PKLR promotes colorectal cancer liver colonization through induction of glutathione synthesis

Alexander Nguyen,1 Jia Min Loo,1 Rohit Mital,1 Ethan M. Weinberg,1 Fung Ying Man,1 Zhaoshi Zeng,2 Philip B. Paty,2 Leonard Saltz,2 Yelena Y. Janjigian,3 Elisa de Stanchina,4 and Sohail F. Tavazoie1

1Laboratory of Systems Cancer Biology, The Rockefeller University, New York, New York, USA. 2Department of Surgery, 3Department of Medicine, and 4Antitumor Assessment Core, Memorial-Sloan Kettering Cancer Center (MSKCC), New York, New York, USA.

Colorectal cancer metastasis to the liver is a major cause of cancer-related death; however, the genes and pathways that govern this metastasis event remain poorly characterized. Here, using a large-scale in vivo RNAi screen, we identified liver and red blood cell pyruvate kinase (PKLR) as a driver of metastatic liver colonization. PKLR expression was increased in liver metastases as well as in primary colorectal tumors of patients with metastatic disease. Evaluation of a murine liver colonization model revealed that PKLR promotes cell survival in the tumor core during conditions of high cell density and oxygen deprivation by increasing glutathione, the primary endogenous antioxidant. PKLR negatively regulated the glycolytic activity of PKM2, the major pyruvate kinase isoenzyme known to regulate cellular glutathione levels.

Glutathione is critical for metastasis, and we determined that the rate-limiting enzyme of glutathione synthesis, GCLC, becomes overexpressed in patient liver metastases, promotes cell survival under hypoxic and cell-dense conditions, and mediates metastatic liver colonization. RNAi-mediated inhibition of glutathione synthesis impaired survival of multiple colon cancer cell lines, and pharmacological targeting of this metabolic pathway reduced colonization in a primary patient-derived xenograft model. Our findings highlight the impact of metabolic reprogramming within the niche as metastases progress and suggest clinical potential for targeting this pathway in colorectal cancer.

Introduction

Colorectal cancer is the third leading cause of cancer death in the United States (1). While surgical resection of localized disease can be curative, mortality is primarily caused by metastatic progression, with the liver being the primary site of relapse. Only 10 percent of patients diagnosed with liver metastatic colorectal cancer live beyond 5 years (2, 3). This poor long-term survival outlook highlights the limitations of standard cytotoxic therapies used, such as 5-fluorouracil and oxaliplatin. While recent nuanced changes to therapeutic regimens and the addition of targeted therapies have modestly improved the management of metastatic disease (3), there is a great need for therapies with significant efficacy against liver metastatic colonization and progression.

To clinically impact colorectal cancer, the process of liver colonization must be better characterized (4–6). Metastatic colonization, the formation of micrometastases and subsequent macrometastases, has been observed to be a significant rate-limiting step during metastatic liver growth (7). This selective pressure may be in part attributable to the liver microenvironment, which is marked by hypoxic regions, a unique circulatory architecture, and significant metabolic activity (8). The genes and pathways most suitable for therapeutic inhibition would be those whose expression is necessary for colonization events. Furthermore, it remains unclear whether cancer cells use different mechanisms for early or late colonization events (9). Late colonization events, which contribute to the continued progression of macrometastases, represent a stage with significant clinical need, given that patients who develop liver metastases exhibit a poor prognosis and that approximately 20 percent of patients present with distant metastatic disease at the time of diagnosis (1).

The identification of key regulators necessitates a systematic, functional approach to characterize the colonization process. Large-scale in vivo RNAi screens have enabled the unbiased characterization of pathological and developmental processes (10). Furthermore, the application of these screens under pathological conditions, as well as under conditions of normal growth, has allowed for the identification of genes with specificity to the disease process being studied. Here, we used a large-scale RNAi drop-out screen (54,591 total hairpins targeting 14,095 human genes) to identify candidate genes required for colonization of the liver by multiple colon cancer cell lines. Our interest in identifying key regulators and pathways led us to focus on kinases as important cell signaling molecules. We found that liver and red blood cell pyruvate kinase (PKLR) promotes colon cancer cell metastatic colonization of the liver, while not promoting basal growth in culture. Analysis of PKLR expression in patient samples revealed a significant association with metastatic disease in multiple data sets. PKLR promotes cell survival in the tumor core, a phenotype that can be recapitulated in vitro under conditions of high cell density and hypoxia. Under these conditions, PKLR is required to maintain levels of the major endogenous antioxidant, glutathione, and support cancer cell survival. PKLR negatively regulates the glycolytic activity of the predominant pyruvate kinase expressed in cancer, PKM2, which is known to

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Analysis of shRNA profiling in tumors and cultured cells revealed significant depletion of shRNAs across all cell lines (Figure 1B and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI83587DS1). For the majority of samples, liver tumor profiles displayed high similarity, despite differing parental cell line origins (Figure 1C). To identify broadly relevant metastasis regulators, genes were considered as putative promoters of liver colonization if at least two shRNAs targeting a given gene were depleted in tumors derived from all 3 cell lines and in both independent transduction replicates. This analysis yielded 556 candidate promoters of liver colonization (Supplemental Figure 1B). Using similar criteria, 719 genes appeared to be required for survival in cultured cells, while 187 genes appeared to be required in both in vivo and in vitro conditions (Supplemental Figure 1, C and D). To confirm that the analyzed shRNAs were effective in suppressing colonization, a secondary library was generated using top-scoring shRNAs that targeted the 556 candidate genes. This library was transduced into a highly metastatic in vivo–selected colon cell line, LS-LVM3b (13). These populations were then subjected to the experimental procedures performed for the primary screen. Analysis revealed that, of these shRNAs, 89% were markedly depleted from tumors (Figure 1D).

