further culture. The fluorescence was measured at different time points. Scale bar, 20 µm. Data shown are representative of three independent experiments. D, the expression of PCK1 was analyzed by Western blot analysis. Tumor cells cultured in 2D rigid dish were used as control. Data shown are representative of three independent experiments.
PCK1 is a Metabolic Feature of TRCs

Given that PCK1 is the key enzyme in catalyzing the rate-limiting step in gluconeogenesis, a further test was conducted to see whether tumorigenic cells used PCK1 to carry out gluconeogenesis. Fructose-1,6-bisphosphatase (FBP1) and glucose-6-phosphatase (G6pase), two enzymes to convert fructose 1,6-bisphosphate to fructose and 6-phosphate glucose-6-phosphate to glucose, respectively, were not expressed by those TRC cells (Supplementary Fig. S3A), suggesting that PCK1 cannot be used for gluconeogenesis in TRCs. Cell growth and proliferation are fed through glucose metabolism. Here, the rates of glucose consumption were found to be much higher in B16 TRCs, compared with the corresponding bulk tumor cells (Fig. 2A, left). However, knockdown of PCK1 expression by siRNA resulted in the decrease of glucose consumption in B16 TRCs (Fig. 2A, right), suggesting that PCK1 promote the use of glucose in TRCs. Carbon flow from glucose to lactate facilitates glucose consumption. Here, B16 TRCs exhibited higher lactate release rates compared with control, and the knockdown of PCK1 decreased their release of lactate (Fig. 2B). Although PCK1 could not mediate gluconeogenesis in TRCs, whether PCK1 induced a retrograde carbon flow in TRC cells was unclear. Here, we tested the biosynthesis of serine, considering its derivation from glyceral-3-phosphate. We found that the knockdown of PCK1 resulted in slight decrease of serine levels in B16 TRCs (Fig. 2C, left). However, glycine, the metabolite of serine, was significantly decreased after PCK1 knockdown (Fig. 2C, right). In parallel, we found the levels of G-3-P, a production of glyceroneogenesis, were increased in TRCs, compared with bulk B16 cells (Fig. 2D, left). Similarly, knockdown of PCK1 resulted in the decrease of G-3-P in TRCs (Fig. 2D, right). Together, these data suggest that the expression of PCK1 enhances the carbon flow from glucose to lactate as well as the flow toward glycerol and serine in TRCs.

Tumor cells consistently express glucose transporter 1 (GLUT1) that initiate glucose metabolism. Here, we additionally analyzed the expression of GLUT1, and found that GLUT1 was decently expressed in TRCs (Supplementary Fig. S3B). PCK1 knockdown did not affect its mRNA expression and cellular membrane localization (Supplementary Fig. S3C). Besides, other enzymes involving glycolysis, Krebs cycle, and lipid metabolism, including hexokinase 2, phosphofructokinase, pyruvate kinase isozyme type M2, lactate dehydrogenase, pyruvate dehydrogenase alpha, citrate synthase, succinate dehydrogenase, ATP citrate-lyase, and fatty acid synthase were analyzed. However, we did not find the differential expression between TRCs and control tumor cells (Supplementary Fig. S4). In addition, knockdown of PCK1 seemed not to affect the expression of those enzymes (Supplementary Fig. S4). Thus, PCK1 enhancing glucose carbon flow in TRCs seems not to be attributable to increasing glucose taking up or regulating enzyme expression.

To further investigate the role of the above unexpected upregulation of PCK1 in TRCs, we treated the 3D soft fibrin gel–cultured TRCs with 3-mercaptopurinolic acid (3-MPA), an inhibitor of PCK1, to inhibit the enzymatic activity of PCK1. We found that the inhibition of PCK1 enzymatic activity resulted in decreases in the size and number of spheroids of TRCs (Supplementary Fig. S5A). Moreover, we harvested the second generation of B16 TRCs by culturing the first generation of TRCs in another 3D soft fibrin gel to generate purer TRCs, and transfected them with PCK1 siRNA. Consistently, the growth of B16 TRCs in the soft gels was significantly inhibited by PCK1 knockdown (Fig. 3A). We did not observe that PCK1 knockdown affected B16 TRC apoptosis (Supplementary Fig. S5B). Interestingly, when we added glycine and G-3-P to PCK1 siRNA-transfected TRC culture system, respectively, we found...
The impaired growth of TRCs could be partially rescued (Supplementary Fig. S5C). On the other hand, when we forcedly overexpressed PCK1 (PCK1-OE) in TRCs, the increased growth was found in soft 3D fibrin gels, compared with the control TRCs (Fig. 3B). These data together suggest that PCK1 plays an important role in promoting tumorigenic TRC growth.

To further dissect the role of PCK1 in TRCs, we cultured B16 TRCs in two-dimensional (2D) rigid dishes to induce TRC differentiation. Consistent with the previous result of Fig. 1C, the expression of PCK1 was gradually downregulated along time (Supplementary Fig. S6A). Given tumorigenic cells with a small number that can repopulate the whole tumor in vivo, we speculated that targeting tumorigenic cells might cause shrinkage of tumor cell population in culture. When culturing bulk tumor cells in 2D rigid dish in the presence or absence of PCK1 inhibitor 3-MPA, we found that although 3-MPA under given concentration did not affect tumor cell proliferation, the number of bulk tumor cells was indeed significantly decreased along the culture time (Supplementary Fig. S6B and S6C), implying a potential critical role of PCK1 in B16 tumorigenic cells. Moreover, when we put the same number of 3-MPA–treated tumor cells back to 3D soft fibrin gels, the spheroid formation was significantly increased, relative to the control (Fig. 3C). This unexpected result might be due to the inhibition of PCK1 activity that also inhibited TRC differentiation, resulting in more TRC enrichment in the population. In addition, we constructed PCK1 siRNA-expressing vectors. When B16 cells were transfected with these plasmids, tumor cell clone formation was not observed in the presence of selection drugs.