Given the off-target limitations of RNAi (14), we applied additional scoring metrics to select candidate genes most suitable for thorough validation. To better rank the candidate hits, we scored all genes from the primary genome-scale liver colonization screen by applying the established RIGER algorithm, which accounts for variability in number of shRNAs for each gene (15). These scores were used to generate gene percentile scores, providing a continuous scale to assess the relative importance of genes. As expected, the top 556 genes regulate glycolytic flux in order to generate reducing power required for antioxidant responses (11). Suppressing glutathione synthesis via RNAi or small-molecule inhibition suppressed metastatic colonization, suggesting potential for clinical utility. Our findings reveal the use of multiple pyruvate kinase isozymes by colon cancer for enhanced regulatory control of glycolysis and antioxidant generation. Furthermore, the microenvironment conditions that render colon cancer cells vulnerable to cell death highlight the metabolically demanding conditions that exist during liver colonization and reveal potential for therapeutic targeting of these pathways.

**Results**

Large-scale in vivo shRNA screen identifies promoters of liver colonization. To study colorectal cancer colonization in the liver molecularly, an in vivo shRNA screen (12) was performed to identify regulatory genes (Figure 1A). Three human colorectal cancer cell lines, representing common mutational subtypes (Kras wild-type/mutant, Braf wild-type/mutant, MSS/MSI) seen in patients, were transduced with subpools of shRNA-encoding lentiviral particles totaling 54,591 total hairpins targeting 14,095 human genes. These cell populations were then inoculated into a total of 204 mice by direct liver injection for the selection of cells capable of colonizing the liver. In parallel, cells split from these same cell populations were grown in culture to select for cells capable of basal growth and survival. Once tumors developed, processing of genomic shRNA inserts from liver tumors, cultured cells, and the original parental population was performed to allow for quantification of shRNA genomic sequence depletion. Loss of shRNA representation from tumors would suggest that depletion of the genes targeted suppressed the ability of colon cancer cells to colonize the liver.

Analysis of shRNA profiling in tumors and cultured cells revealed significant depletion of shRNAs across all cell lines (Figure 1B and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI83587DS1). For the majority of samples, liver tumor profiles displayed high similarity, despite differing parental cell line origins (Figure 1C). To identify broadly relevant metastasis regulators, genes were considered as putative promoters of liver colonization if at least two shRNAs targeting a given gene were depleted in tumors derived from all 3 cell lines and in both independent transduction replicates. This analysis yielded 556 candidate promoters of liver colonization (Supplemental Figure 1B). Using similar criteria, 719 genes appeared to be required for survival in cultured cells, while 187 genes appeared to be required in both in vivo and in vitro conditions (Supplemental Figure 1, C and D). To confirm that the analyzed shRNAs were effective in suppressing colonization, a secondary library was generated using top-scoring shRNAs that targeted the 556 candidate genes. This library was transduced into a highly metastatic in vivo–selected colon cell line, LS-LVM3b (13). These populations were then subjected to the experimental procedures performed for the primary screen. Analysis revealed that, of these shRNAs, 89% were markedly depleted from tumors (Figure 1D).
candidate genes were markedly enriched among the top percentile scores, providing confidence in the candidate liver colonization gene list (Supplemental Figure 1E). Interestingly, creatine kinase, brain-type (CKB), which we previously identified as a promoter of the initiation phase of liver colonization (13), scored as a promoter by the RIGER algorithm (78th percentile) in this independent screen, despite being targeted by only 2 hairpins in the primary screen.

**PKLR promotes liver colonization but not tumor growth**. Our interest in uncovering critical regulatory mechanisms led us to focus on kinases. We assessed the gene percentile scores of kinases from the 556 candidate promoters. The top-scoring kinases included many known regulators of colorectal cancer progression, including *IGF1R* (16) and *DDR2* (17), which displayed high gene percentile scores in the screen performed in cultured cells, consistent with their known roles in vitro proliferation (Figure 2A).

Surprisingly, one of the top-scoring genes identified was *PKLR*, which had been commonly believed to be expressed only in liver, kidney, and red blood cells (18). Interestingly, *PKLR* displayed a low gene percentile score under culture conditions, suggesting a selective requirement for this gene during liver colonization.

To confirm these findings, cell lines were generated with additional independent shRNAs not previously used in the aforementioned screens that provided adequate knockdown (13% and 17% relative to control, Supplemental Figure 2A). In vitro proliferation assays confirmed that PKLR depletion did not reduce cell viability in culture (Figure 2B). PKLR depletion also did not reduce subcutaneous tumor growth (Figure 2C). Importantly, in concordance with the in vivo screen results, PKLR-depleted cells were
PKLR expression is associated with metastatic disease in patients with colorectal cancer. In mammalian cells, pyruvate kinase is encoded by 4 isozymes: M1, M2, liver (PKL), and red blood cell (PKR). While the M1, PKL, and PKR isozymes are described to exhibit tissue-specific expression, the pyruvate kinase M2 isoform is highly expressed across cancer types (19). PKL and PKR have only been described to promote glycolysis in their respective tissues, catalyzing the final rate-limiting step, which involves the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, generating pyruvate and ATP. Although PKLR had originally been described to be solely expressed in liver, kidney, and red blood cells (20), PKL expression has also been observed in epithelial cells in the intestinal epithelium of rats (21, 22). Analysis of the Human Protein Atlas data (23) revealed PKL mRNA expression in normal colon tissue and PKLR protein expression in normal colonic glandular tissue and colorectal cancer tumors (http://www.proteinatlas.org; Supplemental Figure 3). To determine whether PKLR expression is associated with liver metastasis, we obtained patient samples at MSKCC and compared PKLR transcript levels in primary tumors to levels in liver metastases. In 70 samples, PKLR mRNA was significantly upregulated in liver metastases relative to primary tumors (Figure 3A). This upregulation was also observed in multiple independent data sets of unmatched (Figure 3B and C) and matched (Figure 3D and E) samples from patients with colorectal cancer (24–27). These samples were from a total of 311 patients. Additionally, in primary tumors, PKLR expression was associated with the presence of metastatic disease (Figure 3F and G) as well as the development of liver metastases (Figure 3H; refs. 28–30). These tumors totaled 284 in sum. Taken together, PKLR expression in human samples supports a positive role for PKLR in liver metastatic progression in agreement with our experimental data.

PKLR promotes cell survival in the metastatic tumor core. To characterize the metastatic defect, PKLR-depleted cells were assessed for cell death in vivo. To assess cell death, an in vivo caspase-3/7–dependent reporter was used to measure apoptotic cell significantly less effective than control cells in colonizing the liver following direct liver inoculation, with many livers remarkably showing an absence of tumor growth (Figure 2D). To validate this colonization defect in the context of metastasis, cells were inoculated via the portal circulation to assess metastatic activity upon hematogenous arrival in the liver. PKLR-depleted cells (expression was knocked down to 9%) relative to that in control SW620; Supplemental Figure 2, B and C) displayed significantly reduced liver metastatic capacity in metastasis assays showing an absence of tumor growth (Figure 2D). To validate this colonization defect in the context of metastasis, cells were inoculated via the portal circulation to assess metastatic activity upon hematogenous arrival in the liver. PKLR-depleted cells (expression was knocked down to 9%) relative to that in control LS-LVM3b, expression was knocked down to 58% and 42% relative to that in control LS-LVM3b, and expression was knocked down to 58% and 42% relative to that in control SW620; Supplemental Figure 2, B and C) displayed significantly reduced liver metastatic capacity in metastasis assays in multiple cell lines (Figure 2, E and F). These results reveal that PKLR promotes metastatic liver colonization of colon cancer cells.

Figure 3. PKLR expression is upregulated in human colorectal cancer liver metastases and is associated with metastatic outcomes in primary tumors. (A) PKLR expression, as measured by quantitative RT-PCR in primary and liver metastasis tumor samples obtained at MSKCC. (B) PKLR expression, as measured by microarray in primary and liver metastasis tumor samples (27). (C) PKLR expression, as measured by microarray in primary and liver metastasis tumor samples (25). (D) PKLR expression, as measured by microarray in matched primary and liver metastasis tumor samples (24, 26). Increased expression is indicated in red, while decreased expression is indicated in gray. (F and G) PKLR expression, as measured by microarray in primary tumor samples of patients with or without metastases present at time of sampling (29, 30). (H) PKLR expression, as measured by microarray in primary tumor samples of patients who were monitored over time for the development of liver metastases (28). Numbers of samples (N) or paired sets (n) are indicated in figure. For box-and-whisker plots, the boundaries of the box are the first and third quartiles, the bands inside are the median, and the ends of the whiskers are the minimum and maximum values. *P < 0.05, **P < 0.01, ***P < 0.001, 1-sided Mann Whitney test (A–C and F–H), or, where appropriate, paired 1-sided t test for matched samples (D and E).
variable angiogenic capacity (33), suggesting that cancer cells farther from the vasculature may be particularly sensitive to nutrient and oxygen deprivation. To identify a phenotypic defect in vitro, PKLR-depleted cells were grown under various conditions individually and were found to exhibit no survival defects under physiologic levels of glucose, hypoxia (1% O2), anoxia (<0.01% O2), high cell density, or low pH (Supplemental Figure 4, A–E). Moreover, PKLR-depleted cells did not exhibit defects in well-established prometastatic phenotypes, including matrigel invasion capacity, anchorage-independent growth, and migration (Supplemental Figure 4, F–H). While individual stressors elicited no phenotypic defect alone, we reasoned that the tumor core features multiple stressors that simultaneously impact cancer cells. To better recapitulate the microenvironment in the tumor core, cells were seeded concurrently at a high cell density and under hypoxia. Under these conditions, PKLR-depleted cells displayed reduced survival — decreased numbers of live cells and concomitant increased numbers of dead cells (Figure 5A). To characterize whether the survival defect occurs continuously or whether nutrient depletion led to a population collapse, cells were assayed daily for viability under high cell density and hypoxia. Cells with reduced PKLR expression began to display a survival defect as early as 1 day under burden during metastatic liver growth. At early as well as during late time points, the apoptosis signal was significantly increased in PKLR-depleted cells in vivo (Figure 4A). Calculation of cumulative apoptotic burden over time revealed a significantly elevated rate of apoptosis (Figure 4B), indicating that PKLR promotes metastatic cell survival continuously. To confirm these findings, livers were resected and processed to allow for visualization of apoptotic markers. Livers bearing PKLR-depleted cells displayed significantly fewer tumor nodules (Figure 4C), confirming our assessments by gross pathology. Interestingly, while tumor nodules generated from control cells appeared to be highly cellular (Figure 4E), the majority of PKLR-depleted tumor nodule cores appeared devoid of cells and were composed of extracellular cleaved caspase-3 (Figure 4, D and E). While the decreased number of total nodules with PKLR depletion suggests a role in early colonization, enhanced apoptosis in nodule cores suggests a pathophysiological requirement for PKLR during later stages of colonization as well.