However, control siRNA-transfected tumor cells easily formed clones, further supporting that PCK1 is required for maintaining tumorigenic cells. To validate the above data in vivo, PCK1 siRNA-transfected B16 TRCs (5 × 10^5) were intravenously or subcutaneously injected to mice. Three weeks later, most control siRNA-transfected TRCs formed tumor nodules in the lungs and grew much larger tumors in the skin (Fig. 3D). In contrast, PCK1 siRNA-transfected TRCs did not form lung tumor nodules and grew much smaller tumors in skin (Fig. 3D). Together, these data suggest that PCK1 confers B16 TRCs the ability to repopulate a tumor in vivo.

Finally, we investigated how PCK1 expression was regulated in TRCs in 3D soft fibrin gels. B16 tumor cells use αvβ3 integrin to sense extracellular mechanical force in 3D fibrin gels (9). Here, we found that blockade of αvβ3 integrin with the inhibitor downregulated PCK1 expression in TRCs in a dose-dependent manner; however, blockade of β1 integrin did not affect PCK1 expression (Fig. 4A). αvβ3 integrin engagement may trigger integrin-linked kinase/extracellular signal-regulated kinase (ERK) signaling pathways (18). We found that the inhibition of the ERK1/2 pathway by U0126 did not downregulate the expression of PCK1 (Fig. 4B). However, blockade of P13K decreased the expression of PCK1 (Fig. 4B). Intriguingly, inhibition of AKT, the classical downstream signal molecule of P13K, did not affect the expression of PCK1 (Fig. 4B). Thus, αvβ3 integrin engagement probably activates the non-classical P13K pathway to induce PCK1 expression in 3D soft fibrin gels. DNA methylation is an important event that regulates gene expression (19). The analysis of methylation of
PCK1 promoter region showed high methylated sequences in TRCs and rigid dish-cultured B16 cells and no difference was found between them (Supplementary Fig. S7). Besides DNA methylation, histone methylation, i.e., histone H3 lysine9 (H3K9) methylation is also very important in regulation of gene expression. G9a and SUV39h1 are two methyltransferases that mediate the methylation of H3K9 (20). Knockdown of G9a or SUV39h1 by siRNA resulted in the upregulation of PCK1 expression, we additionally blocked PI3K pathway and detected the expression of G9a and SUV39h1. We found that PI3K inhibitor did not affect the expression of G9a, but caused the upregulation of SUV39h1 expression (Fig. 4D), suggesting a potential link between the PI3K signaling pathway and H3K9 methylation. On the basis of the above data, we proposed that in 3D soft fibrin gels, TRCs use αvβ3 integrin to transduce extracellular mechanical signaling, leading to activating PI3K and subsequently influencing histone methylation, so to regulate the expression of PCK1.

In summary, in the current study, we use biomechanical method to select and amplify melanoma tumorigenic cells (TRCs) to study their metabolic features. Our data show that the key gluconeogenic enzyme cytosolic phosphoenolpyruvate carboxykinase, by virtue of its differential expression in B16 TRCs, not only promotes TRC glycolysis but also mediates the retrograde carbon flow to serine and G-3-P, thus conferring TRCs with the ability to repopulate a tumor. We describe this novel metabolic pathway in a schematic (Supplementary Fig. S8). All in all, these findings disclose that upregulation of PCK1 is a critical metabolic feature of tumorigenic TRCs, thus providing a potential target for melanoma treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Luo, R. Ma, J. Liu, N. Wang, B. Huang
Development of methodology: S. Luo, J. Liu, J. Ma, B. Huang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Li, S. Luo, R. Ma, J. Liu, P. Xu, H. Zhang, K. Tang, X. Liang, Y. Sun, T. Ji, B. Huang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Luo, B. Huang
Writing, review, and/or revision of the manuscript: S. Luo, N. Wang, B. Huang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Luo, R. Ma, J. Liu, B. Huang
Study supervision: S. Luo, J. Liu, B. Huang

Grant Support
This work was supported by the National Basic Research Program of China (2014CB542100, 2012CB932500), the National Science Fund for Distinguished Young Scholars of China (61225021), the National Natural Science Foundation of China (81472653, 81472735), and the Special Fund of Health Public Welfare Profession of China (201302018).

Received September 9, 2014; revised January 21, 2015; accepted January 22, 2015; published OnlineFirst February 24, 2015.

References
Upregulation of Cytosolic Phosphoenolpyruvate Carboxykinase Is a Critical Metabolic Event in Melanoma Cells That Repopulate Tumors

Yong Li, Shunqun Luo, Ruihu Ma, et al.

Cancer Res  Published OnlineFirst February 24, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-2615

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2015/02/25/0008-5472.CAN-14-2615.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.