**PKLR promotes cell survival under concurrent conditions of high cell density and hypoxia.** The tumor core represents a unique microenvironment with limited nutrient supply, distinct stromal composition, and altered cell-to-cell architecture (31). Liver metastases are susceptible to stress-induced apoptosis (32) and demonstrate Figure 4. PKLR promotes metastatic cell survival in the tumor core. (A) 5 x 10^5 SW620 cells were inoculated by portal circulation injection, and apoptotic cell burden in the liver was monitored using DEVD-luciferin bioluminescence relative to live cell bioluminescence over time (n = 5). *P < 0.05, **P < 0.01, 1-sided t test between indicated sample and shControl. (B) Cumulative apoptotic/live cell burden over time was determined by calculating the area under the curve for each mouse. Linear regression lines are shown. ***P < 0.001, ANCOVA testing for difference in slope between indicated sample and shControl. (C) Livers were extracted in the previous experiment after 28 days, and nodules per liver section were quantified (n = 4). The average value from 3 liver sections for each mouse was used. *P < 0.05, 1-sided t test between indicated sample and shControl. (D) For each liver, proportion of the indicated nodule type is shown. ***P < 0.001, 2-sided Fisher’s exact test on total nodule count for each PKLR shRNA compared with shControl. (E) Representative images of nodule types. DAPI (blue) was used to label nuclei, cleaved caspase-3 (red) was used to label apoptosis, and luciferase and human vimentin (green) were used to label cancer cells. Scale bar: 50 μm.
these conditions, indicating that the effect is continuous (Figure 5B). Given the continuous survival defect observed in PKLR-depleted tumors, cells were assessed for apoptosis and necrosis by flow cytometry after 1 day under concurrent high cell density and hypoxia. PKLR-depleted cells exhibited fewer live cells, a greater proportion of early apoptotic cells, and a greater proportion of late apoptotic cells under these conditions (Figure 5, C and D). These results indicate that PKLR functions to enhance cell survival under cell-dense, hypoxic conditions, simulating pathophysiological features pertaining to metastatic tumor growth in the liver.

**PKLR negatively regulates PKM2 pyruvate kinase activity.** Interestingly, the in vitro survival defect of PKLR-depleted cells was observed using cell culture media supplemented with excess pyruvate, which can be readily imported into cells (34), arguing against the canonical glycolytic function of PKLR in these colon cancer cells. Additionally, the M2 isoenzyme of pyruvate kinase has been described to function as the predominant isoform observed in colorectal cancer tissue and is the only isoform encoded by the PKM gene observed to be expressed in the cell lines used (35–37). To determine whether altered pyruvate kinase activity contributes to glycolytic deregulation, glycolytic flux was assessed under conditions of hypoxia and high cell density each are known to affect mitochondrial metabolism (36). Previous studies have demonstrated an advantage conferred by decreasing PKM2 glycolytic activity in cancer cells, resulting in a shift toward alternative pathways that promote biosynthetic processes (39, 40) and intracellular reducing power under oxidizing conditions such as hypoxic stress (11). Given the differing biochemical properties of pyruvate kinase isozymes (38), PKL could function to alter PKM2 pyruvate kinase activity. Indeed, immunoprecipitated PKM2 complexes associated with PKL exhibited reduced pyruvate kinase activity relative to PKM2-only complexes (Figure 6D), which corresponded to decreased glycolytic enzyme complex formation (Supplemental Figure 6A). Conversely, immunoprecipitated PKM2 complexes from PKLR-depleted cells grown in cell-dense, hypoxic conditions exhibited enhanced pyruvate kinase activity relative to complexes from control cells (Figure 6E), consistent with an observed increase in glycolytic enzyme complex formation (Supplemental Figure 6, B and C). Additionally, cell lysates from PKLR-depleted cells displayed an increase in total pyruvate kinase activity (Figure 6, F and G) as well as a decreased PEP-to-pyruvate ratio following incubation under hypoxic, cell-dense conditions (Supplemental Figure 6D). To determine whether altered pyruvate kinase activity contributes to glycolytic deregulation, glycolytic flux was assessed under conditions of hypoxia and high cell density. PKLR depletion resulted in increased glucose consumption as well as lactate excretion (Figure 6, H and I). Our findings reveal that, in colon cancer cells, PKLR expression can be used to negatively regulate PKM2 pyruvate kinase activity and deregulate glycolytic metabolism to allow for cancer progression.

**PKLR increases glutathione levels.** Decreased PKM2 activity can be advantageous for cancer cells, as glycolysis is critical for regulating a metabolic shift toward increased biosynthetic processes and intracellular reducing power (11, 39–41). Additionally, conditions of hypoxia and high cell density each are known to affect cellular redox status (11, 42, 43). To determine whether the survival defect could be due to an impaired antioxidant response, PKLR-depleted cells were assessed for depletion of intracellular glutathione, the main endogenous antioxidant produced in cells, under hypoxic, cell-dense conditions. Indeed, PKLR-depleted cells demonstrated decreased glutathione levels (Figure 7, A and
LS174T cells were subjected to immunoprecipitation using anti-HA beads followed by Western blot analysis. The experiment was conducted at least 3 times. (C) Cell lysates from LS174T cells were subjected to immunoprecipitation using anti-HA beads followed by Western blot analysis. The experiment was conducted at least 3 times. (D) Sequentially immunoprecipitated PKM2 complexes from transfected HEK293T cells (n = 3) were assayed for pyruvate kinase activity. Fusion protein expression was confirmed by Western blotting. The experiment was conducted twice. *P < 0.05, 2-sided t test. (E) Immuno- precipitated PKM2 complexes from LS174T cells (n = 3), subjected to 16 hours under high cell density and hypoxia, were assayed for pyruvate kinase activity. Activity was normalized to immunoprecipitated HA-PKM2, as measured by quantitative Western blotting. The experiment was conducted 3 times. ***P < 0.001, 2-sided t test.

PKM2 expression was confirmed by Western blotting. The experiment was conducted twice. **P < 0.01, 1-sided t test between indicated sample and shControl. (F) Glucose uptake rates and (I) lactate excretion rates were determined from conditioned media of LS174T cells grown in high cell density and hypoxia for 24 hours. Data shown are from 3 biological replicates. *P < 0.05, 1-sided t test between indicated sample and shControl.

B). To demonstrate that PKLR functions through PKM2 to elicit a change in glutathione levels, PKLR-depleted cells were treated with a PKM2 small-molecule inhibitor (ref. 44 and Supplemental Figure 7A). Treatment with the PKM2 small-molecule inhibitor partially restored glutathione levels under hypoxic, cell-dense conditions (Figure 7C). To confirm that glutathione depletion is responsible for the cell survival defect, PKLR-depleted cells incubated in hypoxic and cell-dense conditions were incubated with the antioxidant N-acetyl cysteine (NAC), a prodrug to a glutathione precursor, and glutathione monoethylester, a membrane-permeable GSH analog. Treatment with either of these compounds rescued cell viability and decreased apoptosis in vitro (Figure 7D and Supplemental Figure 7B). Consistent with these in vitro findings, treatment of mice bearing PKLR-depleted cells with NAC partially rescued metastatic liver colonization capacity (Figure 7E), while nearly completely suppressing apoptosis and apoptotic rate in vivo (Figure 7F).

GCLC as therapeutic target for metastatic liver colonization. Given the susceptibility of liver metastases to glutathione depletion, we reasoned that glutathione synthesis might serve as a critical pathway used for metastatic progression. GCLC, the catalytic subunit of glutamate cysteine ligase, is responsible for the rate-limiting reaction of glutathione synthesis (45). Consistent with our experimental observations suggesting a role for glutathione in colorectal cancer progression, GCLC expression levels were significantly higher in human colorectal cancer liver metastases relative to primary tumors (Figure 8, A and B, and Supplemental Figure 8A). Using GCLC-depleted cells (expression was knocked down to 27% and 13% relative to control levels in LS174T cells and to 41% and 26% relative to control levels in SW480 cells; Supplemental Figure 8, B and C), functional studies revealed GCLC to promote metastatic liver colonization (Figure 8, C and E), suppress apoptosis in vivo (Figure 8D), and promote cell survival under hypoxic, cell-dense conditions (Figure 8F and Supplemental Figure 8D), consistent with the observed phenotypes seen upon PKLR depletion. Additionally, GCLC depletion did not significantly alter proliferation under optimal cell culture conditions (Supplemental Figure 8E). These findings suggested that small molecules that block glutathione synthesis may exhibit metastasis-suppressive effects (46). To test this hypothesis, colon cancer cells were injected into the portal circulation of mice that were provided L-buthionine-(S,R)-sulfoximine (BSO), a micromolar inhibitor of glutathione synthesis, in their drinking water. Therapeutic delivery...
The advantageous expression of liver-specific pyruvate kinase in cancer cells, which allows for metastatic growth in the liver, suggests that Paget’s “seed and soil” hypothesis can be explained in part by cancer cell utilization of pathways endogenous to host cell types within the metastatic niche (50). The PKL isozyme, as the sole enzyme for glycolytic pyruvate production of BSO suppressed metastatic colonization of colon cancer cell lines and increased cancer cell apoptosis in vivo (Figure 9, A–D). Importantly, BSO also suppressed metastatic colonization by a patient-derived primary colon cancer graft (Figure 9E). These results highlight the importance of cancer cell glutathione levels for metastatic survival in the liver and identify this metabolic pathway for potential therapeutic targeting.

Discussion
Glycolytic deregulation has proven to be critical for cancer progression. The activity of PKM2, a central regulator of glycolysis, is tightly regulated to balance the need for high-energy compounds and the need for anabolic molecules required for cancer cell growth, proliferation, and antioxidant defense (11, 36, 39, 47, 48). Cancer cells take advantage of this regulation — PKM2 activity can be inhibited through destabilized subunit interactions and resultant loss of homotetramer structure, which is required for maximum enzymatic activity (48). Our findings reveal that colon cancer cells use an additional means of PKM2 regulation, namely expression of an additional isoenzyme, PKL. While PKL has been shown to promote glycolysis in hepatocytes, in which it is the only pyruvate kinase isoenzyme expressed (49), we found that PKL negatively regulates pyruvate kinase activity in colon cancer cells by antagonizing PKM2 and allows for maintenance of the primary endogenous antioxidant glutathione. This enables metastatic cell survival, particularly in the tumor core.

Tumors are inherently cell dense, and liver parenchyma is a highly cellular organ; thus, as oxygen concentration decreases near the tumor core, the core is especially vulnerable to the combination of cell density and hypoxia. Additionally, death in the tumor core could be detrimental to tumors as a whole, since dying cells could recruit immune effectors that would impact progression of remaining cells in the tumor periphery. While these conditions in the core may be relevant to other sites of metastasis or contexts during cancer progression, we observe these conditions as an important selective pressure during colorectal cancer liver colonization.
and regulation of biosynthetic pathways, is essential for hepatocytes. This importance is highlighted by regulation of PKL activity and expression in response to glucagon, insulin, and dietary carbohydrates (49). Since hepatocytes, such as those lining the portal circulation, experience significant oxidative stress as a result of hypoxemic blood supply and xenobiotic metabolism (8), antioxidant supply and regulated glutathione regeneration is critical for hepatocyte survival and function. Previously, we have shown that the initiation of colon cancer metastatic colonies requires colon cancer cells to metabolically convert liver-derived creatine to phosphocreatine for use as an energetic source during low-energy states (13). While early metastatic colonization can be achieved through utilization of organ-specific nutrients, such as creatine and phosphocreatine, cancer cell activation of a metastatic pathway endogenous to the liver niche appears to contribute to continued progression of metastatic colonies.

Colon cancer cells modulate glutathione levels to allow for survival in the liver through expression of PKLR. The importance of glutathione levels is highlighted by further independent enhancement of glutathione levels in liver metastases through an increase in transcript-level expression of GCLC, the rate-limiting reaction in glutathione synthesis. While GCLC function and PKLR function appear to be critical regulatory steps that are broadly used by colon cancer metastases, as suggested by clinical mRNA expression association, other mechanisms may be additionally used to ensure sustained glutathione levels, such as activation of hepatocyte glutathione efflux to allow for the import of glutathione precursors from plasma (51). Additionally, the utility of glutathione is not limited to colon cancer or liver colonization, as glutathione appears critical for other cancer types and stages of progression (11, 52). Nevertheless, sufficient glutathione levels are important for metastatic cell survival and represent a potential target for clinical therapies.

We found that therapeutic inhibition of glutathione synthesis can suppress liver colonization by increasing cancer cell apoptosis, suggesting that targeting this pathway has the potential to induce cell death within metastatic nodules. While our studies showed efficacy at doses that mice tolerated well, the development of more potent inhibitors of GCLC may allow for enhanced efficacy (53) and suitability for clinical use. Given that small-molecule activation of PKM2 is being investigated as a strategy to suppress tumor growth (54), our findings suggest that glycolytic activation...
CO2, and dissociation and dilution of cells before reaching confluency. (EP) bioluminescence and gross pathology are shown. *7 × 105 CLR1 primary cells were provided drinking water containing BSO. Metastatic colonization was measured by liver bioluminescence after 35 days (n = 5). Representative liver bioluminescence and gross pathology are shown. (Figure 9. Targeting of glutathione synthesis as a therapeutic strategy. (A) 5 × 105 LS174T cells were inoculated by portal circulation injection, and mice were provided drinking water containing 20 mM BSO. Metastatic colonization was measured by liver bioluminescence after 21 days (n = 5). Representative liver bioluminescence and gross pathology are shown. *P < 0.05, 1-sided Mann Whitney test between indicated sample and shControl. (B) DEVD-luciferin bioluminescence relative to live cell bioluminescence was measured in livers at day 7 from mice in A (n = 5). *P < 0.05, 1-sided t test. (C) Liver tumor mass was measured in mice in A after 21 days. ***P < 0.001, 1-sided t test. (D) 5 × 105 SW480 cells were inoculated by portal circulation injection, and mice were provided drinking water containing BSO. Metastatic colonization was measured by liver bioluminescence after 35 days (n = 5). Representative liver bioluminescence and gross pathology are shown. *P < 0.05, 1-sided Mann Whitney test between indicated sample and shControl. (E) 7 × 105 CLR1 primary cells were inoculated by portal circulation injection, and mice were provided drinking water containing 20 mM BSO. Liver tumor mass was measured after 28 days (n = 8). Representative gross pathology is shown. Scale bar: 1 cm. ***P < 0.001, 1-sided t test between indicated sample and shControl.

Methods

Additional details are provided in the Supplemental Methods.

Cell culture. LS174T, LS-LVM3b, SW620, WiDr, and SW480 cell lines were propagated as previously described (13) and were obtained from ATCC, except for the in vivo–selected LS-LVM3b line, which was derived in the laboratory. These lines were selected to represent the mutational spectrum most commonly seen in patients (Kras wild-type/mutant, Braf wild-type/mutant, MSS/MSI). Cells in culture were routinely tested for mycoplasma contamination. Standard cell culture media/mutant, Braf wild-type/mutant, MSS/MSI). Cells in culture were routinely tested for mycoplasma contamination. Standard cell culture media.

Stable and transfected cell lines. Virus was generated using the ViraSafe lentiviral packaging system (Cell Biolabs). shRNA plasmids used were obtained from the Sigma-Aldrich TRC library. Indicated shRNAs (Sigma-Aldrich) are as follows: shControl (SHC002), PKLR sh83 (TRCN0000006383), PKLR sh84 (TRCN0000006384), PKLR sh39 (TRCN0000199139), PKLR sh40 (TRCN0000194740), GCLC sh64 (TRCN0000333564), and GCLC sh62 (TRCN0000344862). Because shRNAs displayed varying efficacy depending on the cell line used, multiple shRNAs were tested for target mRNA depletion, and the best shRNAs were used for experiments. To generate overexpression, N-terminal FLAG tag or HA tag was added to cDNA of PKL/PKR or PKM2, respectively, and cloned into pBabe-Puro or pBabe-Hygro expression vector. Transduction was performed with 8 μg/ml polybrene. Transfection of HEK293T cells was performed using Lipofectamine 2000 (Invitrogen).

shRNA screening. 10 subpools of lentiviral particles from the TRC1 human shRNA pooled library, consisting of 71,444 shRNA clones representing 14,523 genes, were transduced into luciferase-expressing LS174T, WiDr, and SW620 colon cancer cells. Two transductions for each cell line were performed as biological replicates at a low titer (MOI <1) to reduce the likelihood of multiple shRNAs in a single cell. 48 hours after transduction, transduced cells were selected with puromycin for 48 hours to remove untransduced cells. After antibiotic selection, the remaining cells were allowed to recover for a week prior to subsequent experiments. A portion of the selected cells was set aside, and genomic DNA was extracted. This was the reference pool of genomic DNA prior to the selective pressure of liver colonization. A second population of cells was used for in vivo experiments, while a third was kept in culture for the duration of the in vivo experiment. For direct liver injections, an average of 7 mice were inoculated with the 800,000 cells from transduced populations, allowing for a 783× depth of coverage. After 3 to 4 weeks, when tumors had developed, as measured by bioluminescence, mice were sacrificed, and tumors were resected. Cells from the various conditions were processed by isolation of genomic DNA using the DNeasy Kit (Qiagen). A total of 5 μg of genomic DNA isolated from tumors from different mice was pooled, and PCR amplification of shRNA inserts was performed using...
10 reaction tubes and pooled. A first amplification was performed using touchdown PCR (Fwd_rd1: TGGGACATCATATGGGCTTAC-CGTAAC; Rev_rd1: AAGGAAAGGATCTGTGTCCCTG). The PCR product from all 10 reactions (~350 bp) was gel purified and pooled. Subsequently, a second round of amplification was performed with Illumina sequencing-specific primers (Fwd_rd2: AATGATACGGC-GACCACCGAGATCTCACTCCTTTCCCTACAGCGCTCTTC-CGATCTGTATTCTTGCTTATATATCTTTGGAAAGGAC, Rev_rd2: CAAGCAGAGGCGACAAGCTGCTCTTCCGATCGTGGA-TGAATTCGCACTTTTGGCTCAGTG). The subsequent PCR product was gel purified (~300 bp) in preparation for high-throughput sequencing on an Illumina HiSeq2000. Once the sequencing results were obtained, the data were filtered to normalize samples by total reads and to remove shRNAs that were not included in all reference samples. 54,591 shRNA clones representing 14,095 genes were used in the final data analysis. For each sample, shRNA ratios to the reference cell line were calculated, and z scores were calculated using peak median absolute deviation (15) to normalize for the global loss of shRNAs under these experimental conditions. Given the effects of off-target RNAi silencing, genes were scored as hits only if at least two shRNAs in each cell line were absent from the final tumor samples in both independent transduction replicates. The secondary library was generated by cloning a top-scoring shRNA from the genome-wide screen into plko.1 and producing a viral library that was subjected to parallel transduction. The primary library was generated by cloning a top-scoring shRNA from the genome-wide screen into plko.1 and producing a viral library that was subjected to parallel transduction. The secondary library was generated by cloning a top-scoring shRNA from the genome-wide screen into plko.1 and producing a viral library that was subjected to parallel transduction.
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**Coimmunoprecipitation and Western blotting.** Cells were lysed using 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, protease inhibitor (Roche), and phosphoSTOP (Roche). Immunoprecipitation was performed using anti-FLAG M2 magnetic beads (Sigma-Aldrich) or anti-HA magnetic beads (Pierce) for 1 hour at 4°C. For FLAG-based immunoprecipitation, beads were washed twice with wash buffer containing 1 M NaCl, followed by a wash with wash buffer containing 50 mM NaCl. For HA-based immunoprecipitation, beads were washed twice with lysis buffer. Elution was performed using either 3× FLAG peptide (Sigma-Aldrich) or HA peptide (Pierce). For immunoprecipitation from transfected HEK293T cells, cells were transfected with plasmids encoding HA-PKM2, FLAG-PKM2, or FLAG-PKL using Lipofectamine 2000 (Invitrogen). 48 hours later, cells were lysed with lysis buffer containing 100 μM fructose 1,6-bisphosphate and incubated with anti-FLAG M2 magnetic beads (Sigma-Aldrich) for 1 hour at 4°C. Beads were washed twice with lysis buffer, and elution was performed using 3× FLAG peptide (Sigma-Aldrich). Eluate was then incubated with anti-HA magnetic beads (Pierce) for 1 hour at 4°C. Beads were washed twice with lysis buffer, and elution was performed using HA peptide (Pierce). For Western blotting, samples were denatured, separated by SDS-PAGE, transferred to PVDF membrane (Pierce or Millipore), blocked, and probed with primary antibody. The following antibodies were used: anti-FLAG (1:1,000; Sigma-Aldrich, clone M2), anti-PKM2 (1:1,000; Cell Signaling, D78A4), anti-PKLR (1:75; Santa Cruz, clone E2), and anti-HA (1:1,000; Cell Signaling, 6E2). For PKM2 and HA detection, fluorescent secondary antibodies (1:10,000; Santa Cruz, clone E2), and anti-HA (1:1,000; Cell Signaling, 6E2). For PKM2 and HA detection, fluorescent secondary antibodies (1:10,000; Cell Signaling, D78A4). The assay was monitored for pyruvate-dependent conversion of NADH to NAD+ by fluorescence on Biotek Synergy Neo. For whole-cell lysates, a Pyruvate Kinase Activity Assay (Biovision) was used according to the manufacturer’s instructions by fluorescent measurement. Activity was normalized to the relative live cell population, as measured by live cell count.

**Metabolite measurement assay.** For pyruvate, PEP, and ATP assays, 3 × 10⁶ cells were seeded in 60-mm plates in triplicate and grown under 1% O₂ for 24 hours in recommended media, without supplemental sodium pyruvate. Cells were washed with PBS containing 100 μM phloretin and lysed and deproteinized using perchloric acid precipitation (Biovision). Pyruvate, PEP, and ATP Assay Kits (Biovision) were used by fluorometric methods on Synergy Neo (Biotek) to quantify intracellular metabolite levels. For lactate and glucose assays, 10⁶ cells were seeded in 6-well plates in triplicate and grown under 1% O₂ for 24 hours when conditioned media was sampled. Levels of glucose were measured using the Amplex Red Glucose Assay (Invitrogen), and levels of lactate were measured using the Lactate Assay Kit (Biovision). Metabolite consumption/excretion per cell per hour was calculated as previously described (40).

**Statistics.** Sample size in mouse experiments was chosen based on the biological variability observed with a given genotype. Non-parametric tests were used when normality could not be assumed. Mann Whitney test and t test were used when comparing independent shRNAs to shControl. One-tailed tests were used when a difference was predicted to be in one direction; otherwise, a two-tailed test was used. Bootstrapping was performed by sampling with replacement in R. Analysis of covariance (ANCOVA) testing was performed using aov() command in R. A P value less than 0.05 was considered significant. Error bars represent SEM unless otherwise indicated.

**Study approval.** Approval for the study was obtained through the MSKCC Institutional Review Board/Privacy Board (protocol 10-018A), the MSKCC Institutional Animal Care and Use Committee (protocol 04-03-009), The Rockefeller University Institutional Review Board (protocol STA-0681), and The Rockefeller University Institutional Animal Care and Use Committee (protocol 15783-H). Written consent was obtained from all human participants who provided samples for patient-derived xenografts.

**Author contributions**

AN, JML, and SFT designed the research. AN, JML, RM, EMW, and FYM performed the experiments. ZZ, PBP, LS, YYJ, and ES obtained, curated, and provided access to clinical samples and patient-derived grafts. AN and SFT wrote the manuscript.

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Address correspondence to: Sohail F. Tavazoie, Laboratory of Systems Cancer Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10065, USA. Phone: 212.327.7208; E-mail: stavazoie@mail.rockefeller.edu.

Jia Min Loo’s present address is: Cancer Therapeutics and Stratified Oncology, Genome Institute of Singapore, Singapore.


